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Pathophysiology of Cholestatic Liver Diseases: New Insights into the Mechanisms of Bile Infarct Formation

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Abstract

Cholestatic liver diseases can be induced for many reasons including obstructions, e.g. by stones or tumors. An early consequence of obstructive cholestasis is the formation of bile infarcts, which refer to clusters of dead hepatocytes due to bile salt accumulation. Although these infarcts were described long time ago (in 1876 by Charcot and Gombault), the leading mechanism is still unclear. Some hypotheses suggested direct killing by accumulation of bile salts up to toxic levels. Others claim indirect cell death via immune cell infiltration and inflammatory cytokine release. However, the sequence of events leading to the formation bile infarcts are still unclear. In the recent issue of *Hepatology*, Ghallab and his colleagues have recorded in a time-resolved manner the key events leading to bile infarct formation and the subsequent systemic changes, using two-photon based intravital imaging. This mini-review highlights the results of this study and discuss the time-resolved events in acute and chronic cholestasis, as well as the link between biliary bile salts and hepatocyte death.

Keywords: Cholestasis; intravital imaging; bile canaliculi; bile salt toxicity; bile leakage; bile duct ligation.

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Introduction

Bile is a toxic body fluid which is present in a detergent concentration in the biliary tract (Woolbright et al., 2015). It serves multiple functions, including (i) fat digestion within the intestinal tract via its detergent action, (ii) excretion of most of the harmful xenobiotics, bilirubin and bile salts, and (iii) elimination of cholesterol (Boyer, 2013). Thus, the biliary tree can be considered as a drainage system of most harmful molecules, particularly bile salts. This raise the question, what are the consequences of bile leakage in case of cholestatic liver diseases? Cholestasis can be mimicked in experimental animals by ligation of the common bile duct at the position between the gallbladder and the duodenum (Tag et al., 2015). This procedure recapitulates at least some features of obstructive cholestatic liver disease progression in human. In the acute stage after bile duct ligation (days 1-3), bile salt concentrations increase sharply in the biliary tree (Ghallab et al., 2018). This coincide with development of clusters of dead hepatocytes, a lesion named 'bile infarcts' (Figure 1). In the chronic phase, bile infarcts are not any longer seen, but ductular reaction and periportal fibrosis develop (Ghallab et al., 2018) (Figure 1). Although bile infarcts are described long time ago in obstructive cholestasis (Rolleston, 1905), the exact leading mechanism is still unclear. This can partly be because of the lack of technologies that allow direct spatio-temporal visualization of bile transport. In this mini-review, we focus on the advantages of intravital imaging in getting new insights into cholestatic liver disease progression.

Bile salt toxicity

Bile salts are amphipathic molecules that are synthesized in hepatocytes from cholesterol in a series of enzymatic reactions. This is initiated by CYP7A1 enzyme and occurs predominantly in the pericentral compartment of the liver lobule (Fickert and Wagner, 2017, Norlin and Wikvall, 2007, Russell, 2003). Following conjugation in hepatocytes, bile salts are secreted into bile canaliculi, mainly via the bile salt export pump (BSEP). From there, bile flux occurs towards the downstream interlobular and large bile ducts and finally drains into the duodenum via the common bile duct (Fickert and Wagner, 2017). Following de-conjugation and enzymatic modifications by gut microbiota, approximately 95% of the formed secondary bile salts are reabsorbed in the terminal ileum and reach the liver back via the portal vein (de Aguiar Vallim et al., 2013, Schneider et al., 2018). The concentrations of bile salts differ in various compartments of the body. In the systemic blood, the total bile salt concentration is approximately 2-8 µM. In hepatocytes, bile salt concentrations are less than 50 µM. In contrast, the biliary concentration of bile salts is approximately 20-40 mM, which is further concentrated in the gallbladder up to 100 mM (Fickert and Wagner, 2017). Based on *in vitro* experiments, bile salts display concentration dependent effects (Jansen et al., 2017) (Table 1). At concentrations below 25 µM, they act as signaling molecules (Chen et al., 2001, Schreuder et al., 2010). At concentrations between 50-200 µM, they may induce apoptosis (Schoemaker et al., 2004). At concentrations around 200 µM, they may induce pro-inflammatory genes (Allen et al., 2011). At concentrations between 200-2000 µM, they may trigger necrosis (Woolbright et al., 2015), and at higher concentrations they act as detergents (Woolbright et al., 2015).

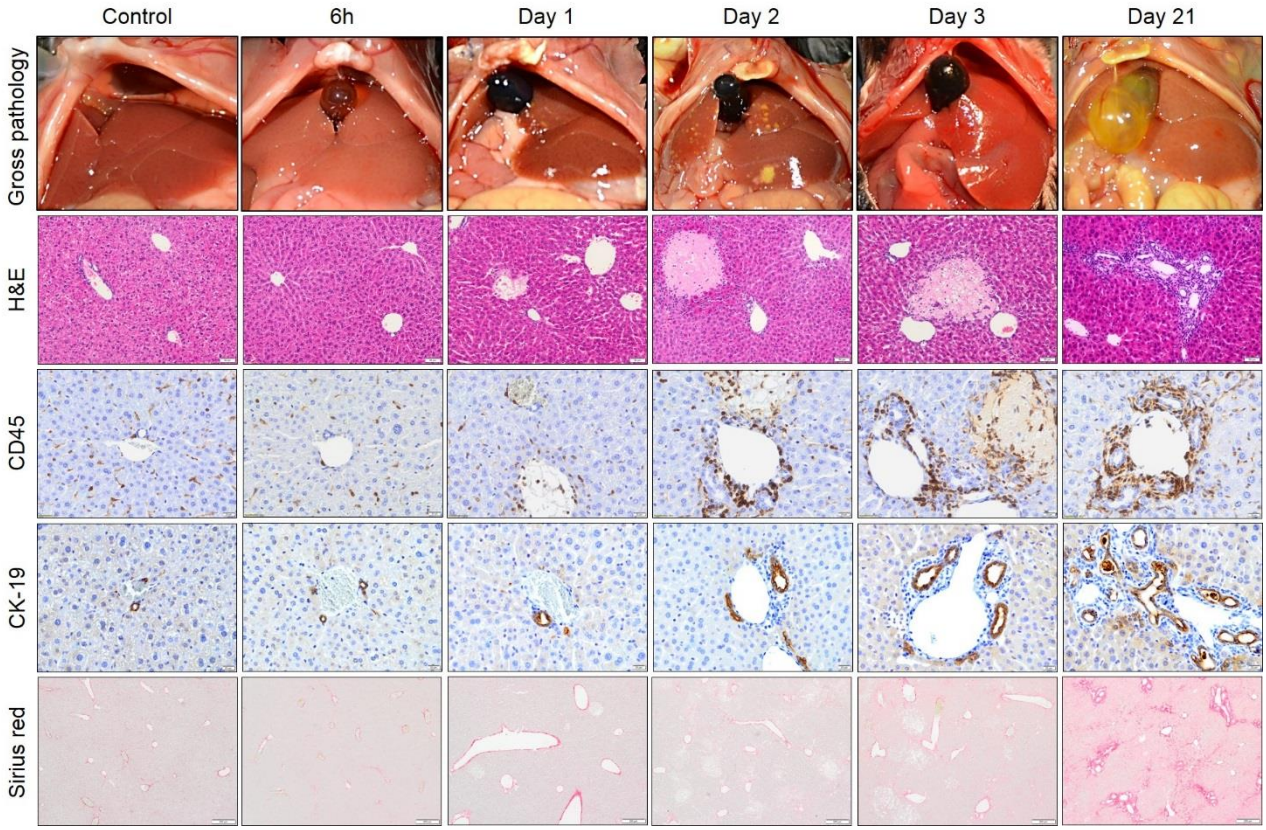

Figure 1. Pathogenesis of obstructive cholestasis following common bile duct ligation (BDL). The gross pathology shows accumulation of dark toxic bile in the acute stage (>day 3) and white/yellow bile in the chronic phase (day 21) after BDL. The HE staining shows bile infarcts on days 1 to 3 post BDL. In the chronic phase, periportal immune cell infiltration (CD45), ductular reaction (CK-19) and fibrosis (Sirius red) can be seen. [Source: Ghallab et al., 2018].

In healthy livers, cholangiocytes are protected against the detergent concentrations of bile salts via several mechanisms: (i) bile salts in bile are packed into *mixed micelles* with phospholipids and cholesterol, which help to neutralize bile salt toxicity (Coleman et al., 1979, Fickert and Wagner, 2017, Jansen et al., 2017, Puglielli et al., 1994, Schoemaker et al., 2004). (ii) *The biliary $HCO3^-$ umbrella*. Penetration of bile salts into cholangiocytes occurs at acidic pH, because it depends on their protonation (Beuers et al., 2012, Chang et al., 2016, Fickert and Wagner, 2017, Jansen et al., 2017). Thus, secretion of HCO^{3-} by cholangiocytes to their apical membrane help in alkalizing the pH leading to deprotonation of bile salts, and thereby protection of cholangiocytes. (iii) At the canalicular level, hepatocytes are protected against bile salt leakage by *tight junctions* and *peri-canalicular cytoskeleton* (Das et al., 2009, Jansen et al., 2017, Liu et al., 2015, Masyuk et al., 2001). These data open the following questions: what happen in case of breach of these natural barriers? How these toxic/detergent bile salt concentrations will affect the neighboring parenchyma? What are the systemic consequences in case of bile leakage into blood?

Table. 1. Concentration-dependent effects of bile salts [Source: modified from Jansen et al., 2017].

Concentration [µM]	Effect	Reference
<25	FXR- and TGR5-signaling	Chen et al., 2001, chreuder et al., 2010
50-200	Apoptosis	Schoemaker et al., 2004
200	Pro-inflammatory genes	Allen et al., 2011
200-2000	necrosis	Woolbright et al., 2015
>2000	Detergent action	Woolbright et al., 2015

Intravital two-photon based imaging reveals mechanism of bile infarct formation

Bile infarcts refer to damage of the liver parenchyma due to bile leakage in case of obstructive cholestasis. This was described more than a century ago by Charcot and Gombault (Rolleston, 1905). However, the mechanism behind this damage, also called Charcot-Gombault necrosis, is not yet clear. Several hypotheses were previously published with the goal to illustrate the mechanism of hepatocyte death in cholestatic livers (Cai and Boyer, 2018). This includes (i) induction of hepatocyte necrosis or apoptosis via accumulation of bile salts above certain thresholds (Schoemaker et al., 2004, Woolbright et al., 2015). (ii) Other publications illustrated that hepatocyte death in cholestasis occurs indirectly as a consequence of immune cell infiltration, and inflammatory cytokine release (Allen et al., 2011, Cai et al., 2017). This hypothesis was supported by decreased bile infarct formation in bile duct-ligated mice where the inflammatory response is mitigated due to depletion of inflammatory cells, gene knockout of cytokine or adhesion molecules or drug treatments (Gujral et al., 2003). Although these arguments which support these investigations, detailed spatio-temporal events that lead to bile infarct formation in cholestatic livers are still to be elucidated.

A recent study by Ghallab and colleagues (Ghallab et al., 2018) have investigated the sequence of events that precede and follow the formation of bile infarcts in mice with obstructive cholestasis. A key event that triggers bile infarct formation is the rupture of the apical hepatocyte membrane. A central technique in this study is two-photon based intravital imaging. Combination of the infra-red excitation laser, broad spectrum wavelength (680-1080 nm), long-distance objectives with high numerical apertures, and sensitive detectors provided by two-photon microscopy, together with good anesthesia and excellent animal preparation allow to perform long-term intravital imaging of intact livers (Reif et al., 2017). Fast recordings in the millisecond range are also possible. The image resolution is close to 200 nm, which is enough to image also at the subcellular level (Ghallab and Hengstler., 2018, Ghallab et al., 2018, Jansen et al., 2017, Koppert et al., 2018, Reif et al., 2017). Applying this technology in imaging bile salt transport in the liver during the *acute stage after bile duct ligation* (days 1-3) (Ghallab et al., 2018) allowed to record the following sequence of events: (i) firstly, individually

dispersed hepatocytes loss their mitochondrial membrane potential; (ii) this is followed by rupture of the apical hepatocyte membrane, and (iii) flooding of the neighboring hepatocyte with bile, which leads to (iv) cell death; (v) the dead cells create a biliary-sinusoidal shunts leading to release of bile into the neighboring sinusoids; (vi) this triggers a domino effect of further death events of neighboring hepatocytes leading to formation of clusters of dead cells (the bile infarcts); (vii) formation of bile infarcts is followed by immune cell infiltration, particularly neutrophils (Figure 2). Of course, this does not mean that immune cell infiltration is not relevant in the pathogenesis of acute cholestasis, but this intravital imaging (Ghallab et al., 2018) reveals that they are not involved in the initial event. Infiltration of immune cells might aggravate the initial damage triggered by rupture of the apical hepatocyte membrane. These data suggest that the limited number of hepatocytes which are killed in the acute phase of obstructive cholestasis serve to reduce bile salt concentrations in the biliary tree. The lost hepatocytes can be efficiently replaced within few days by the enormous regeneration capacity of the liver (Ghallab et al., 2016, Leist et al., 2017, Schliess et al., 2014).

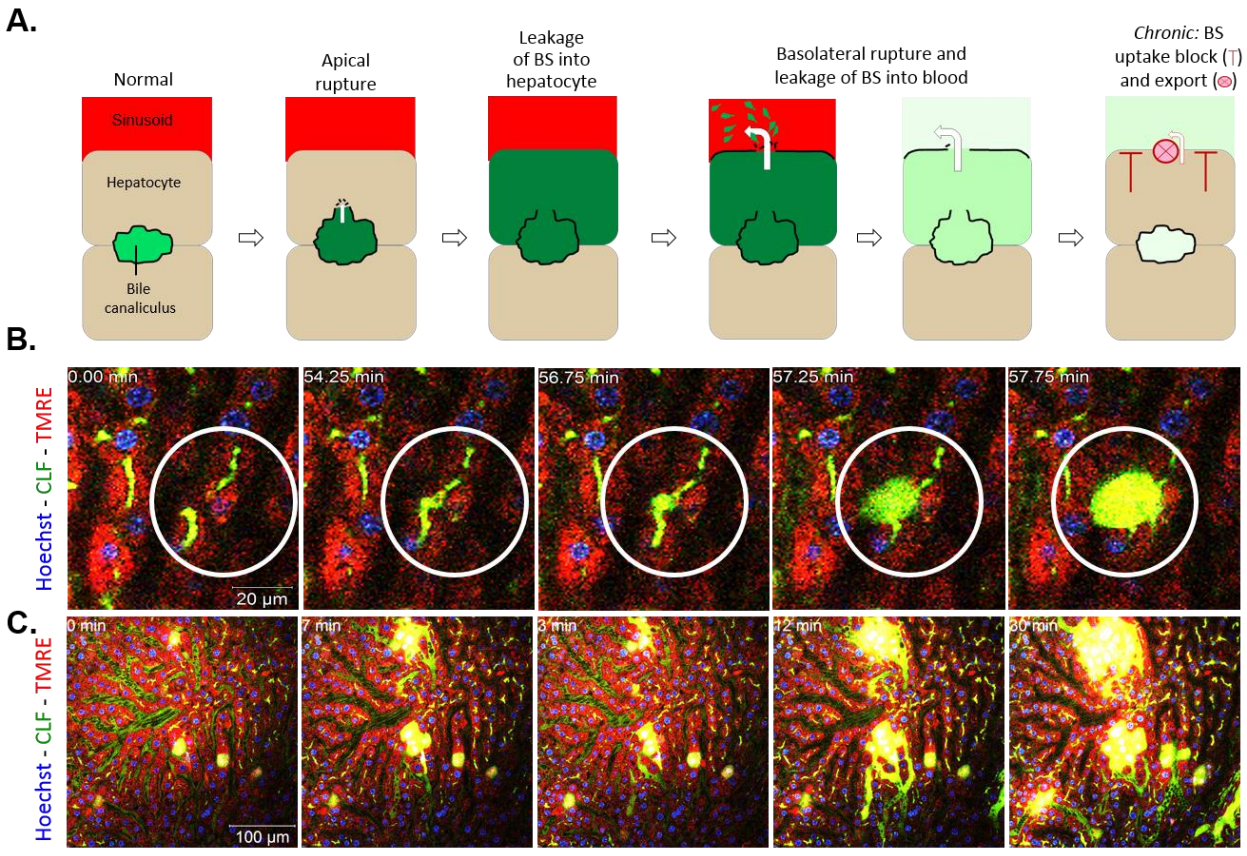

Figure 2. Apical membrane rupture is the key event triggering bile infarct formation. A&B. A schedule and stills of intravital imaging recording the events before and after rupture of the apical membrane on day one after bile duct ligation. **C.** Domino effect of bile infarct formation starting from individually dispersed bile flooded cells. [Source: modified from Ghallab et al., 2018].

In the chronic phase after bile duct ligation (> day 21), there is no bile infarcts, and the concentrations of bile salts in the biliary tree decrease to control levels or even less (Ghallab et al., 2018). This is because several adaptive mechanisms are established by the liver at this stage to prevent accumulation of the toxic bile salts. This includes adaptation at the hepatocyte and at the biliary tree levels. ***At the hepatocyte level.*** Chronic cholestasis leads to downregulation of the basolateral uptake transporters (NTCP, Oatp) (Geier et al., 2007, Ghallab et al., 2018, Slitt et al., 2007). This can be directly seen by the strongly reduced uptake of green fluorescent bile salt analogues on day 21 after bile duct ligation (Ghallab et al., 2018). In addition, the efflux transporters, MRP3 and MRP4, are strongly upregulated at the sinusoidal membrane, allowing the transport of bile salts from hepatocytes to blood (Geier et al., 2007, Ghallab et al., 2018). Furthermore, CYP7A1 expression in hepatocytes, the key enzyme in bile salt synthesis, is reported to be downregulated in cholestatic patients (Schaap et al., 2009). ***At the biliary tree level.*** The biliary tree can be organized into three main topological domains: the large and common bile ducts, the interlobular bile ducts, and the canalicular network (Jansen et al., 2017). (i) *The large bile ducts* are distinguished by having diameters more than 100 µm. In obstructive cholestasis these large ducts respond by further increase of their diameters, allowing to accommodate the large volume of bile (Jansen et al., 2017). (ii) *The interlobular bile ducts* are defined by having diameters between 10-100 µm. In cholestasis, these ducts undergo extensive remodeling which leads to branching and corrugation; a process named ductular reaction (Jansen et al., 2017). This occurs in harmony with upregulation of the sodium dependent transporter, ABST, and the basolateral organic solute transporter Ostα/β in cholangiocytes. These transporter changes allow to remove bile salts from the biliary tree and efflux into the peri-biliary plexus; a process named cholehepatic shunt (Geier et al., 2007, Jansen et al., 2017, Slitt et al., 2007). Thus, remodeling of the interlobular bile ducts allows to maximize the bile resorption capacity via the cholehepatic shunt. (iii) *The Bile canaliculi* are the most upstream domain of the biliary tree formed between the apical membranes of two neighboring hepatocytes. In healthy livers, the diameter of bile canaliculi is approximately 1 µm (Jansen et al., 2017). In contrast, in obstructive cholestasis, the diameter increases up to 10-20 µm (Ghallab et al., 2018). However, the relevance of these canalicular alterations was not clear. A recent study by Gupta et al. illustrated blebbing of the canalicular hepatocyte membrane as an early response after bile duct ligation (Gupta et al., 2017). This is followed by subsequent separation and bile discharge into blood via the sinusoidal membrane. Others (Rahner et al., 1996) have illustrated para-cellular leakage of bile due to leakiness of the tight junctions. Both mechanisms were not observed in the recent intravital imaging study of (Ghallab et al., 2018), which shows that apical membrane rupture leads to hepatocyte killing, and thereby creating a shunt between bile canaliculi and blood sinusoids.

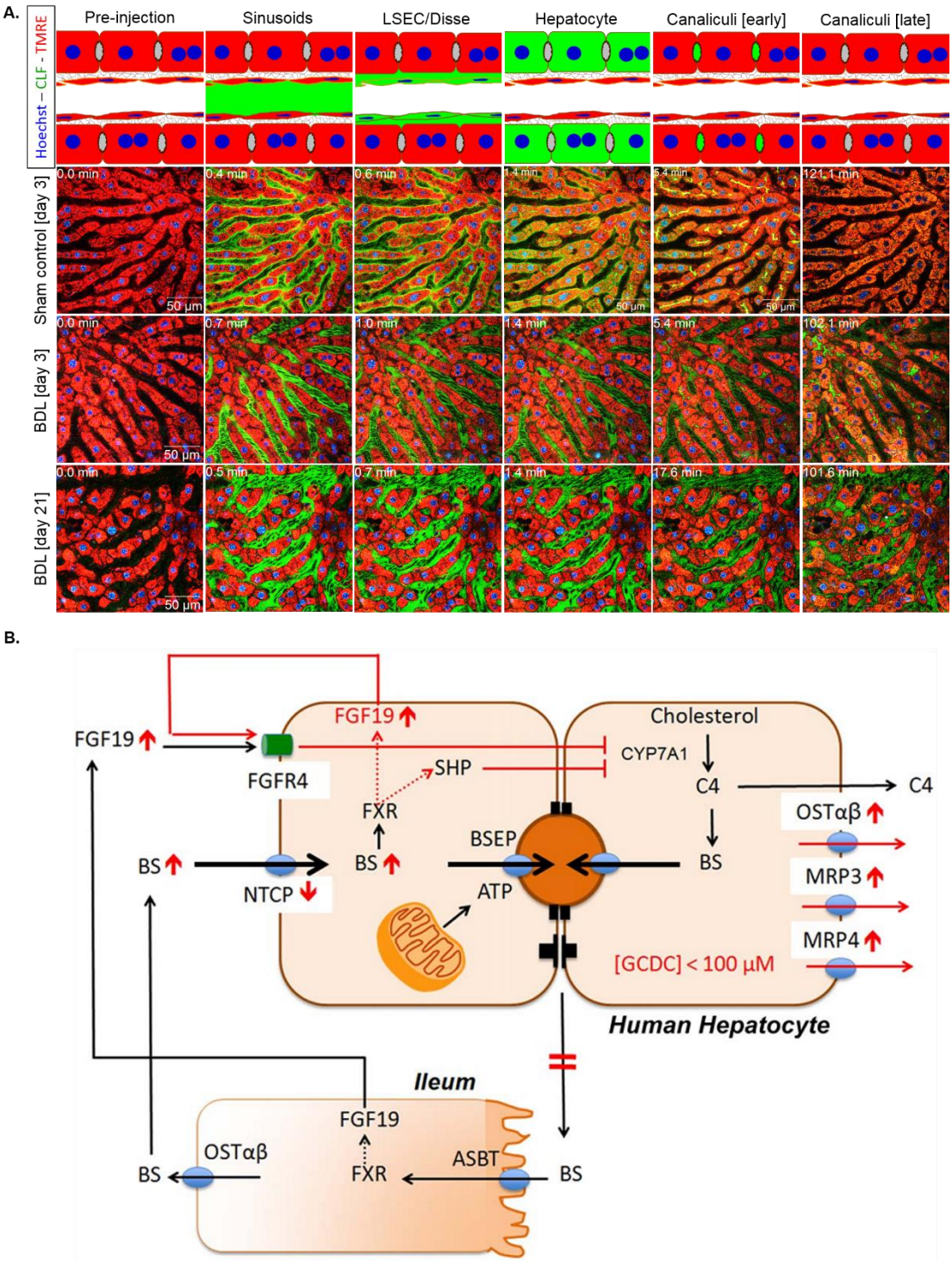

Figure 3. Cholestasis-induced adaptation of transporter and enzyme expression. A. Stills of intravital imaging showing efficient uptake and secretion of the green fluorescent bile salt analogue CLF in controls. In contrast, the uptake from blood is strongly reduced after bile duct ligation, particularly on day 21. **B.** A schedule summarizing the adaptive changes at the transporter and enzyme expression level in cholestasis. [Source: Ghallab et al., 2018; Jansen et al., 2017].

Conclusions

Intravital imaging of cholestatic livers revealed that rupture of the apical hepatocyte membrane is the key event that leads to bile infarct information. Furthermore, the time resolved events (summarized in Figure 4) in the acute and the chronic phase after bile duct ligation should be considered during cholestatic liver disease treatment. Although all discussed mechanisms aim at protecting the liver against bile salt accumulation, this results in sharp elevation of bile salt concentrations in the blood. Therefore, the impact of this challenge on other body organs, particularly the kidneys, requires further detailed studies.

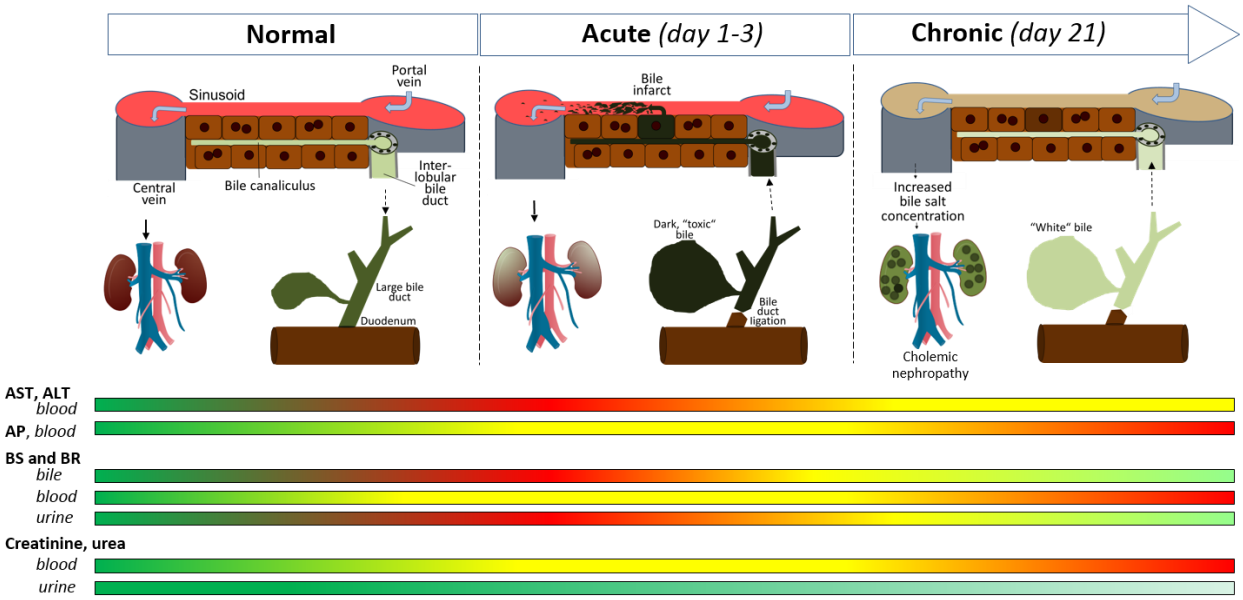

Figure. 4. Mechanism of bile infarct formation. The schedule shows the rupture of the apical hepatocyte membrane on days 1 to 3 post bile duct ligation, as a key event on bile infarct formation, and the subsequent systemic changes. ALT: alanine transaminase; AST: aspartate transaminase; AP: alkaline phosphatase; BS: bile salts; BR: total bilirubin. [Source: Ghallab et al. 2018]

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