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On the Mechanisms of Biliary Flux

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Since the late 1950s, transport of bile in the liver has been described by the "osmotic concept," according to which bile flows into the canaliculi toward the ducts, countercurrent to the blood flow in the sinusoids. However, because of the small size of canaliculi, it was so far impossible to observe, let alone to quantify this process. Still, "osmotic canalicular flow" was a sufficient and plausible explanation for the clearance characteristics of a wide variety of choleretic compounds excreted in bile. Imaging techniques have now been established that allow direct flux analysis in bile canaliculi of the intact liver in living organisms. In contrast to the prevailing osmotic concept these analyses strongly suggest that the transport of small molecules in canalicular bile is diffusion dominated, while canalicular flow is negligibly small. In contrast, with the same experimental approach, it could be shown that in the interlobular ducts, diffusion is augmented by flow. Thus, bile canaliculi can be compared to a standing water zone that is connected to a river. The seemingly subtle difference between diffusion and flow is of relevance for therapy of a wide range of liver diseases including cholestasis and NAFLD. Here, we incorporated the latest findings on canalicular solute transport, and align them with extant knowledge to present an integrated and explanatory framework of bile flux that will undoubtedly be refined further in the future. (Hepatology 2021;74:3497-3512).

The mechanism of transport of glandular secretions such as saliva, bile, and exocrine pancreatic juice is fundamental to their role in physiology. Recently, reports from two groups have rekindled the decades-old scientific debate on the mechanisms by which biliary fluid and biliary constituents exit the liver.[1-3] Bile acids (BAs) are secreted by the action of ATP-dependent transporter proteins to reach high concentrations in the liver canalicular network, but what transports them out of the canalicular network, into the bile ducts, and finally out of the liver? Conventional wisdom was in favor of an osmotically driven mechanism according to which the BAs and other solutes, such as glutathione, bilirubin and many other organic anions, secreted by hepatocytes, draw water into the bile canaliculi to create bulk *canalicular* fluid flow. In sharp contrast, work done by the present authors demonstrated the conspicuous lack of measurable flow in canaliculi.[1] Instead, BAs appear to move by molecular diffusion in a virtually stagnant canalicular fluid until they reach the bile ducts. Only in the ducts is fluid flow evident, caused by local inorganic

Abbreviations: HU, Hounsfield unit; BA, bile acid; CFTR, cystic fibrosis transmembrane conductance regulator; IP3, inositol 1,4,5-triphosphate; KO, knockout; TGR5, thioredoxin glutathione reductase 5.

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ion secretion, drawing water from the cholangiocytes into the ductular lumen. Moreover, an osmotic potential generated by the high concentration of solutes diffusing from the canalicular network into the ducts may contribute to water influx from cholangiocytes into the ductular lumen, although this mechanism has not yet been directly proven. Therefore, the ducts, much more than the canaliculi, may represent the anatomical site where bile flow originates. Although this distinction in the anatomical site of origin of bile flow may seem like splitting hairs, it has important consequences for the understanding and development of therapy of various liver diseases. Solving this conundrum requires a multidisciplinary excursion into fluid mechanics, molecular diffusion, and the development of new techniques that allow direct measurement of these processes in the biliary tract of intact functioning livers. After discussing the fundamental biophysical theory and presenting recently published data in this context, we here present a framework for bile flux and discuss its implications on pathophysiology and therapeutic developments.

The Trajectory of a BA Through the Liver

Two functional domains must be differentiated within the liver to correctly describe bile flux (Fig. 1A). (1) The bile canalicular network, formed by the apical membranes of adjoining hepatocytes, into which BAs and other bile constituents are actively secreted, with the pericentral part as the closed end and the canals of Hering as the connections to the bile ductules (note that this network represents a "dead-end" compartment; and (2) the bile ducts, formed by cholangiocytes, which progressively merge into larger ducts downstream, eventually exiting the liver as a single extrahepatic bile duct. Functional differentiation of these anatomical structures has traditionally been attempted using compounds that were postulated to enter bile specifically either through the canalicular network (e.g., mannitol) or through the bile ducts (e.g., C14 - HCO_3^-). Macroscale observations of the concentrations of these compounds as they appear in secreted bile were used to extrapolate the contribution of the different functional domains to bile transport. Today, intravital microscopy enables direct differentiation of these two anatomical sites through reporter mouse strains such as Hnf1beta-CreER-tdTomato,[4] which express red fluorescence in cholangiocytes but not hepatocytes (Fig. 1A). Transport of bile constituents can be traced using fluorescent probes that are excreted into bile.

Osmotic Theory of Bile Flux

The theoretical concept for an osmotically driven bile flux was proposed in 1959 by Sperber.[5] The core reasoning for this theory is that BAs are osmotically active solutes and therefore have osmotic potential. Earlier seminal studies had already shown that hydrostatic pressure does not affect bile formation.[6,7] BAs are secreted across the canalicular membrane of the hepatocytes through the ATP-dependent bile salt export pump, whereas other solutes are secreted by members from the same superfamily of ABC-transporters, such as ABCC2, ABCG2, and ABCB1. Because the canalicular membrane is impermeable to these solutes, the concept postulates the establishment of an osmotic gradient that draws water from the hepatocytes into the canalicular lumen. Moreover,

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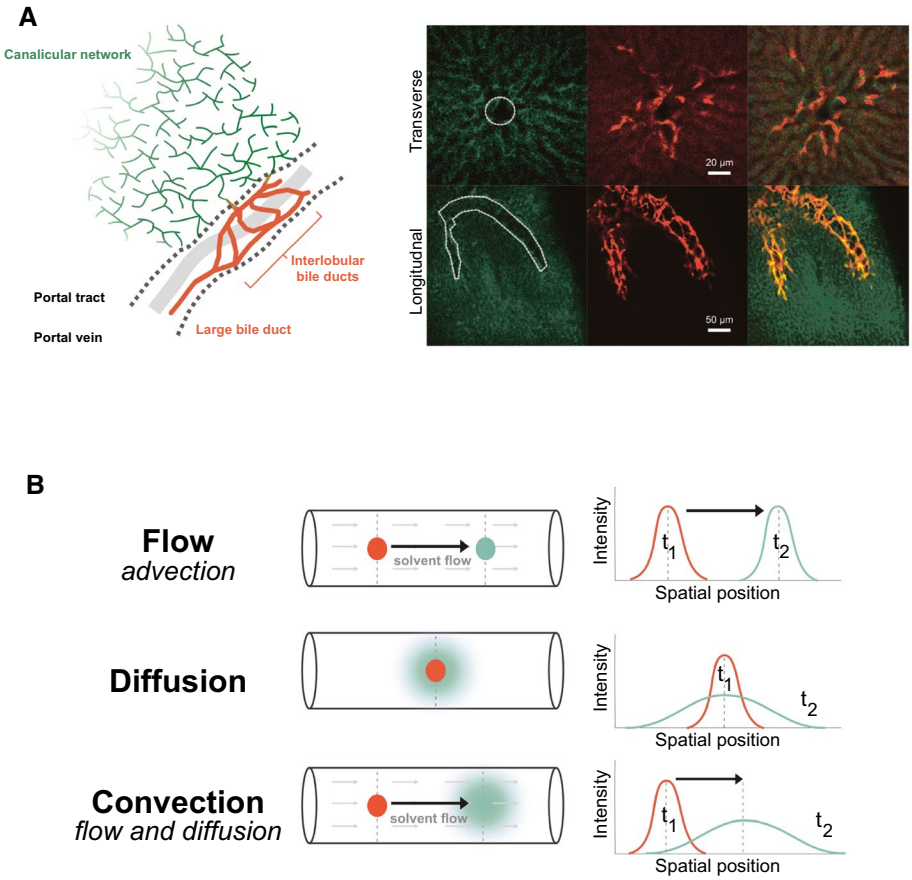


FIG. 1. (A) Schematic representation (left) of liver tissue showing the canalicular network between hepatocytes and the interlobular ducts forming a plexus around the portal vein. Intravital imaging (right) of the same structures using a bile salt analog cholyllysyl fluroscein (green) in a reporter mouse exhibiting duct-specific tdTomato fluorescence in cholangiocytes of the ductular epithelium. (B) Schematic representation of the independent and combined effect of diffusive or advective flux mechanisms over time on a cluster of particles in a cylindrical tube. Graphs show the expected distribution of particles in space at two time points for each of the three cases.

because the canalicular network is closed at the pericentral side, the increase of fluid volume in the network leads to directed flow toward the periportal bile duct.[8] A large body of experimental evidence has accumulated over seven decades that measures the macroscopic clearance of compounds from blood, and their appearance in bile. These clearance kinetics and concentrations in bile, along with changes in bile volume measured under a variety of treatment conditions, have been used to infer the osmotic theory of canalicular bile flux. The main support for this hypothesis was the clear observation that bile flow strongly depends on the amount of solute (BAs and other choleretic compounds) secreted by the hepatocytes. However, as a molecular and cell biological understanding of bile transport has developed, including the discovery that BAs are signaling molecules, alternatives to the concept of osmotically driven canalicular bile flow appear possible. Below we summarize the current state of knowledge with respect to the canalicular and ductular bile flux.

Bile Acid–Dependent and Independent Flow

The primary utility of the osmotic theory is that it posited an explanation for macroscopic extrahepatic bile flow measurements in response to intravenous administration of compounds such as BAs, reviewed comprehensively by Boyer.[8] Intravenous

administration of excess BAs in animals and humans leads to an increased extrahepatic bile flow, as well as higher output concentration of BAs in bile. There is an *almost* linear relationship between output BA concentrations and related bile acid–dependent flow (i.e., the volume of extrahepatically excreted bile). Mathematical extrapolation of this relationship to the zero-BA concentration (y-intercept) reveals a nonzero bile flow. This offset has been interpreted as a BA-independent flow, which was thought to represent two other osmotically active processes: (1) the secretion of non-BA organic anions, which is substantial given the 30%-50% decrease in BA-independent bile flow in Abcc2-deficient rats,[9] and (2) ductular secretion of bicarbonate. Classical experiments by Hardison and Wood, in which they replaced bicarbonate by tricine, suggested that bicarbonate contributes about 50% to BA-independent bile flow in the rat.[10] Subsequent work on the effect of the hormone secretin—which acts on cholangiocytes of the ductular epithelium—further substantiated the paradigm that ductular flow is also osmotically generated, but caused by the secretion of inorganic ions such as HCO_3^-.

Several reports[8,11] have shown that there is a clear (albeit pseudolinear) correlation between excreted BA concentrations and extrahepatic bile flow. It was recognized early on that the osmotic coefficient of a particular BA does not necessarily correlate with its choleretic potential.[12] BAs are hydrophobic molecules that undergo a phase transition to form micelles at concentrations exceeding their critical micellar concentrations (CMCs). These micellar suspensions have a much lower osmolarity than dilute solutions of Bas.[13] The CMC of various BA derivatives is between 5 mM and 20 mM,[14] which is further reduced in the presence of other amphiphilic bile constituents (e.g., phospholipids) that favor micelle formation. Indeed, it is this micellization that provides the emulsifying properties of bile used in digestion. Because BAs in canalicular bile exist primarily in the low-osmolarity micellar (and not solution) phase, the contribution of BAs to canalicular osmotic potential is limited; however, each BA molecule is accompanied by a counter cation that is, osmotically, fully active. The entry of counter cations has always been assumed to occur largely through tight junctions.

Canalicular secretion of other solutes by hepatocytes such as glutathione could generate much more osmotic force. Each glutathione molecule that is secreted has to be accompanied by two counter cations. The glutathione concentration (GSH + GSSG) in rodent bile is about 4mM.[15] With associated counterions this would contribute a 12-mOsm osmotic potential. Breakdown of glutathione by apical gamma-glutamyltransferase (as in humans) would generate approximately 20 mOsm—sufficient for flow generation. However, the crucial question of the anatomical site at which water enters the biliary network remains unanswered.

Canalicular Versus Ductular Water Flow

Recent studies with intravital microscopy strongly suggest that little if any water is drawn into the canaliculus during solute secretion, because no measurable flow occurs in this anatomical compartment.[1] This requires a closer look at water transport at these anatomical sites. It has always been assumed that water enters the canaliculus through aquaporins in the canalicular membrane and paracellularly through claudin in tight junctions. The issue on claudins is complicated; however, it appears that most claudins except claudin-2 are impermeable to water.[16] Claudin-2 is expressed in hepatocytes, but interestingly only in pericentral and not in periportal hepatocytes, whereas under normal conditions the main solute flux occurs in the periportal zone. Claudin-2 knockout (KO) mice show a 50% reduction in bile flow in comparison to wild-type mice.[17] However, claudin-2 is also expressed in cholangiocytes, where expression was also knocked out. Hence, while paracellular claudin-2-mediated water flow apparently contributes to bile flow, it is not possible to rule out that the water is added in the ductules.

Aquaporins are expressed in the hepatocyte; Huebert et al.[18] showed that the abundance of aquaporins in the hepatocyte is AQP8 >> AQP9 > AQP0. Interestingly, they observed that AQP8 is largely localized in intracellular and subapical vesicles. AQP8 is also detected in mitochondria. In a study with hepatocyte couplets, it was demonstrated that

when these cells were stimulated with cAMP, there was a strong insertion of AQP8 into the canalicular membrane. Conversely, AQP9 is largely localized in the basolateral membrane.[19] These observations may suggest that, at least under basal conditions, there is relatively little water flow through aquaporins in the canalicular membrane. Furthermore, and again under basal conditions, there appears to be little water transport through hepatocellular claudin-2, as this is only localized in the pericentral region.

A quite broad spectrum of aquaporins is expressed in cholangiocytes; as Masyuk and LaRusso[20] describe: "Rat cholangiocytes express more AQPs than any cell type reported to date, seven AQPs from the known thirteen." Of these, AQP1 and AQP4 have been studied functionally. AQP1 is localized in both the apical and basolateral membrane, and inhibition of its expression leads to significantly decreased water transport in isolated bile duct units.[21] Similar to the situation with AQP8 in hepatocytes, the presence of AQP1 in the apical membrane of cholangiocytes requires activation through cAMP as a downstream signal of secretin signaling.[22] AQP4 is localized in the basolateral membrane of cholangiocytes.[23]

Thus, based on data from the literature, it is not possible to conclude whether the major water entry into the biliary network occurs at the level of hepatocytes, cholangiocytes, or both. Because the recent data using intravital microscopy show that there is no or only very low canalicular flow, the possibility remains that water influx into the canaliculus is very low or absent; and instead, solutes secreted into bile canaliculi at hyperosmotic concentrations diffuse into the bile ducts, and only there may draw water through claudin and aquaporins of cholangiocytes to give rise to ductular flow.

Mannitol Clearance

An experiment that was interpreted to support the osmotic canalicular concept involves polar uncharged solutes, which are not subject to active transport and enter the bile by diffusion such as erythritol and mannitol.[24] When co-administered with BAs, the relative clearance of these compounds is increased through a BA-induced increase in bile flow, albeit with a delay. This increased clearance could not be replicated through secretin-induced bile flow at the level of ductular cholangiocytes.[25] These findings were interpreted as follows: Because mannitol enters bile through canaliculi, and its clearance can only be enhanced through BA-induced, and not secretin-induced flow, the relative concentrations of mannitol-BA represent canalicular (rather than ductular) bile transport. While ingenious, these experiments were later challenged by other studies. First, permeability studies as well as clearance studies of mannitol showed that its entry into bile was not necessarily exclusively canalicular and could be affected by secretin-induced ductular flow in dogs and guinea pigs.[26,27] Second, mannitol is far from an inert tracer but enhances the biliary excretion of certain compounds while triggering reabsorption of water in bile ducts.[28] These complexities undermine the utility of mannitol clearance as a straightforward tracer of canalicular bile flow, as initially postulated.

Testing the Osmotic Theory of Canalicular Bile Flux

As the pioneers of the osmotic theory were fully aware, it was, until recently, not possible to test the osmotic concept of canalicular flow by direct canalicular measurements of water influx or unidirectional flow into canaliculi.[31] Macroscale phenomena such as extrahepatic bile output or the clearance of compounds into extrahepatic bile are equivalently well-explained by water excretion into canaliculi or into ducts. Testing the central tenet of the osmotic theory requires a demonstration of flowing bile in liver canaliculi that has only now become possible through the use of functional intravital imaging.[1]

Quantification of Flow and Diffusion in Micro-vessels

Although the described osmotic concept defines bile canaliculi as the source of fluid flow, it is important to consider that this is not the only mechanism by which flux of solutes occurs, as diffusion also leads

to considerable solute movement at physiological temperatures. Therefore, a comprehensive concept must include the contribution of both these processes. Based on the general principles of diffusion and advection (Box 1A), we can describe a localized cluster of particles in a liquid-filled cylindrical tube (Fig. 1B). Due to advection, the agglomeration will simply move along the tube with the fluid as a single cohesive mass, quantitatively represented by a translation of the center of mass in the direction of

Box 1 Features of diffusion and flow and differentiation of both flux mechanisms by FLAP

A. Biophysical basics of diffusion, advection and convection

	Diffusion	Advection
Molecular behavior	Individual solute particles exhibit Brownian motion due to thermal energy	Particles are carried with moving fluid driven by the pressure gradient along the fluid
Ensemble behavior	Symmetric spatial dispersion (spreading) into the fluid from high concentration to low concentrations	Directional movement of particles in the direction of fluid flow, independent of their concentration
Rate and direction	Rate of diffusion is proportional to the concentration gradient Direction is along the concentration gradient	Velocity of advection is proportional to the pressure gradient Direction is along the fluid flow
Constants and Units	3D-diffusion coeffient (length^2/time)	1D-velocity (length/time)
Typical examples	Globular proteins ~1 μm^2/s; Small molecules (200-1000 Da): ~400 μm^2/s; Diatomic gases: O2: 2000 μm^2/s for	Capillary blood flow: ~100 μm/s

Convection

- Combined diffusion and advection of solute in a moving solvent
- Relative contribution of diffusion and advection varies over space and time
- Relative contribution can be represented as dimensionless Peclet number (P) = $\frac{velocity \times length}{Diffusion\ co\text{-}efficient}$

B. Analysis of diffusion and advection by 'Fluorescence loss after photoactivation'

- Utilizes the property of certain chemicals, such as 5-chloromethylnitrobenzyl-fluorescein (CMNBF), to become fluorescent upon irradiation with UV-light.
- FLAP can be used to create fluorescent molecules inside biological structures such as blood vessels, bile ducts, and bile canaliculi in the liver of an anesthetized living mouse.
- The UV-light dose required for photoactivation is only 0.1-0.5 mJ/μm^2, which does not alter hepatocyte transport functions, mitochondrial activity or induce cell death for the duration of the experiment.
- CMNBF is secreted by hepatocytes into bile canaliculi, thus making it suited for bile flux analysis.

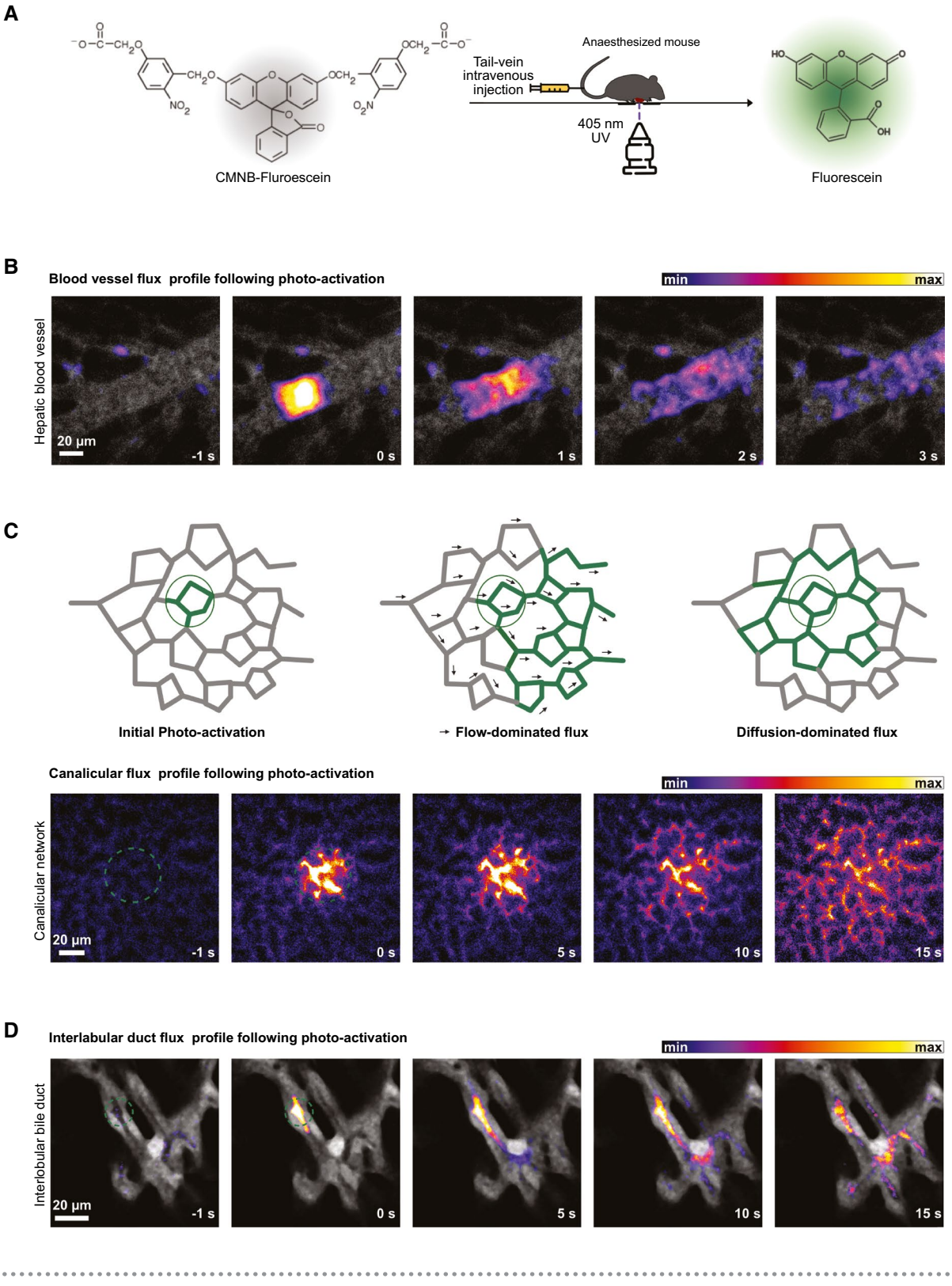
fluid flow. On the other hand, diffusion will cause the cluster to disperse in all available directions (i.e., particles spread away from a stationary center of mass). Diffusion is entropic and thus inevitable for solutes at physiological temperatures. If bulk flow is present, dispersion and translation of the cluster occur simultaneously in a process named convection (Fig. 1B). Fluid advection is generally more efficient at transporting molecules over long distances but counters a build-up of high concentrations. For example, capillary blood flow of approximately 100 μm/s is much more efficient over large distances (>/~1 mm) than solute diffusion with coefficients up to 400 $\mu m^2/s$, as the time required for particle movement increases linearly with the distance for advection but increases as the square of the distance for diffusion.

Specific methodologies are required to probe the described biophysical processes in the context of bile flux in the canalicular network and biliary ducts of live animals. Rheological methods such as ultrasound or MRI are available but lack either the resolution or the specificity required to probe diffusive and advective mass transfer in very small structures such as bile canaliculi and interlobular bile ducts. Therefore, fluorescence loss of photoactivation (FLAP) was recently introduced[1] that allowed the quantification of flow and diffusion in microconduits (Box 1B). As an illustrative example, photoactivation of CMNB-fluorescein–dextran in a blood vessel of the liver is shown in Fig. 2A,B. After photoactivation for 1 second of a 20-μm-radius circle in the blood vessel infused with CMNB-fluorescein–dextran, a mass of fluorescein is created in the middle of the blood stream; subsequently, two features can be observed: (1) the center of mass of fluorescein shifts along the direction of the blood flow; and (2) the mass disperses due to diffusion.

While in a blood vessel with large diameter, luminal photoactivation is possible, photoactivation exclusively within the much smaller lumen of bile canaliculi (0.5-1.5-μm diameter) is difficult. Hence, a 20-μm region of the canalicular network rather than individual canaliculi is activated. A region of the canalicular network represents a constrained space, wherein molecular flux may only proceed through the pre-existing paths allowed by the canalicular conduits. Nonetheless, dispersion of photoactivated material by diffusion or translation of its center of mass by advection will occur similarly but merely with the condition that the material may only travel along the paths allowed by the network topology (Fig. 2C). Following photoactivation of such a region of the canalicular network, the symmetrical spreading expected due to diffusion was observed. The lack of any detectable shift in the center of mass over several minutes indicated the absence of detectable advection.

Interlobular bile ducts represent conduits of approximately 5-10 μm in diameter. This size and geometry allow activation within the lumen. Importantly, a shift in the center of mass of the fluorescent material was also seen along one direction in the longitudinal axis, indicating the presence of advection. Together, these imaging data are in line with a concept in which small molecules in bile move through the canalicular network by diffusion (Fig. 2C), whereas fluid flow sets in at the interlobular ducts (Fig. 2D).

Solute dissipation in relatively small regions of the canalicular network (20-μm diameter) revealed the local flux mechanism. The relationship of this local flux mechanism to the larger context of *lobular* compound clearance is influenced by the lobular topology (Box 2A), in which distinct gradients will be established by diffusion and flow (Box 2B). At fields of view of 500 × 500 μm, the entire lobular canalicular network and the associated interlobular bile ducts can be visualized and photoactivated (Fig. 3B).[1] The topology of the lobule was determined using the hepatocyte nuclear factor 1β (HNF1β) reporter mice, in which the interlobular bile ducts show red fluorescence. Photoactivation experiments with CMNB-fluorescein demonstrated that initially homogenous fluorescence over the lobular canalicular network evolves into a **PC(high)**-PP gradient (Fig. 3B). Furthermore, the center of mass remained stationary at the center of the initial homogenous activation profile. This is in agreement with a diffusion-dominated flux mechanism. Performing a similar activation in an interlobular ductular mesh shows a clear directional preference of the photoactivated material (Fig. 3C). Fluorescence is transported to only some of several branches of the ductular mesh, revealing the presence of a directional flow in ducts.


A
CMNB-Fluroescein
Anaesthesized mouse
Tail-vein
intravenous
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405 nm
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B
Blood vessel flux profile following photo-activation
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max
Hepatic blood vessel
20 µm
-1 s
0 s
1 s
2 s
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Initial Photo-activation
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Interlabular duct flux profile following photo-activation
min
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Interlobular bile duct
20 µm
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0 s
5 s
10 s
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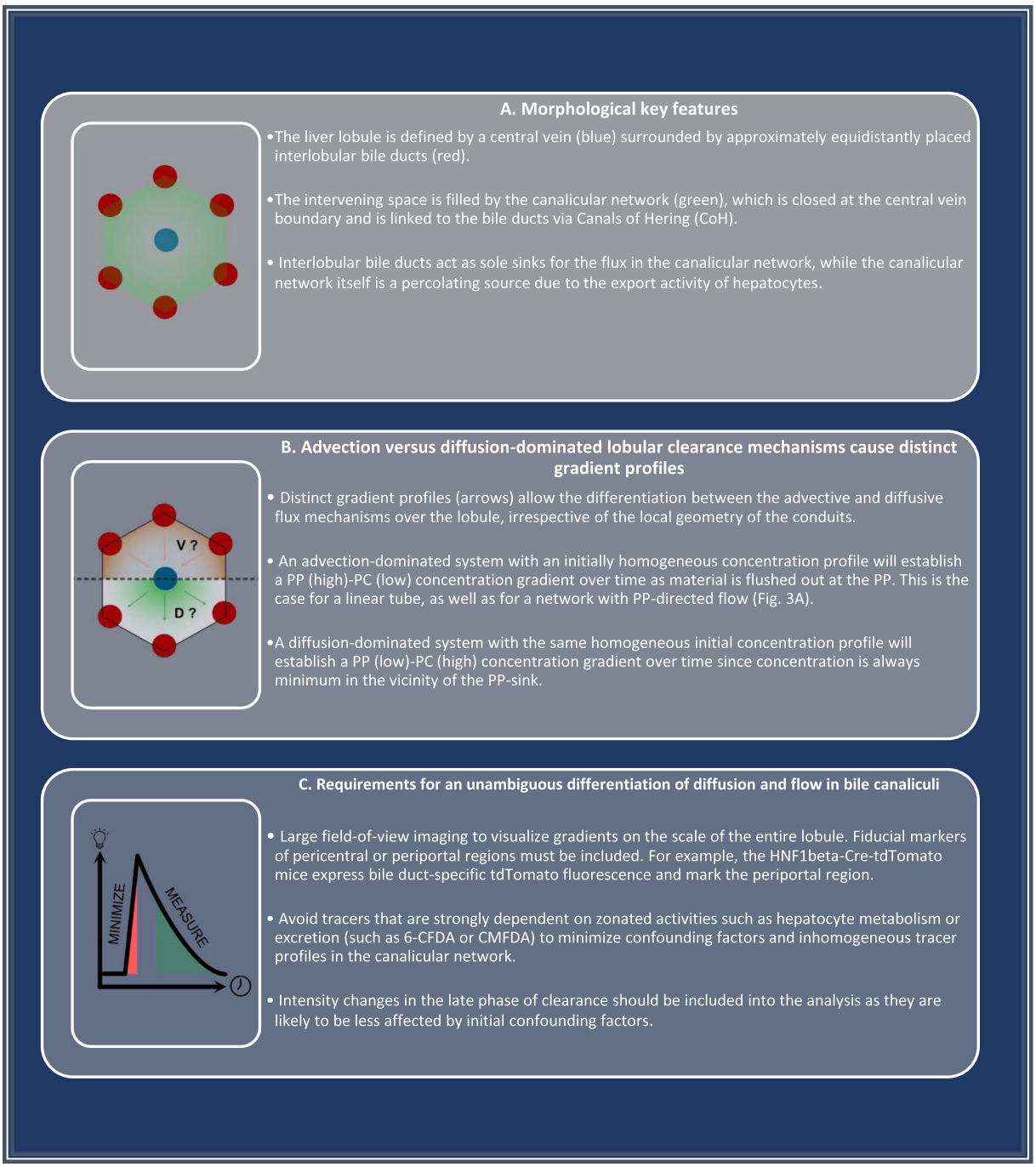
FIG. 2. (A) Schematic of an intravital imaging experiment with CMNB-fluorescein in a mouse, showing its uncaging to a fluorescent form (fluorescein) with 405-nm irradiation using a confocal scanning microscope. (B) Photo-activation of CMNB-fluorescence in a blood vessel showing the convective flux of fluorescent material due to blood flow and diffusion. (C) Schematic representation (top) of the expected distribution of fluorescent material in a section of the canalicular network through diffusion (omnidirectional) or through flow (preferentially in the direction of flow). Photo-activation of CMNB-fluorescence in a section of the canalicular network (bottom) empirically demonstrating the diffusive flux of fluorescent material due to blood flow and diffusion. (D) Photo-activation of CMNB-fluorescence in a section of the interlobular bile duct empirically demonstrating the convective flux of fluorescent material toward the southern extension of the duct.

Box 2 Differentiation of diffusive and flow mechanisms in the biliary tract



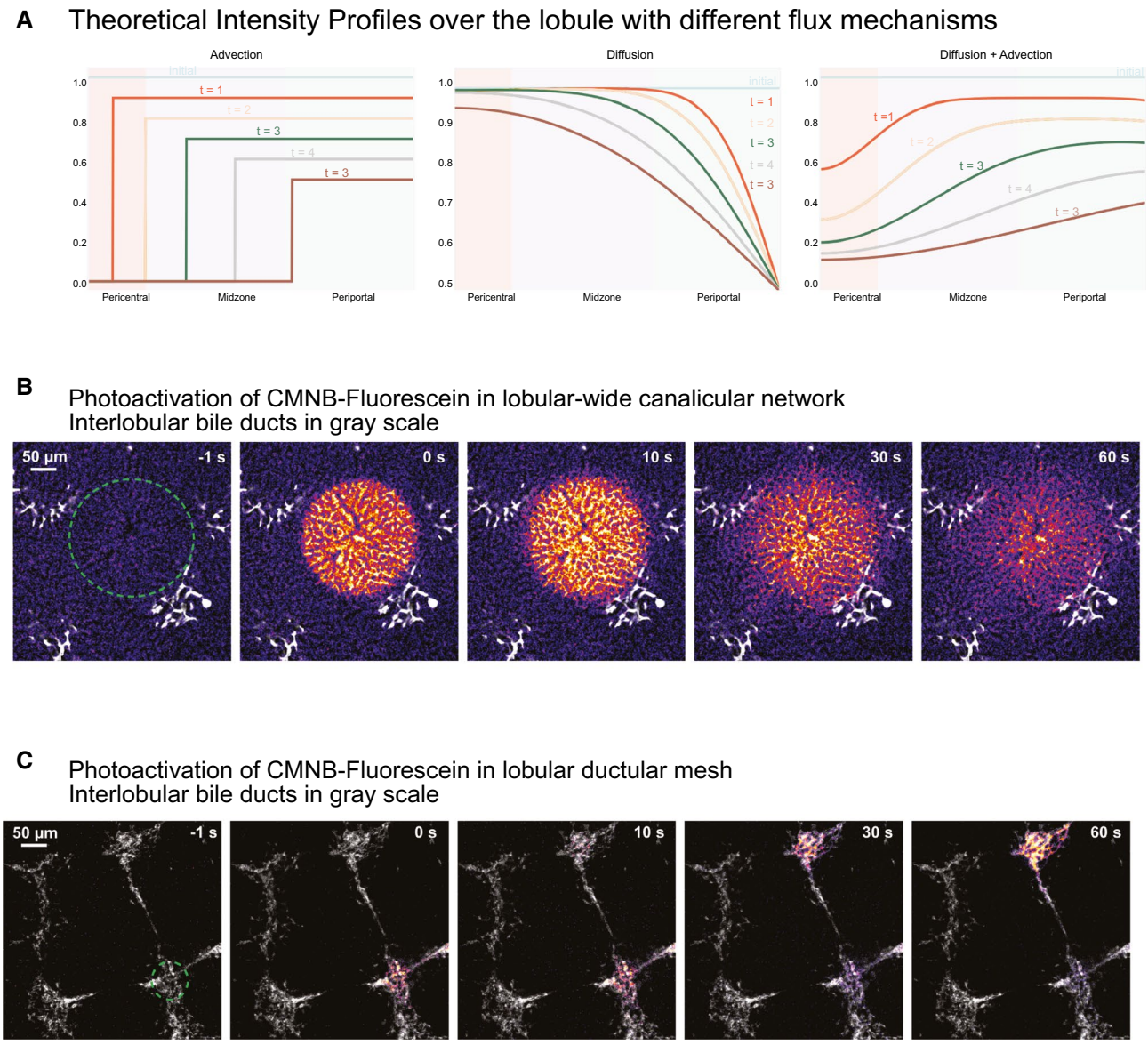


FIG. 3. (A) Schematic representation of fluorescence intensity profiles in a liver lobule due to the effect of water-influx driven pericentral-to-periportal advection (left), diffusion with a periportal sink (center), and their combination (right). Advection and convection generate a gradient with higher intensities at the periportal zone, whereas diffusion leads to gradients with higher intensity in the pericentral zone. (B) Lobule-wide photoactivation of CMNB-fluorescein showing a gradient with higher intensity in the pericentral zone of the canalicular network, implicating diffusion as the primary flux mechanism. (C) Activation of a section of the interlobular ductular plexus showing a directional movement toward one of the northern extensions of the plexus, implicating a convective flux mechanism.

For an unambiguous differentiation of diffusion and flow, specific experimental requirements are essential (Box 2C). Tracers with zonated metabolism or secretion may add confounders that complicate this differentiation. For example, if zonated secretion generates a **PC(high)**-PP gradient, both the diffusion and flow will result in a higher clearance in the PC than PP region, making it difficult to differentiate between both flux mechanisms. Photoactivatable tracers allow a better control of initial tracer intensity, thereby reducing the effect of these confounders.

While photoactivation provides an intuitive and easily visualized evidence of diffusion-dominated canalicular flux, orthogonal corroboration of the results

was achieved by intravital raster image correlation spectroscopy.[1] This inherently local technique does not use photoactivation, is not affected by lobule topology or zonation, and confirmed that no advection was detectable in the canalicular network, in contrast to interlobular ducts with a basal advection velocity of about 1 μm/s. Similar to the photoactivation experiments, administration of secretin or trichloroacetic acid (TCA) to the mice stimulated advection in ducts but not in canaliculi.

Alternative Conceptual Framework for Bile Flux

Diffusion-dominated canalicular bile flux has profound implications on our understanding of biliary excretion, and consequently, on the development of therapy for liver diseases. We are compelled to contend with a framework of bile flux in which bile constituents transit through two distinct functional zones: (1) the canalicular network, where bile constituents are concentrated in a stagnant water zone; and (2) the biliary ducts, where biliary fluid is modified and driven out of the liver through secretion of water. The challenge now is to explain the effects of various compounds on extrahepatic bile flow.

Constituents of bile secreted in the canalicular zone can act as signals to the ductular zone, thereby influencing biliary output (Fig. 4). Moreover, the ductular zone receives systemic signals from the blood as well. Physiological homeostasis of biliary output is therefore regulated through the convergence of these signals at the ductular zone.

The best characterized canalicular "signals" to ducts are BAs, whose signaling activity was confirmed by the discovery of thioredoxin glutathione reductase 5 (TGR5) on the luminal membrane of cholangiocytes. The discovery that BAs function as signaling molecules provides a further explanation on how BA is secreted by hepatocytes into canaliculi, which then reach the ducts by diffusion and influence bicarbonate and water secretion by cholangiocytes. BAs are agonists for TGR5, a G-protein coupled receptor with sub-micromolar binding affinity to BAs.[29] *In vitro* studies showed that TGR5 activation with BAs stimulated production of cAMP in ductular cells expressing this protein exogenously,[30] like the action of the hormone secretin, which is known to stimulate ductular flow. Corroborating these studies, TGR5-KO mice show a reduction in bile flow, whereas synthetic TGR5 agonists (INT-777) increased extrahepatic bile flow.[30] Increased bile flow after partial hepatectomy is conspicuously absent only in TGR5-KO mice, corroborated by increased biliary inorganic ion concentrations (Na^+, HCO_3^-, and Cl^-) after partial hepatectomy in wild-type, but not in TGR5-KO mice.[31]

The TGR5 protein is juxtaposed on the plasma membrane with the cystic fibrosis transmembrane conductance regulator (CFTR)—a chloride channel known to be activated by BA exposure. Genetic loss of function of the CFTR chloride channel leads to debilitating loss of water transport in several epithelial tissues, including the biliary epithelium.[31,32] Thus, BA-TGR5 agonism at the bile-facing side of cholangiocytes activates adenylate cyclase, causes elevated cAMP, and in turn activates CFTR to secrete Cl^- into the ductular lumen.[33] This extrusion of Cl^- is a precondition for Cl^-/HCO_3^- exchange.[34] Secreted bicarbonate creates an osmotic gradient for the movement of water across cholangiocyte apical membranes.[13,15] Through this pathway, the BA signal at the luminal side converges with that of the hormone secretin, which also leads to CFTR activation but from the blood-facing side of cholangiocytes.

Bile contains numerous other candidates that act as signals from the canalicular zone to the ductular zone. Intracellular calcium release is a common activator of ion channels and is stimulated by ATP and histamine, as well as BAs. ATP released from hepatocytes[35] activates the purinergic receptors P2X/P2Y on cholangiocytes, leading to intracellular Ca^{2+} release and subsequent chloride channel activation in the ducts.[36] Similarly, histamine released into the canaliculi can activate the histamine receptor H1 on cholangiocytes, leading to intracellular Ca^{2+} release through the inositol 1,4,5-triphosphate (IP3)/cyclic adenosine monophosphate response element-binding protein (CREB) pathway.[37] In general, Ca^{2+} signaling activates the anoctamin 1 Cl^- channel, leading to Cl^- efflux into the biliary lumen, thus contributing to the Cl^-/HCO_3^- osmotic gradient.[38] Systemic signals such as secretin, acetylcholine, and vasoactive intestinal peptide, which reach the basolateral membrane of cholangiocytes through the bloodstream, have been comprehensively reviewed.[8] Canalicular signals and systemic signals typically converge at the

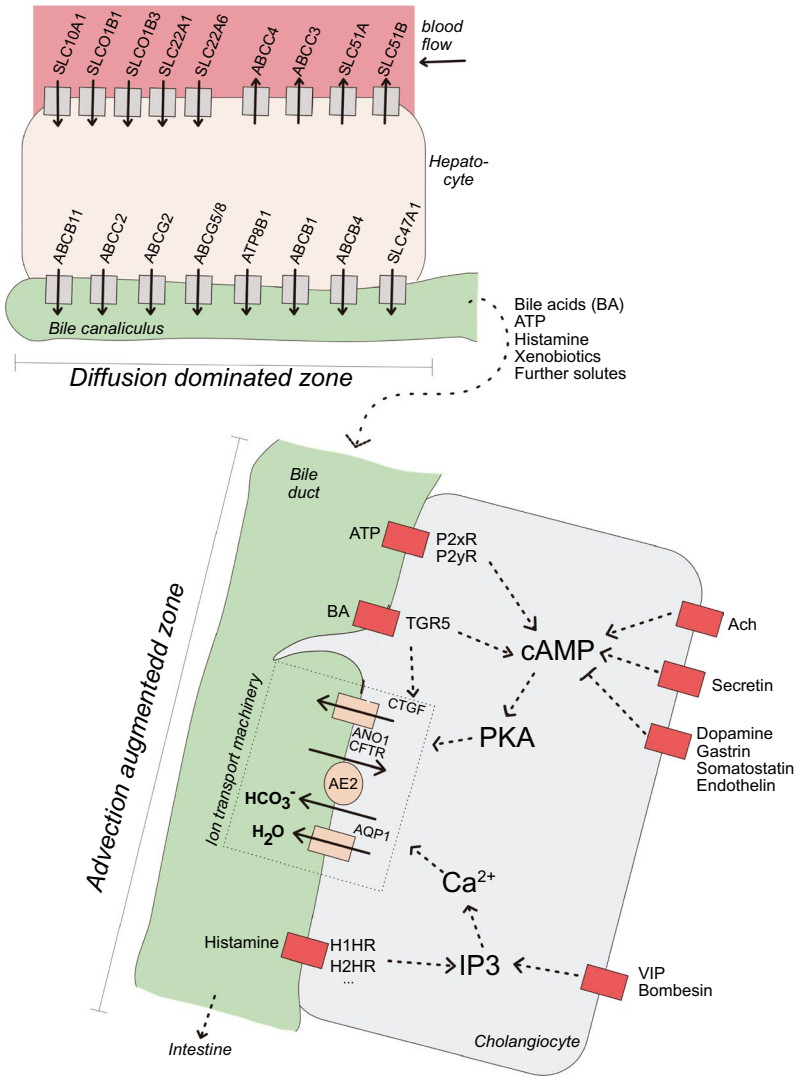


FIG. 4. Schematic representation of an updated framework for bile flux. Hepatocytes express several transport proteins to secrete small molecules to the blood and bile. The canalicular network is a diffusion-dominated zone where substances are excreted by hepatocytes through transport proteins to reach high concentrations in the canalicular lumen. Some of these substances such as BAs, ATP, or histamine diffuse to the bile duct and stimulate specific receptors on the cholangiocyte apical membrane (H1R, P2X, P2Y, and TGR5), which in turn trigger signaling through second messengers (cAMP, Ca^{+2}, IP3, and PKA). Similar stimulation can occur from blood-borne factors (e.g., secretin, vasopressin) through the cholangiocyte basolateral membrane. This intracellular signaling leads to ion-efflux into the ductular lumen, concomitantly driving osmotic water efflux, thus generating bile flow. Abbreviations: ANO1, anoctamin 1; CTGF, connective tissue growth factor; PKA, protein kinase A; and VIP, vasoactive intestinal peptide.

level of second messengers (cAMP, Ca^2+, and IP3) to activate ion channels and thus regulate water secretion by cholangiocytes. Finally, the hyperosmotic ductular fluid itself can signal through cell volume sensors,[39] which trigger intracellular Ca^{2+} signaling pathways.

These signaling mechanisms can therefore induce the formation of hyperosmotic fluid in the ductular lumen and drive water influx, regardless of whether canalicular bile is hyperosmolar. Through these signaling mechanisms, it is possible to link choleresis to biliary ion concentrations, Na^+ (~150 mM), Cl^- (~103 mM), and bicarbonate (~31 mM) in human bile that are actively generated by cholangiocytes. All known bile flow–inducing agents lead to a substantial increase in the rate of HCO_3^- and Cl^- ion secretion to bile following stimulation.[34,40] In a normal

physiological state, these inducers, including BAs themselves, are always present. Therefore, a basal water influx into ducts is always expected. BA-independent flow, therefore, merely reflects the contribution of inducers other than BAs on ductular bile flow.

In this integrated framework, in which cholangiocytes respond to bile and blood signaling factors to modulate extrahepatic bile flow (Fig. 4), all previous findings fall into a cohesive and rational concept (Box 3), without invoking a "canalicular bile flow." BA-dependent flow is now interpreted as the *excess* water influx into bile ducts induced by BA secretion. Ducts generate water flow to act as sinks for the high concentration of compounds in canalicular networks. The driving force for ductular water influx is the overall hyperosmotic solute concentration within the ducts. In a sense, this framework is a modification of the original osmotic theory—essentially identifying

Box 3 Interpreting observations in the conceptual framework of bile transport through the liver

Observation	Old interpretation	Contradiction	New interpretation
Bile acid-dependent flow	Osmotic water influx into the canalicular network due to bile acids	Absence of measurable advection in the canalicular network	Bile acid stimulated signaling induces *ductular* flow
Bile acid-independent flow	Osmolytes other than bile acids (GSH, HCO_3) cause water influx in canaliculi as well as ducts.	Absence of measurable advection in the canalicular network; absence of experimental evidence that GSH and other compounds draw water osmotically into canaliculi.	BAIF occurs at the level of ducts and not canaliculi. Modulation of ductular flow occurs through signaling from biliary and blood factors such as secretin, ATP, histamine, vasopressin, GSH etc. These factors stimulate ion excretion that in turn draws water into the ductular lumen.
Mannitol clearance	Traces canalicular bile "flow" since mannitol permeability is solely canalicular.	Permeability in ductular epithelium was demonstrated.	Traces total contribution of diffusive canalicular and advective ductular flow
Canalicular microperistalsis	Required for "pumping" of canalicular bile	Peristaltic rate is too slow (3 contractions / hour)	Represents standard apical membrane dynamics and vesicle trafficking
Bile plug formation in canaliculi when canalicular secretion is impaired	No explanation		Transport of bile acids in canaliculi is diffusion dominated and requires high concentrations of bile acid for efficient clearance, with larger lobules being more susceptible due to shallower diffusion gradients. Cholestasis results in enhanced cholesterol and phospholipid secretion, possibly contributing to the "solute load" in bile.

the anatomical site of water influx to be the flow-augmented ducts, whereas the diffusion-dominated canaliculi are concentration build-up zones.

Caveats

Evidence for this concept of bile flux originated primarily from genetic mouse models that lack one or more of the receptor/ion transport machinery, and microscopy for direct measurement of flux, as well as measurement of extrahepatic bile flow. Naturally, none of these experiments were performed in humans. Direct flux analysis in canaliculi and ducts has so far only been performed in mice, with the influence of only two choleretic compounds, TCA and secretin.[1] Similar experiments need to be done with the vast number of known non-BA choleretics, with an emphasis on the cAMP-activated situation, given the strong cAMP-dependent aquaporin insertion into the apical membrane of hepatocytes and cholangiocytes. Moreover, experiments should also be tailored to study high rates of solute secretion to investigate potential canalicular flow when pericentral, claudin-2-expressing hepatocytes, are also involved. In such experiments, particular attention should be given to the apical membrane localization of aquaporins under stimulated and basal conditions. Moreover, analysis of further mammalian species is required to study whether the principle observed in mice can be generalized.

Pathophysiological and Clinical Implications

Diseases that compromise cholangiocytes, such as primary biliary cirrhosis and primary sclerosing cholangitis, cause reduced bile flow at the larger bile ducts due to destruction/obliteration, stenosis, and/or blockage of the biliary system. The framework may have several pathophysiological and therapeutic implications, as the primary cell type responsible for excretion of water into the bile are cholangiocytes and not hepatocytes. Conversely, a near absence of water flow in the canalicular space will lead to higher concentrations of biliary solutes, making protective mechanisms against toxic bile salts, such as canalicular phospholipid secretion, even more important than previously anticipated.

Cholangiocytes have been shown to infiltrate into liver lobules, where bile canalicular networks were lost (e.g., due to targeting of the cytoskeletal protein radixin).[41] These proliferating cholangiocytes were shown to form new bile-transporting conduit systems. Thus, ductular reactions may represent an adaptive response to compensate for a cholestatic situation due to insufficient canalicular connectivity.[42] Against the background of the concept of bile flux (Fig. 4), this can be interpreted as an expansion of the flow-augmented zone to compensate for the compromised diffusion dominated zone. Importantly, this compensatory response, if functional, should not be antagonized therapeutically.

The concept of osmotic canalicular bile flow necessitates a fluid pressure gradient in the canalicular network. Because the canalicular network is disrupted in diseases such as NAFLD, the assumption of canalicular bile flow suggests that a pressure build-up may occur within the lobule, analogous to portal hypertension seen in cirrhotic liver diseases. Based on fluid dynamic calculations, the computed increased bile pressures in the disrupted canalicular networks have been proposed to represent a critical pathophysiological component of NAFLD.[3] However, the recently demonstrated absence of directed canalicular flow necessitates re-evaluation of this interpretation. Disruptions in the canalicular network may limit diffusion and eventually lead to micro-cholestasis correlated with disease progression, even in the absence of canalicular pressure build-up.

Bile flow may be restored by therapeutic BAs, such as ursodeoxycholic acid (UDCA), or nor-UDCA, which either act as agonists for TGR5[43] or potently stimulate ductal bicarbonate secretion.[44] However, therapeutic BAs must pass from the sinusoidal blood through hepatocytes to reach cholangiocytes. Transport into and through hepatocytes is known to be compromised in cholestatic conditions.[45] Hence, the development of potent agonists of cAMP/IP3-CREB signaling or synthetic analogues of hormones such as secretin that access the cholangiocyte from the basolateral side have the potential to be more effective choleretic agents. Inducing choleresis through blood-borne agents has the added advantage of allowing a more precise control of the concentration at the site of action at the basolateral membrane. On the other hand, stimulation of cholangiocytes from the luminal side offers the advantage that the signaling compounds

can be enriched in the stagnant canalicular water zone to reach much higher concentrations as would be possible in systemic blood.

Conclusions

The idea of osmotically driven bile canalicular flow has been challenged by the advent of techniques that allow the intravital quantification of advection and diffusion within the lumen of canaliculi and ducts. After abandoning osmotic canalicular flow, an integrated framework emerges, in which bile constituents transit a diffusion-dominated canalicular and a flow-augmented ductular domain. As biliary and blood-borne signals that modulate ductular water influx and in turn extrahepatic bile flow are discovered, this framework may provide a better understanding of normal liver function and point to strategies that restore bile flow or limit liver damage when canalicular diffusion or ductular flow is impaired.

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