Novel insights into toxic and chronic liver disease

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 injury[1]. 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 agent[2]. 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 LC_{50} 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.,* PKCα 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 toxicity[4]. 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 ibuprofen[3] and APAP[5], 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, CCl_4) 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 ($Jnk1^{\Delta hepa}$) were used, whereas an approach with siRNA against hepatocytic *Jnk2* ($siJnk2^{\Delta hepa}$) was completed to obtain animals presenting a lack of *Jnk2* specifically in hepatocytes. Following acute ibuprofen-induced liver toxicity, $siJnk2^{\Delta hepa}$ animals showed poor survival as well as a dramatic increase in liver injury parameters, worsened liver histology and MAPK activation, compared to $Jnk1^{\Delta hepa}$ 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 hepatotoxicity[5]. To this end, a

new murine strain was generated, which presented a concomitant deletion of *Jnk1* and *Jnk2* ($Jnk^{\Delta hepa}$) exclusively at the hepatocytic level. Here we denoted a greater level of liver injury in $Jnk^{\Delta hepa}$ 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 $Jnk1^{\Delta hepa}$ 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 $Jnk^{\Delta hepa}$ animals, as seen in increased inflammation, cell proliferation, and fibrosis progression, compared with $Jnk1^{\Delta hepa}$ or control mice. Exposure to APAP induced an increased oxidative stress response in $Jnk^{\Delta hepa}$ 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 $Jnk^{\Delta hepa}$ mice during APAP treatment, suggesting additional sources other than hepatocytes for its origin. Our study also demonstrated protection against APAP liver toxicity in $Jnk^{\Delta hepa}$ and control animals conferred by the Jnk inhibitor SP600125, but off-target effects were identified[5]. 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 hepatitis[6]. 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 databases[7]. 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 resolution[8]. 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 roles[10]. 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$^{\Delta hepa}$ model, which

characterizes for IKKγ deficiency in hepatocytes and spontaneously develops HCC[11]. For this purpose, mice with deficient *Jnk2* expression in hepatocytes (Jnk2$^{\Delta hepa}$) were generated and later also expanded to Jnk2$^{\Delta hepa}$ and NEMO$^{\Delta hepa}$ double knockouts[12]. 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, siJnk2 prevented HCC progression, which is a feature of 44-week-old NEMO$^{\Delta hepa}$ animals. In early-stage CLD, we evidenced that Jnk2 downregulation in NEMO$^{\Delta hepa}$ 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 system[13]. 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$^{\Delta hepa}$/Fas^{lpr} mice were generated, which carry a mutation in their Fas gene[14]. We here showed that defective Fas signaling (NEMO$^{\Delta hepa}$/Fas^{lpr}) exhibited decreased serum liver injury markers and multifocal hepatic necrosis compared with control livers (NEMO$^{\Delta hepa}$), which exhibited high mitotic index, oval cell proliferation and mild lipidosis. Besides, reduced compensatory cell proliferation in NEMO$^{\Delta hepa}$/Fas^{lpr} livers correlated with decreased cell death and absence of Caspase-3 activation in contrast to NEMO$^{\Delta hepa}$. We further evidenced notably downregulated TNF levels in NEMO$^{\Delta hepa}$/Fas^{lpr} animals concomitantly with decreased liver fibrosis. The significantly reduced presence of CD11b+ F4/80+ cells was representative for NEMO$^{\Delta hepa}$/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$^{\Delta hepa}$/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

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