Interaction network and regulation of the human ABC transporter MDR3/ABCB4

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„Das Lachen ist ein guter Brauch -
Beelzebub, der tut es auch.“

- Wilhelm Busch

Table of contents

Abstract

Bile formation is mainly performed by the three ABC transporters of the bile triumvirate at the canalicular membrane. These are namely ABCB4, ABCB11 and the heterodimer ABCG5/G8. Each of these display a narrow but specific substrate spectrum, which is quite uncommon for ABC transporters. While ABCB4 specifically enriches the outer leaflet with PC lipids, ABCB11 transports conjugated bile acids across the canalicular membrane and ABCG5/G8 secretes cholesterol into the bile canaliculi. Since bile acids exhibit a harsh detergent activity, they would harm hepatocytes and cholangiocytes. However, ABCB4 transports a high amount of PC lipids to enriched microdomains within the outer leaflet, while bile acids pull off patches of these extra lipids and form mixed micelles. Thereby, ABCB4 protects the cells from the harsh detergent activity of bile acids. Furthermore, cholesterol secreted by ABCG5/G8 is incorporated into the mixed micelles to prevent crystallization at higher concentrations. Obviously, the role of ABCB4 in providing sufficient PC lipids is essential not only for bile equilibrium but also human health. ABCB4 deficiency leads to symptoms such as reduction or complete absence of PC lipids in bile and these ABCB4-related diseases are of high clinical relevance. Hence, research studies on ABCB4 involving the understanding of the transporter are imperative. ABC transporters are often considered as single entities but in case of the bile triumvirate, an interplay between the transporters and their substrates is assumed. For ABCB11 and ABCG5/G8, it was demonstrated that lipid composition, cholesterol content and presence of bile acid affects these transporters. In contrast, effect of bile acids or cholesterol on ABCB4 have not be directly demonstrated so far due to the difficulty in measuring lipid lifting in a lipid environment and because of bile acids acting as lipid extractors. Both are individual steps with their own kinetic parameters.

However, a detergent-solubilized and purified ABCB4 presented a tool which could be exploited further to investigate the modulation of ATPase activity of ABCB4 by bile acids and cholesterol. Additionally, the effect of bile acids in the presence of a substrate (DOPC) of ABCB4 and cholesterol was examined. In the current thesis, four essential findings on ABCB4 were obtained: 1) monomeric bile acids modulate ABCB4 in a concentration dependent manner; 2) maximal modulation by bile acids corelates with their cmc; 3) bile acid modulation of ABCB4 changes but persists in the presence

of an actual substrate (DOPC), and 4) Nor-UDCA highly stimulates ABCB4 in the presence of DOPC (and cholesterol), revealing a new and medically-relevant mechanism of action. It also emphasized the importance to study Nor-*urso* versions of bile acids in further detail.

Zusammenfassung

Die Gallenbildung findet ausschließlich an der kanalikulären Membran der Leber-Hepatozyten durch die drei ABC Transporter des Gallen-Triumvirates statt. Diese sind die Transporter ABCB4, ABCB11 und der heterodimere ABCG5/G8. Jeder dieser Transporter besitzt ein kleines, jedoch sehr spezifisches Substratspektrum, was für ABC Transporter eher ungewöhnlich ist. Während ABCB4 spezifisch die äußere Lipidschicht der Membran mit PC-Lipiden anreichert, transportiert ABCB11 konjugierte Gallensäuren über die kanalikuläre Membran und ABCG5/G8 sekretiert Cholesterin in die Gallenkanalikuli. Da Gallensäuren eine starke Detergenzienwirkung aufweisen, würden diese die Membran der Hepatozyten und Cholangiozyten schädigen. Daher sorgt ABCB4 für einen starken Überfluss an PC-Lipiden in den angereicherten Mikrodomänen der äußeren Lipidschicht, sodass die Gallensäuren Stücke dieser überflüssigen Lipide extrahieren und gemischte Mizellen bilden können. Dementsprechend schützt ABCB4 die Zellen vor der Detergnzienwirkung der Gallensäuren. Außerdem werden das von ABCG5/G8 sekretierte Cholesterin in diese gemischten Mizellen eingebunden, um die Kristallisation des Cholesterins bei höheren Konzentrationen zu verhindern. Dementsprechend ist ABCB4 nicht nur für das Gleichgewicht der Gallenflüssigkeit, sondern auch die menschliche Gesundheit verantwortlich. Ein Ausfall von ABCB4 führt zu Symptomen wie Reduzierung oder Fehlen von PC-Lipiden in der Gallenflüssigkeit und deshalb sind ABCB4 verursachte Krankheiten von hoher klinischer Relevanz. Dementsprechend ist auch die nötige Forschung höchst relevant, um den Transporter besser zu verstehen. ABC Transporter werden oft einzeln betrachtet, aber im Falle des Gallentriumvirates wird ein Zusammenspiel der Transporter und jeweiligen Substrate vermutet. Für ABCB11 und ABCG5/G8 wurde bereits gezeigt, dass die Lipidkomposition, der Cholesterinanteil in der Membrane und Gallensäuren die Transporteraktivität beeinflussen. Im Gegensatz dazu, konnte bisher kein direkter Effekt von Gallensäuren oder Cholesterin auf ABCB4 nachgewiesen werden, da es besonders schwer ist, Lipid-Transport in einer Lipidumgebung zu messen, vor allem, wenn Gallensäuren diese zusätzlich extrahieren. Jedoch sind Transport und Extraktion von Lipiden zwei unterschiedliche Schritte mit ihren eignen kinetischen Parametern.

Allerdings bietet ein in Detergenzien solubilsiertes und gereinigtes ABC4 die Möglichkeit, die Modulation der ATPase-Aktivität von ABCB4 durch Gallensäuren und Cholesterin zu untersuchen. Zusätzlich konnte auch der Effekt der Gallensäuren in Anwesenheit eins eigentlichen Substrats von ABCB4 (DOPC) und Cholesterin bestimmt werden. In dieser Arbeit konnten vier neue Erkenntnisse für ABCB4 aufgezeigt werden: 1) monomere Gallensäuren modulieren ABCB4 in Abhängigkeit ihrer Konzentration; 2) die maximale Modulation durch Gallensäuren korreliert mit ihrer entsprechenden kritischen Mizellenkonzentration (cmc); 3) der Effekt der Gallensäuren auf ABCB4 bleibt zwar in Anwesenheit eines eigentlichen Substrates (DOPC) bestehen, verändert sich jedoch deutlich und 4) Nor-UDCA stimuliert ABCB4 äußerst stark in Anwesenheit von DOPC (und Cholesterin), was einen neuartigen und medizinisch relevanten Mechanismus dieser Gallensäure beschreibt. Dies unterstreicht die Wichtigkeit Nor-*urso*-Versionen von Gallensäuren in Zukunft zu studieren.

1 Introduction

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CHEMICAL REVIEWS

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Structure and Function of Hepatobiliary ATP Binding Cassette Transporters

Tim Kroll,[#] Martin Prescher,[#] Sander H. J. Smits, and Lutz Schmitt*

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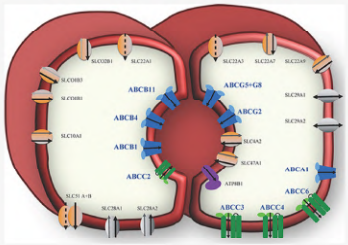
ABSTRACT: The liver is beyond any doubt the most important metabolic organ of the human body. This function requires an intensive crosstalk within liver cellular structures, but also with other organs. Membrane transport proteins are therefore of upmost importance as they represent the sensors and mediators that shuttle signals from outside to the inside of liver cells and/or vice versa. In this review, we summarize the known literature of liver transport proteins with a clear emphasis on functional and structural information on ATP binding cassette (ABC) transporters, which are expressed in the human liver. These primary active membrane transporters form one of the largest families of membrane proteins. In the liver, they play an essential role in for example bile formation or xenobiotic export. Our review provides a state of the art and comprehensive summary of the current knowledge of hepatobiliary ABC transporters. Clearly, our knowledge has improved with a breath-taking speed over the last few years and will expand further. Thus, this review will provide the status quo and will lay the foundation for new and exciting avenues in liver membrane transporter research.



CONTENTS

Special Issue: Transporters, Porins, and Efflux Pumps

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1. GENERAL INTRODUCTION

The biology and pathobiology of the liver are of central importance for human health as it is the major metabolizing and detoxifying organ of the human body. It is mainly composed of hepatocytes or parenchymal cells, which make up approximately 80% of the volume of the liver. Cholangiocytes, liver sinusoidal (LSEC), as well as macrovascular (MaVEC) endothelial cells, hepatic stellate cells (HSC), and Kupffer cells (KC), and immune cells form the remaining 20%.[1]

The liver represents a central metabolic hub for the regulation of glucose and lipid metabolism as well as protein synthesis. This metabolic function is performed specifically by hepatocytes, which are also responsible for the *de novo* synthesis of bile acids from cholesterol and their enterohepatic circulation, while metabolizing enzymes are mainly concentrated in the liver and intestine.[2]

Because of its physiological importance, hepatotoxicity or drug induced liver injury (DILI) is likely the most important factor in failure of drug development.[3] On top of this, transporters mainly of the ATP binding cassette (ABC) transporter[4] and solute carrier (SLC)[5] families play pivotal roles in absorption, distribution, targeting, or elimination. This obviously influences drug pharmacokinetics and -dynamics. For example, the liver mediates mainly detoxification reactions mediated by phase one (oxidation, reduction, hydrolyzation) and phase two biotransformations (conjugation with different chemical groups for higher solubility such as glutathione conjugation), and the final products of these reactions are often found as glucuronidated or sulfated conjugates, which are the substrates of efflux processes (phase three) mediated by ABC transporters of the C subfamily[6] (see section The ABCC Subfamily of the Liver - Drug and Toxin Efflux). This highlights how these processes interplay within the liver. Therefore, it should not come as a surprise that the international transport consortium (ITC)[5] has included hepatobiliary membrane transport proteins in drug screening studies. The ITC includes members of academia, industry, and the Food and Drug Administration (FDA). These transporters are of emerging clinic importance with respect to drug uptake and/or clearance. In 2013,[5] MATE (multidrug and toxin extrusion, SLC47A), the ABCC subfamily, more precisely ABCC2, ABCC3, ABCC4, and ABCB11 (bile acid export pump, BSEP), were added to the already existing list that was composed of the ABC transporters ABCB1 (or multidrug resistance protein 1 (MDR1)), ABCG2 (or breast cancer resistance protein, BCRP), and the SLCs (the organic anion transporter peptides OATP1B1 and OATP1B3, SLCO1B1, and SLCO1B3, respectively), OCT2 (organic cation transporter 2, SLC22A2), as well as OAT1 and OAT3 (organic anion transporter, SLC22A6 and SLC22A7, respectively). Furthermore, it is not only about the kinetics of drug−drug interactions that might be modulated by these transporters, it is also about the dynamics of these interactions, i.e., the interplay between drug concentration and biological effect. This emphasizes the importance of hepatobiliary membrane transporters in the physiology and pathophysiology of the human liver. Here, we will focus only on structural and functional aspects of hepatobiliary ABC transporters and try to summarize our current knowledge of the mode of action and how these primary active transporters coexist and function in the membrane.

1.1. Transport across the Plasma Membrane of the Liver

Over the last 25 years, cloning, transfecting cell lines, and functional characterization of hepatobiliary transporters has moved the field from a model of passive diffusion (import as well as export) of all liver relevant compounds across the plasma membrane to a rather detailed knowledge about the molecular players, the substrate spectrum, and mechanistic aspects of how liver cells communicate with their environment, a process mediated by active transport proteins. The importance of the liver is also reflected by the number of these membrane transport systems that are present in both the sinusoidal (basolateral) and the canalicular (apical) membranes of hepatocytes (Table 1 and Figure 1). Membrane import systems are enriched in the sinusoidal membrane, while the major drug efflux pumps and the transporters exporting the components of bile are localized in the canalicular membrane. Another way to classify these transport systems are the families of membrane transport proteins, to which they belong. These are either ABC transporters or SLCs and one member of the P-type ATPase family, ATP8B1.

Members of the first family are enriched in the canalicular membrane, while SLCs are predominately found in the basolateral membrane (Figure 1). Since hepatobiliary ABC transporters are the focus of this review, a detailed discussion of these transport systems will be given in the section An ABC of the Plasma Membrane of the Human Liver. The family of human SLCs covers more than 65 subfamilies with more than 450 different transporters. These include passive transporters (uniporter), ion-coupled transporters (symporters), and exchangers (antiporters). The liver is not only essential for metabolism, detoxification, and *de novo* bile acid synthesis, but also important with respect to enterohepatic circulation.[8] Here, bile acids are imported, recycled, and reused from the bloodstream into the hepatocyte predominantly by NTCP (sodium taurocholate cotransporting polypeptide, SLC10A1), a 56 kDa cotransporter, cloned in 1991,[9] which imports taurocholate (TC) at a stoichiometry of one TC: two Na^+. Nowadays, we know that not only TC, but all bile acids, steroid, and thyroid hormones as well as to a certain extend xenobiotics are substrates of NTCP.[10,11] NTCP also represents the entry point for the hepatitis B and D viruses[12] making it an interesting target for drug development. Subsequently, three OATPs (OATP1B1, OATP1B3, and OATP2B1) were cloned and functionally characterized.[13−17] Although they are also capable of functioning as bile acid import proteins, their substrate spectra are much broader and include a large variety of organic anions.[18] Important, however, is that all these compounds are transported together with bicarbonate.[19]

Pharmacokinetics, i.e., the relationship between drug concentration and resident time in the body, are strongly coupled to the capability of the liver to extracts drugs. This is mainly accomplished by SLCs of the OATP (SLCO, former SLC21), OAT (SLC22), and OCT (SLC22) subfamilies. OCTs are electrogenic uniporters that transport organic

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Table 1. Summary of Hepatobiliary Membrane Transport Proteins in Plasma Membrane of Humans, for Which the Presence on the Protein Level Was Verified According Referenced Papers or if Written in Italics to Human Protein Atlas (http://www.proteinatlas.org) or the UniProt Database (https://www.uniprot.org)[a]

member	substrate(s)	cell type	subcellular localization	function	PDB entry	disease	references
				ABC transporters			
ABCA1	phospholipids and cholesterol	hepatocytes	basolateral membrane	transport of phospholipids and excess of free cholesterol and HDL formation	5XJY	Tangier disease	92, 118
ABCB1	drugs	hepatocytes	canalicular (apical) membrane	decrease of intracellular drug levels	4M1M (Apo), 6QEE (inhibitor bound), 6C0V (ATP bound), 4Q9H, 5KO2, 5KPD (EQ mutant)	overexpression tumors, Alzheimer's disease	220, 234, 235, 520−523
ABCB4	phosphatidylcholine lipids	hepatocytes	canalicular (apical) membrane	protection of the biliary tree	6S7P (ATP bound)	mild chronical cholesasis, ICP, DILI, LPAC, PFIC-3	149, 240, 265, 270, 524−529
ABCB11	bile salts	hepatocytes	canalicular (apical) membrane	secretion of conjugated bile salts into the bile	6LR0	DILI, ICP, BRIC-2, PFIC-2, BSEP V444A linked to PBC, chronic hepatitis C, cholangiocarcinoma and sepsis-associated cholestasis	271, 328, 436−438, 451, 452, 452, 494, 525, 530
ABCC2	glucuronidated metabolites	hepatocytes	canalicular (apical) membrane	metabolite and toxin export		Dubin and Johnson disease, DILI	124, 172, 189
ABCC3	drugs and conjugated metabolites	hepatocytes	basolateral membrane	back-up system in liver cholestasis, export of metabolites and toxins		overexpression tumors	113, 193
ABCC4	drugs and conjugated metabolites	hepatocytes	basolateral membrane	back-up system in liver cholestasis, export of metabolites and toxins		overexpression in tumors	198, 199, 202
ABCC6	ATP	hepatocytes	basolateral membrane	unknown		*pseudixanthoma elasticum* (PXE)	7, 128, 130
ABCC7[b]	chloride	cholangiocytes	apical membrane	electrolyte (Cl^-) transport in epithelial cells	5UAK (ATP-free), 6MSM (ATP-bound)	cystic fibrosis	86, 87, 90
ABCG2	drugs, cholesterol, urate, porphyrins and sulfated metabolites	hepatocytes	canalicular (apical) membrane	decrease of intracellular drug levels, urate excretion	6VXI (bound tomitoxantrone), 6VXJ (bound to SN38), 6VXH (bound to imatinib), 6HIJ (bound to cholesterol), 6HZM (bound to ATP Mg^{2+})	gout	364−368, 379,531, 532
ABCG5/G8	cholesterol, plant sterolins	hepatocytes	mainly canalicular membrane of	transport of cholesterol and plant sterols like sitosterol	5DO7	sitosterolemia, hypercholesterolemia, gallstone disease	389, 407, 498, 500, 509, 533−535
				P-type ATPases			
ATP8B1	phosphatidyl-serine lipids	hepatocytes and cholangiocytes	canalicular (apical) membrane	flip of PS lipids		PFIC1 (Byler's disease)	35, 36
				Solute Carrier Transporters (SLCs)			
SLC4A2 (AE2)	chloride vs bicarbonate	hepatocytes/*bile duct cells*	*plasma membrane*	secretion of bicarbonate in exchange for chloride ions			536
SLC22A7 (OAT2)	organic anions for unknown exchange	hepatocytes	basolateral membrane	import of organic anions in exchange for unknown ions			22, 537, 538
SLC22A9 (OAT7)	organic anions in exchange for short chain fatty acids	hepatocytes	basolateral membrane	organic anions in exchange for short chain fatty acids			20, 22

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Chem. Rev. 2021, 121, 5240−5288

Table 1. continued

member	substrate(s)	cell type	subcellular localization	function	PDB entry	disease	references
				Solute Carrier Transporters (SLCs)			
SLC47A1 (MATE1)	tetraethylammonium (TEA), 1-methyl-4-phenylpyridinium (MPP), cimetidine, metformin, guanidine, procainamide, cephalexin, cephradine	hepatocytes	*cell membrane*	The stated substrates are transported in exchange for protons.			539−541
SLC22A1 (OCT1)	organic cations	hepatocytes	basolateral membrane				22, 542
SLC22A3 (OCT3)	organic cations	hepatocytes, *bile duct cells*	*plasma membrane*				22
SLC51A (OSTα)	bile acids	hepatocytes	basolateral membrane, *ER membrane*			progressive cholestasis	24
SLC51B (OSTβ)	conjugated steroids	hepatocytes	basolateral membrane,*ER membrane*			progressive cholestasis	24
SLC10A1 (NTCP)	sodium and bile acids	hepatocytes	*basolateral membrane*	symport of sodium and bile acids; mainly for uptake into hepatocytes		hypercholanemia	543, 544
SLC10A2 (ASBT)	sodium dependent bile acid transport	(large) cholangiocytes	apical membrane	uptake of bile acids		Crohn's disease	545, 546
SLCO1B1 (OATP1B1)	bicarbonate, bile acids and organic anions	hepatocytes	basolateral membrane	Antiporter: import of bile acids and organic anions; export of bicarbonate		Involved in Rotor syndrome	547
SLCO1B3 (OATP1B3)	bicarbonate, bile acids and organic anions	hepatocytes	basolateral membrane	Antiporter: import of bile acids and organic anions; export of bicarbonate		Involved in Rotor syndrome	547
SLCO2B1 (OATP2B1)	bicarbonate, bile acids, organic anions and cations	hepatocytes	basolateral membrane	Antiporter: import of bile acids, organic anions and cations; export of bicarbonate		Involved in Rotor syndrome	547
SLC28A1 (CNT1)	concentrative 1:1 sodium/nucleoside	hepatocytes	apical and basolateral membrane	sodium and nucleoside import			548, 549
SLC28A2 (CNT2)	concentrative 1:1 sodium/nucleoside	hepatocytes	apical and basolateral membrane	sodium and nucleoside import			548, 550
SLC29A1 (ENT1)	nucleosides and nucleobases	hepatocytes	apical and basolateral membrane	facilitated transport, maintain equilibrium of nucleosides and nucleobases			548
SLC29A2 (ENT2)	nucleosides and nucleobases	hepatocytes	apical and basolateral membrane	facilitated transport, maintain equilibrium of nucleosides and nucleobases			548, 550

[a]SLC: Solute carrier transporters, OATP: organic anion transport peptides, OAT: organic anion transporters, OCT: organic cation transporters, NTCP: sodium taurocholate co-transporting polypeptide, MATE: multidrug and toxic compound extrusion. [b]ABCC7 or CFTR is included even though it is not an ABC transporter. ABCC1 is expressed in fetal liver, and expression is typically absent in adult liver, but often expressed again in hepatocellular carcinomas; therefore, ABCC1 was not included in the table. Please note that we have not included three dimensional structures of homologous proteins. Even though P4-type ATPases were found in liver tissue of the mouse,[519] we only included ATP8B1 due to its characterized role in the human canalicular membrane.

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Chem. Rev. 2021, 121, 5240−5288

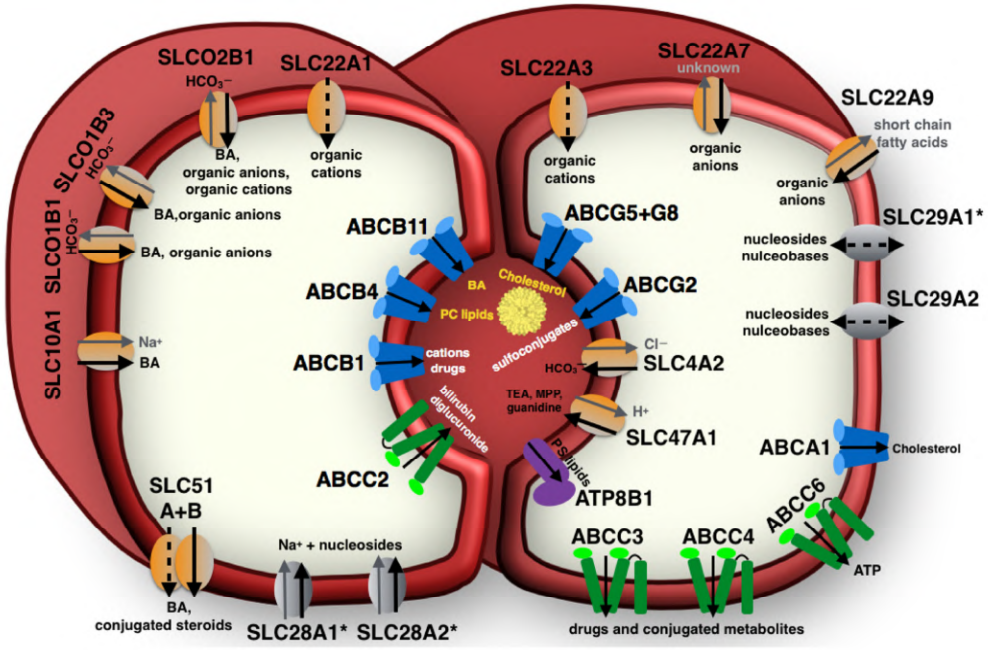

Figure 1. Scheme of two hepatocytes forming a bile canaliculi. All transporters, which are localized in the plasma membrane of human hepatocytes, are highlighted. ABC transporter with exception of the ABCC subfamily (colored in green) are illustrated in blue. ABCB4, ABCB11, and ABCG5/G8 translocate compounds, which form a mixed micelle (light yellow) in the bile canaliculus. ABCC transporters mostly secrete drugs and conjugated compounds such as bilirubin diglucuronide in the case of ABCC2. The exception is ABCC6, which most likely is an exporter for ATP.[7] The P-type ATPase ATP8B1 is shown in magenta. This transporter secretes phosphatidylserine (PS) lipids. The members of the solute carrier (SLC) superfamily are illustrated in orange and gray for the nucleoside transporters (SLC28 and SLC29), since those integrated in both the canalicular and basolateral membrane. Further SLCs can be divided into exchangers (SLC4A2, SLC22A7 SLC22A9, SLC47A1, SLCO1B1, SLCO1B3, SLCO2B1) indicated by a black and a gray arrow in opposite directions, facilitators (SLC22A1, SLC22A3, SLC29A1, SLC29A2, SCLS1A+B) indicated by a dashed arrow and cotransporters (SLC10A1, SLC28A1, SLC28A2,) with black and gray arrows in the transport direction. For further information, see also Table 1. *SLC transporter marked in this are present in both membranes (canalicular and basolateral), but due to space limitations only shown in the basolateral membrane here.

cations along their concentration gradient, a so-called facilitated diffusion. OATs, on the other hand, transport organic anions in a sodium ion independent manner. However, to reach the intracellular concentrations, active transporters are required. So far, sulfo-, but not glucuronide- or glutathione-conjugated anions have been identified as substrates of OAT7,[20] while for example prostaglandins or nonsteroidal drugs as well as creatine and cGMP are substrates of OAT2.[21] OCT1 and OCT3 are sodium ion independent, electrogenic facilitators (uniporters) that transport a large variety of structurally unrelated organic cations.[22,23] Finally, the hetero-dimeric organic solute transporter α–β (OST α/β, SLC51) catalyzes facilitated diffusion of bile acids and conjugated steroids across the apical membrane of hepatocytes. This process strictly depends on the gradient of these molecules.[24] Heterodimerization of SLC51 is mainly required for proper trafficking and stability. Interestingly, data bank searches identified so far no paralogues in other organisms. The three ABCC transporters, ABCC3, ABCC4, and ABCC6, of the basolateral membrane will be discussed in more detail in sections ABCC3 (MRP3) — The Back-up System for ABCC2 in Liver Cholestasis, ABCC4 (MRP4) — The Little Brother of ABCC1 in the Liver, and ABCC6 — The ABC Transporter that Transports ATP, respectively.

Moving to the canalicular (apical) membrane of hepatocytes, two additional members of the SLC family are present, AE2 (anion exchanger isoform 2, SLC4A2) and MATE1 (SLC47A1). Isoform 1 of AE is better known as the band 3 protein found in erythrocytes. AE1 and AE2 both exchange chloride ions against bicarbonate ions. In cholangiocytes, AE2 participates in the so-called "biliary bicarbonate umbrella", a mechanism that protects these cells against damage by bile acids.[25] MATE1 like the bacterial prototype NorM[26,27] is a drug/H^+ antiporter that extrudes drugs and protects in concert with ABCB1 and ABCG2 (see sections The Multidrug Efflux Pump ABCB1 and ABCG2 - Brother in Arms of ABCB1) the canalicular membrane against a myriad of structurally unrelated xenobiotics. The last, non-ABC transmembrane protein of the canalicular membrane is ATP8B1 or FIC1. It belongs to the subfamily 4 of P-type ATPase[28,29] and flips lipids of the phosphatidylserine (PS) family from the outer to the inner leaflet of the membrane.[30,31] In *S. cerevisiae*, the functional homologue is Drs2 and Drs2-related ATPases,[32,33] well-studied lipid flippases.[34] Similar to Drs2, ATP8B1 plays a pivotal role in the initiation and maintenance of the transversal lipid asymmetry of the plasma membrane and mutations in ATP8B1 lead to PFIC1 (progressive familial intrahepatic cholestasis type I).[35,36] Likely, dysfunction of ATP8B1 results in a loss of membrane asymmetry and thereby loss of protection against the detergent action of bile acids.[37]

Cloning and functional analysis of hepatobiliary transporters are and were extremely important and represent powerful tools

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Chem. Rev. 2021, 121, 5240−5288

Table 2. Conserved Sequence Motifs of ABC Transporters[a]

motif	consensus sequence	canonical	degenerated
Walker A	GXXGXGKST	ABCA1, ABCB1, ABCB4, ABCB11, ABCC2, ABCG5	NBD1 of ABCG8
C-loop	LSGGQ/R	ABCA1, ABCB1, ABCB4, ABCB11, ABCC2, ABCG8	NBD2 of ABCB11
			NBD2 of ABCC1
			NBD2 of ABCC2
			NBD2 of ABCC3
			NBD2 of ABCC4
			NBD2 of ABCC5
			NBD2 of ABCC6
			NBD2 of ABCC7
			NBD2 of ABCG5
Walker B	ΘΘΘΘDE	ABCA1, ABCB1, ABCB4, ABCC2, ABCG5, ABCG8	NBD1 of ABCB11
			NBD1 of ABCC1
			NBD1 of ABCC2
			NBD1 of ABCC3
			NBD1 of ABCC4
			NBD1 of ABCC5
			NBD1 of ABCC6
			NBD1 of ABCC7
D-loop	SALD	ABCA1, ABCB1, ABCB4, ABCB11, ABCC2, ABCG5, ABCG8	
H-loop	XHX	ABCA1, ABCB1, ABCB4, ABCB11 ABCC2,ABCG5, ABCG8	

[a]A definition of canonical/degenerated is provided in the section A General Introduction to ABC Transporters. X: any amino acid, Θ: any hydrophobic amino acid. One has to stress that already a mutation in one of the conserved sequence motifs renders a transporter degenerated. For simplicity, the motif that is corrupted is indicated. If the transporter contains all catalytically relevant amino acids, it is listed at the bottom line of row "canonical".

that increased our molecular knowledge about these systems exponentially. In the case of functional studies, transporters were either heterologously overexpressed or cell lines, which were transiently or stably infected with the respective transporter, were used in combination with various biochemical or biophysical approaches. However, these approaches assume that the transporter acts as an isolated entity. Only recently, these studies were expanded, and cell lines expressing two or more transporters at the same time were introduced.[38−49] This strategy arose in many unforeseen results that clearly demonstrated that hepatobiliary transporters are everything but not isolated entities. Rather an intense and functional important cross talk and interactions between transporters of the same (basolateral or canalicular) or cross talk between transporters of both membranes exist.[38,39,45,50−52] We believe that we have only seen the tip of the iceberg so far and will discuss these systems more in detail in section An ABC of the Plasma Membrane of the Human Liver as many times, these double or triple infected cell lines involved ABC transporters.

1.2. A General Introduction to ABC Transporters in Humans

In 1986, a seminal paper described the relation between bacterial nutrient uptake systems, nodulation systems, cell division proteins and hemolysin B from *Escherichia coli*.[53] This paper and modeling studies[54] coined the term ATP binding cassette (ABC) transporter. These primary active membrane transporters use the energy of binding and hydrolysis of ATP to translocate their substrates across biological membranes against a concentration gradient and are found in all kingdoms of life.

A functional ABC transporter is composed of two membrane spanning units, the transmembrane domains (TMD) and two copies of the power plant, the nucleotide binding domains (NBD), which harbor all conserved sequence motifs that give rise to this family of membrane transport proteins. In contrast to prokaryotes and archaea, in which the four modules are often encoded on separated genes, only fusion of one NBD or TMD (so-called half-size transporter) or a fusion of all four modules (so-called full-size transporter) are present in eukaryotes. Another important difference between ABC transporters of bacterial and archaeal origin and eukaryotic ABC transporters is the directionality of the transporter. With the exception of human ABCA4[55] and plant transporters,[56−59] only exporters have been described in the eukaryotic phyla. Since this review focuses on hepatobiliary ABC transporters, we will not discuss any ABC system that performs an import function.

As stated above, the NBD contains the conserved sequence motifs of ABC transporters. These include the Walker A and Walker B motifs[60] as found in all P-loop NTPases[61] and the hallmarks of ABC transporters, the signature motif and the D-loop. Additionally, two conserved amino acids are located in the so-called Q- and H-loops.[62,63] The consensus sequences of these motifs are summarized in Table 2. The Walker A motif, also called the P- or Gly-rich loop, coordinates the phosphate moiety of the bound nucleotide. This loop is located between a β-strand and an α-helix and is part of the mononucleotide fold.[64] The Walker B motif is formed by four hydrophobic residues forming one strand of the central β-strand of the catalytic subdomain of the NBD, which adopts a fold most similar to RecA[65] or the α subunit of the F_1 ATPase.[66] The β-strand is followed by an Asp and a Glu residue. The Asp residue coordinates the cofactor Mg^{2+} that is indispensable for hydrolytic activity of the NBD, while the following Glu residue forms the catalytic base[67] in the majority of systems.[68−70] This residue polarizes the attacking water molecule that results in nucleophilic hydrolysis of the anhydride bond of the $\beta-\gamma$ phosphate moiety. However, it has to be stressed that the histidine of the H-loop has been shown to play an identical

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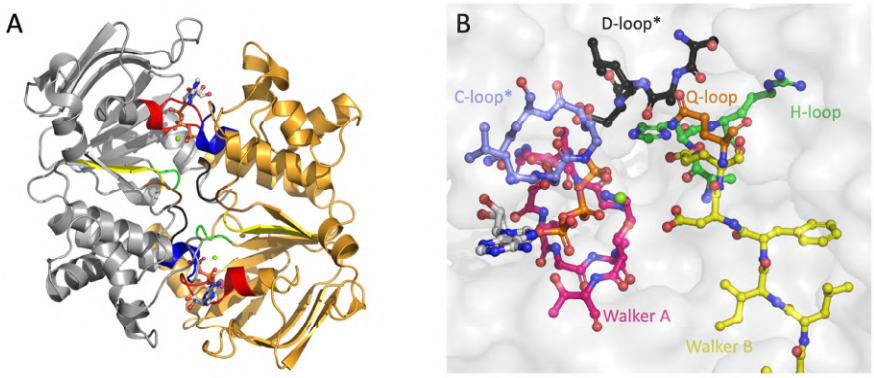

Figure 2. Structural overview of the NBD of ABC transporters. (A) Structure of the ATP-bound dimer of the isolated NBD of the *E. coli* hemolysin B ABC transporter (PDB entry 1XEF).[71] One NBD is shown in gray, and the other one is in orange cartoons. Color coding of the conserved sequence motifs is as follows: Walker A (red), Q-loop (brown), C-loop (blue), Walker B (yellow), D-loop (black), and H-loop (green). The bound ATP molecules are shown in ball-and-stick representation and the cofactor Mg^{2+} as green spheres. (B) Zoom-into into one NBS of the composite dimer. The NBD is shown as a light gray transparent solid. Color coding is as in (A). Water molecule within the NBS have been omitted for clarity. Please note that this is a model of the wild type NBD based on the crystal structure, in which the histidine residue (His 662) was mutated to Ala. * denotes conserved sequence motifs from the opposing NBD in the composite dimer. The interested reader is referred to ref 71 for a more detailed and schematic summary of the interactions of the NBD and bound ATP.

role in for example the isolated NBD of the *E. coli* hemolysin B transporter and is proposed to act as the linchpin of ATP hydrolysis.[71] Here, the His and the Glu residues form a catalytic dyad, in which the His residue polarizes the attacking water molecule, while the Glu residue fixes the side chain of the His residue in a productive conformation.[72] Finally, the conserved Gln residue of the Q-loop connects the catalytic and the helical subdomain of the NBD and interacts with bound ATP.[68,70,73] The hallmarks of ABC transporters, the C- and D-loop are part of the so-called nucleotide binding site (NBS). In the presence of ATP, the NBDs form a head-to-tail dimer with two ATP binding sites. One NBS is formed by the Walker A and B motifs as well as the Q- and H-loop. The binding site is complemented by the C- and D-loop of the opposing dimer[68,70,71,73] (Figure 2). Thus, ATP acts as a "molecular glue"[70] that stabilizes the dimer. Here, the conserved Ser residue of the C-loop interacts specifically with the γ-phosphate of ATP, while the D-loop interacts with residues of the Walker A motif. This inter-NBD interaction within the two NBDs is furthermore strengthened by interactions of the C-terminal helices of the two NBDs.[68,70,73] This architecture therefore explains why preferably ATP binding induces formation of the NBD dimer and is proposed to act as the power stroke[74] of the catalytic cycle. In parallel, a processive clamp model[75] was proposed, which was subsequently refined by suggesting the constant contact model.[76]

ABC transporters are defined by four conserved sequence motifs and two conserved amino acids (Table 2). If an NBD harbors these catalytic essential amino acids, it is termed "canonical". If, however, one or multiple exchanges of conserved amino acids occur, the NBD is called "degenerated". Here, one has to stress that the degree of degeneration, i.e., the number of amino acids exchanged, is highly variable. However, very often a substitution of at least the Glu residue of the Walker B, the catalytic base, is observed. While degeneration of the NBD in bacteria and archaea is rather uncommon with LmrCD,[77] TmrAB,[78] Tm287/288,[79] or PatAB[80] being some of the few known exceptions, in eukaryotic organisms it is rather common. This is for example the case for entire ABCC subfamily of the hepatobiliary ABC transporters (Table 2). In these cases, the C-loop of NBD2 is corrupted. Because of the composite architecture of the NBS, these two exchanges will create an ATP binding site that might bind ATP, but is incapable of hydrolyzing the nucleotide on the biological time scale of a transport cycle. Thus, the entire ABCC subfamily can only hydrolyze ATP at one NBS. If one assumes that hydrolysis and substrate transport are strictly coupled in these transporters, the molecular mechanism will be different between canonical transporters that use two ATP per transport cycle compared to degenerated transporters.[81] For example, the drug efflux pump Pdr5 from baker's yeast, a full-size transporter and member of the G subfamily, contains one completely corrupted NBS; i.e., all catalytically relevant amino acids are exchanged and do not contribute to hydrolytic activity. More importantly, this transporter is uncoupled meaning that ATP hydrolysis and substrate transport are not linked on the molecular level.[82,83]

In humans, 48 ABC transporters have been identified that were grouped in seven subfamilies (ABCA to G) based on the phylogeny of the NBD.[84] As an exception from the "transporter rule", the ABCC subfamily contains ABCC7 or CFTR (cystic fibrosis conductance regulator), which is a chloride channel, and mutants of CFTR are the cause for the most inherited disease among Caucasians, cystic fibrosis.[85] While the structure of this important ABC channel is known by now[86−89] and ABCC7 is present in liver,[90] the second two nontransporter genes ABCC8/9 or SUR1/SUR2 (sulfonylurea receptor) are not expressed in the human liver. ABCC8 is a subunit of the beta cell ATP-sensitive potassium channel and acts as a regulator of these K^+ channels during insulin release, while ABCC9 performs the same function in nonpancreatic cells.[91]

Looking at the transporters, the ABCA subfamily covers 13 members representing the largest transporters with respect to molecular weight among all human ABC transporters. The full-size transporter ABCA1, for example, whose structure has been determined by single particle electron cryomicroscopy (cryo EM),[92] is composed of 2261 amino acids, while ABCB1 the

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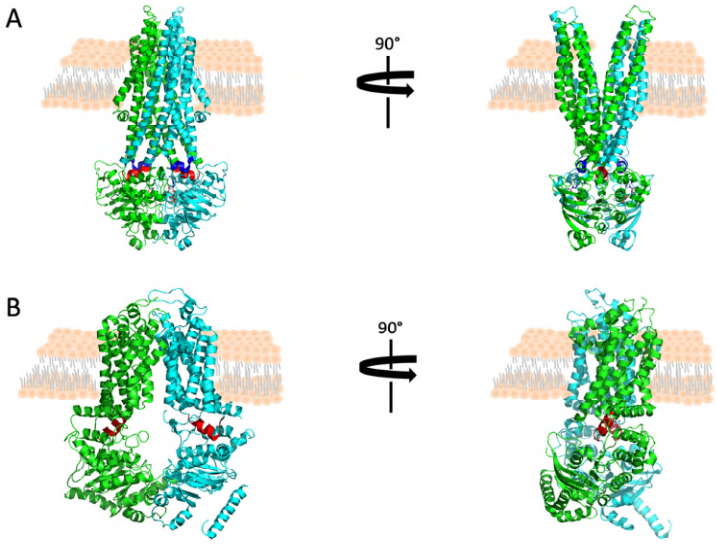

Figure 3. Crystal structures of Sav1866 and human ABCG5/G8 as blueprints for type I and type II exporters. (A) Crystal structure of Sav1866.[108] Monomers are shown in green and cyan cartoons. The CHs are highlighted in blue (CH1) and red (CH2), respectively. The bound ADP is shown in ball-and-stick representation. The putative position of the lipid bilayer is illustrated. (B) Crystal structure of human ABCG5/G8 heterodimer (PDB entry 5DO7).[111] ABCG5 is shown in the green carton and ABCG8 is the cyan cartoon. The single CH of each monomer is highlighted in red.

paradigm of a full-sized multidrug efflux pump is composed of "only" 1280 amino acids. ABCA transporters are involved in lipid and/or cholesterol transport; for example, ABCA1 transports cholesterol and is important in HDL (high density lipoprotein) formation.[93,94] The 11 members of the ABCB subfamily (seven half-sized and four full-sized) comprise various functions including drug, antigenic peptide, lipid, or bile acid transport and are expressed in different organs and cellular membranes. The ones present in liver cells will be discussed in later sections in more detail.

The 12 functional members of the ABCC subfamily are all full-size transporters. Interestingly, additional domains next to the NBDs and TMDs are present in this subfamily. First, ABCC7 contains the so-called R (regulatory) domain, which is located between the NBD1 and TMD2. The R-domain has to become phosphorylated by protein kinase C to act as an ATP-gated Cl^- channel.[95,96] Furthermore, ABCC1 harbors a third transmembrane domain (TMD0) composed of five transmembrane helices (TMH) at its N-terminus. Deletion of TMD0 does not result in impaired ATPase activity or substrate transport. TMD0 is also found in ABCC2, ABCC3, ABCC6, ABCC8, ABCC9, and ABCC10, respectively. However, there are two other ABC transporters of the B subfamily, TAP (transporter associated with antigen processing) or ABCB2/B3 as well as TAP-like (ABCB9) that contain a TMD0.[97,98] In this case and in the cases of ABCC8 and ABCC9,[99] TMD0 mediates protein−protein interactions, while the precise role of TMD0 for the other ABCC transporters remains elusive. In contrast, the linker connecting TMD0 and TMD1, the "lasso motif",[100] is essential for proper trafficking.[101−103] All four members of the D subfamily localize to peroxisomes and are involved in lipid metabolism. The ABCE and ABCF subfamilies do not contain a TMD and are referred to as ABC proteins. We will therefore not include ABC proteins in this review. Finally, the topology of the G subfamily with five half-size members out of which two (ABCG5/G8) form a heterodimer[104−106] is different from the classic one observed in all other ABC transporter subfamilies and often termed "inverse topology".[107] While the classic arrangement is $(TMD\text{-}NBD)_2$ in the case of half-size transporters or (TMD-NBD-TMD-NBD) in the case of full-size transporters, the NBD always precedes the TMD in the case of members of the G subfamily and is thus localized at the N-terminus of the NBD-TMD module.

The first crystal structure of an ABC exporter, the bacterial Sav 1866,[108] revealed the presence of long intracellular helices that continue directly from the TMHs, two coupling helices (CH) and a domain swapping (Figure 3A). This domain swapping subdivides each TMD into two parts. In the case of TMD1, TMH 1, 2, 3, and 6 belong to the first part, while TMH4 and TMH6 form the second part. For TMD2, the first part is composed of TMH10 and TMH11, while the second part is formed by TMH7, 8, 9, and 12. Thus, the first helical part is build up by TMH1, 2, 3, and 6 of TMD1 and TMH11 and 12 of TMD2. Accordingly, the second part is composed of TMH4 and 5 of TMD1 and TMH 7, 8, 9, and 12 of TMD2. This domain swap has important mechanochemical consequences. The intracellular loops (ICL) 1 and ICL2 adopt helical structures and are therefore also termed coupling helix (CH) 1 and 2. CH1 contacts primarily the *cis* NBD, while CH2 exclusive interacts with the *trans* NBD. On the side of the NBD, CH1 contacts a region preceding the C-loop that was termed the X-loop[108] as well as the helix following the Walker A motif. CH2, on the other hand, contacts the Q-loop region. This obviously has large mechanistical implications as the C- and Q-loop regions are the parts of the NBD that undergo the largest motions upon ATP-induced dimerization, suggesting that their motion is directly transmitted to the TMDs through the long and helical ICLs. This allows a direct coupling of NBD dimerization with conformational changes in the TMD. This coupling provides a molecular explanation for the two side access model proposed already in 1966[109] This proposal

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originally suggested for importers was extended to ABC transporters in 2004[74] and suggested how the NBDs catalyze the switch from the inward facing (IF) to the outward facing (OF) conformation. In the IF state, substrate binding site is accessible from the cytoplasm, while it is open to the extracellular space in the OF state. The X-loop furthermore was used as a diagnostic motif to identify multidrug exporters, as one laboratory could experimentally demonstrate that Sav1866 imposed drug resistance when overexpressed in *L. lactis*.[110] The results derived from the crystal structure of Sav1866 were not anticipated and provided a blueprint for human ABC exporters including ABCB1, which structure will be discussed in the section The Multidrug Efflux Pump ABCB1. It therefore came as a surprise when the crystal structure of the cholesterol exporter ABCG5/G8 (Figure 3B) was reported in the apo state.[111] ABCG5/G8 transports sterols including cholesterol and mutations cause sitosterolemia.

As shown in Figure 3B, the overall fold was more reminiscent of ABC import systems such as BtuCD[112] and contained only a single CH between TMH2 and TMH3, which is highlighted in red in Figure 3B. Thus, no domain swapping is present in this transporter. Additionally, a connecting helix (CnH) between the NBD and TMH1 and substantial extracellular domains (ECD) between TMH5 and TMH6 are evident. Missense mutations[105,106] causing sitosterolemia are located within the helical ECDs. Both the CH and the CnH are in proximity to the C-loop and might serve as a read-out platform for motions of the NBD due to ATP-induced dimerization. Furthermore, one has to stress that ABCG5/G8 is an asymmetric transporter; i.e., NBS1 formed by the Walker A and Walker B motifs as well as the Q- and H-loops of ABCG8 and the C- and D-loops of ABCG5 is deficient in hydrolyzing ATP. This is reflected by the architecture of the NBD-TMD interface. The CH, CnH, C-loop, and helix E form a three-helix bundle that present the communication pathway and appear to be more rigid in NBS1 than in NBS2.[111] Helix E, which is defined by the conserved Glu residue 146 (the E146Q mutation is disease causing[106]), packs against CnH and becomes part of the helical bundle that is supposed to transmit conformational changes from the NBD to the TMD.

Thus, structural biology provided us with a framework of structural information, and at least two different folds for human ABC export systems exist. Nevertheless, these two structures are not sufficient to allow detailed insights into the actual transport mechanism or the mode of substrate selection. The following section will now focus on human ABC exporters that are expressed in the liver on the protein level and summarize our current knowledge of their structure and function. Only when necessary, we will sidestep and include other ABC systems to explain more in detail the putative mode of action(s) of ABC transporters of the human liver.

1.3. An ABC of the Plasma Membrane of the Human Liver

Twelve[113] of the 48 human ABC transporters[84] have been identified to be functionally expressed in healthy human liver (Table 1), while 36 ABC transporter were only detected on the mRNA level. ABCB5[114] and B6[115] were predominantly detected in hepatocellular carcinomas. The latter is furthermore only expressed in mitochondria. This review focuses primarily on the healthy liver and transporters of the plasma membrane. Thus, we will not discuss both transporters any further.

Cellular cholesterol efflux and high density lipoprotein (HDL) formation is mediated by ABCA1.[116] On the mRNA level,[117] the protein was clearly detected, but so far it could only be visualized on the protein level in cell culture[118] and in rats.[119] Because of its importance for cholesterol homeostasis in mice[120] and the presence of structural information,[92] we will describe the liver relevant knowledge more in detail in section THE ABCA SUBFAMILY MEMBER OF THE LIVER - ABCA1.

The expression of CFTR was so far only confirmed in cholangiocytes.[90,121] Structural information in different functional states is available[86−89] and presents obviously an important step forward in our understanding and the consequences of disease-causing mutations. However, CFTR is an ATP-gated chloride ion channel and will not be included in this review as already stated.

In hepatocytes, three ABC transporters are located in the basolateral membrane, ABCC3, ABCC4, and ABCC6, respectively. In the canalicular membrane of hepatocytes, six ABC transporters and only five SLCs are located. The functions of the two SCLs, MATE1 and AE2, have already been discussed in the previous section. The canalicular membrane also expresses three nucleoside transporters.[122,123] The ABC transporters can be functionally subdivided into a conjugate transporter (ABCC2), drug efflux pumps (ABCB1 and ABCG2, respectively), and the three transporters involved in bile formation ABCB11, ABCB4, and the heterodimer ABCG5/G8, respectively. Here, a delicate interplay between bile acid export (ABCB11), cholesterol secretion (ABCG5/G8), and PC lipid flop from the inner to the outer leaflet (ABCB4) is emerging. We will discuss the interplay of these three transporters more in detail in section The Bile Triumvirate − ABCB4, ABCB11, and ABCG5/G8. The drug efflux pumps, ABCB1 and ABCG2, respectively, possess sort of a complementary substrate spectrum, which together with ABCC2 imposes a maximal level of protection against xenobiotics, a topic that will be discussed in detail in the next sections. ABCC2 secretes conjugated metabolites, mainly in the form of glucuronide- or glutathione-conjugated.[124] This function is complementary to the function of ABCC3 and ABCC4 located in the basolateral membrane. In contrast, a pathophysiological role was assigned to ABCC6 as mutations to pseudoxanthoma elasticum (PXE),[125,126] a connective tissue disorder. ABCC6 is expressed in kidney and the basolateral membrane of hepatocyte.[127] Here, ATP was identified as the physiological substrate.[128] The extracellular hydrolysis of ATP to AMP and PP_i is a process that generates the calcification inhibitor pyrophosphate (PP_i).[129] Consequently, dysfunctional ABCC6 results in reduced levels of PP_i and misbalanced calcification of connecting tissue.[130]

Stable or transiently infected cell lines are probably the most powerful tools to study transport function, analyze amino acid exchanges, and determine the transport rates and kinetic parameters. The only "drawback", if at all, is the fact that the transporter is treated as an isolated entity in these cell lines. Thus, the laboratories of Dietrich Keppler,[39,41,43,44,47,48] Yuichi Sugiyama,[46,49] Bruno Stieger,[45] and other groups[40,42] developed double- or triple-stable transfected cell lines. In the first published example,[39] OATP1B3 and ABCC2 were stably infected in polarized MDCKII cells. As in hepatocytes, OATP1B3 localized to the basolateral and ABCC2 to the canalicular membrane. The obtained data clearly revealed that uptake and efflux are by no means separated transport

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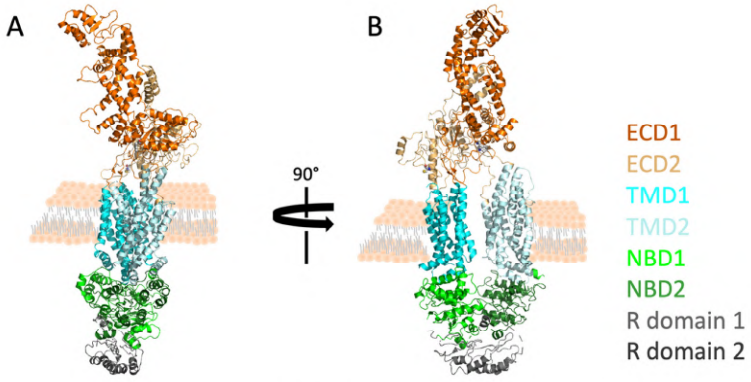

Figure 4. Moving cholesterol across a membrane - ABCA1. (A) Single particle cryo-EM structure of human ABCA1 (PDB entry 5XJY[92]). The R domains are shown in gray and light gray, the NBDs as green, the TMDS as cyan and light cyan, and the two ECDs as orange (ECD1) and light orange (ECD2) cartoons. The putative position of the lipid bilayer is illustrated. (B) As in (A) after a rotation of 90° perpendicular to the membrane as indicated.

processes. Rather, mathematical modeling[38] demonstrated that the cross-talk between both transporters was required to obtain intracellular transport rates that mirrored the situation in the human liver. This obviously imposes another level of complexity in the study of hepatobiliary transport processes. Since this review focuses on hepatobiliary ABC transporter, we will discuss one example[45] more in detail in section The ABC Bile Triumvirate – ABCB4, ABCB11, and ABCG5/G8.

We now turn to a detailed description of the structure and function of hepatobiliary ABC transporters grouped according to their functions outlined above.

2. THE ABCA SUBFAMILY MEMBER OF THE LIVER - ABCA1

Human ABCA1, first cloned in 1994 as ABC1,[131] is essential for cholesterol homeostasis and HDL formation. It transports excess of cholesterol and phospholipids to a proteinaceous acceptor, apolipoprotein A1 (apoA-1).[132] This secretion represents the rate limiting step of HDL biogenesis. Here, HDL absorbs cholesterol and carries it back to the liver. The secretion performed by ABCA1 is therefore crucial to reabsorb cholesterol and necessary for a healthy circulation. In macrophages, an additional anti-inflammatory activity was observed.[133] Thus, these two functions, cholesterol homeostasis and anti-inflammatory actions, protect pancreatic cells and regulate insulin secretion. A deficiency in ABCA1 is the basis of a rare genetic disorder, Tangier disease, that goes in hand with a severe reduction in HDL levels and increased levels of triacylglycerides.[134] As a consequence of the impaired function of ABCA1, cholesterol remains intracellular, the size of many body tissues increases significantly, and the risk of coronary artery diseases rises. As a result of increased intracellular cholesterol levels, cholesterol reacts with a fatty acid to form an ester that is deposited intracellular. These depositions are mainly found in tonsils, but also lymph nodes, bone marrow, liver, and spleen and complicate blood circulation due to a reduction of the diameter of the blood vessels. The highest mRNA levels of human ABCA1 were detected in adrenal glands, fetal tissue, liver, lung, and placenta.[117] However, the subcellular location in human liver has not been demonstrated on the protein level. The situation is different in rats. Here, high levels of human ABCA1 were detected in endothelial cells and hepatocytes, while the level was lower in Kupffer cells.[119] In knockout mice, hepatic ABCA1 is apparently involved in very-low-density lipoprotein (VLDL) as increased levels of triacylglycerides-rich VLDL particles were detected. This pointed toward a new relationship between increases in triacylglyceride levels and reduced amounts of HDL.[135,136]

The structure of human ABCA1, determined by single particle cryo-EM, at 4.1 Å resolution (PDB entry 5XJY and Figure 4) suggested the presence of a lateral access mechanism for cholesterol transport,[92] which deviated from the classical two side-access model.[109] Similar to the ABC transporters of the C subfamily, only one coupling helix and six TMHs are present per TMD. Next to the two NBDs and two TMDs, two small R domains following the NBDs are present. The most prominent feature of ABCA1, however, is two large extracellular domains (ECD) located between TMH1 and TMH2 (ECD1) and TMH7 and TMH8 (ECD2). The ECDs are suggested to interact with apoA1[137,138] and/or substrate[139] in the ABCA subfamily. All structures of ABC transporters determined in the nucleotide-free state adopted the inward facing conformation with separated NBDs. In striking contrast, the nucleotide-free structure of human ABCA1 is in a conformation most similar to the outward-facing conformation.[92] ECD1 (583 amino acids) and ECD2 (270 amino acids) adopt a twisted conformation, in which ECD1 is placed above TMD2, while ECD2 is placed above TMD1. Both adopt a novel protein fold and are stabilized by three (ECD1) and one (ECD2) disulfide bond, respectively. Interestingly, a hydrophobic tunnel is present in the ECDs, which might serve as an existing tunnel for cholesterol or phospholipids. This proposal is supported by the fact that many of the mutations identified in Tangier disease map to the ECD, especially the two well-characterized ones, W590S and C1477R.[140,141]

3. THE ABCC SUBFAMILY OF THE LIVER - DRUG AND TOXIN EFFLUX

3.1. The ABCC Subfamily - Similarities and Differences

The ABCC subfamily consists of 12 functional members. Next to an ATP-dependent chloride anion channel, CFTR/ABCC7, and two sulfonylurea receptors, SUR1/ABCC8 and SUR2/ABCC9, nine multidrug resistance-associated proteins (MRPs) belong to the ABCC family.[142] A 13th member of the ABCC

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Table 3. Sequence Identity of Human ABCC Subfamily Members Which Are Present in the Liver and Liver Excluded ABCC1 (MRP1)

	symbol	identifier	name	amino acids	long				short	
					ABCC1	ABCC2	ABCC3	ABCC6	CFTR	ABCC4
long	ABCC1	P33527-1	MRP1	1531	100					
	ABCC2	Q92887-1	MRP2	1545	48	100				
	ABCC3	O15438-1	MRP3	1527	58	47	100			
	ABCC6	O95255-1	MRP6	1503	45	38	43	100		
short	ABCC7	P13569-1	CFTR	1480	28	28	27	27	100	
	ABCC4	O15439-1	MRP4	1325	39	37	36	33	35	100

family (ABCC13) is a pseudogene for which no functional properties are known.[143]

The members of the ABCC subfamily can be further divided into short and long members. The term long ABCCs derives from an additional N-terminal domain called TMD0 present in these transporters.[144] Five ABCCs, namely, ABCC1 (MRP1), ABCC2 (MRP2), ABCC3 (MRP3), ABCC6 (MRP6), and ABCC7 (CFTR), belong to the long MRPs. The others, including ABCC4 (MPR4), belong to the short MRPs (other examples are ABCC5, ABCC11, and ABCC12). For the sake of completeness, ABCC8 and -9 are no MRPs, but are long members of the ABCC subfamily. ABCC10 or MRP7 is not expressed in the liver, but also belongs to the long MRPs.

All members of the ABCC family consist of at least four domains typical for ABC transporters, namely, two TMDs and NBDs. The NBDs, although highly conserved in all ABC transporters, are degenerated in the ABCC subfamily (Table 2). This means that the first NBS has little or no ATPase activity, whereas the second NBS (NBS2) has a much higher ATPase activity.[6,145] Interestingly, NBD1, which forms NBS1 with the exception of the C- and D-loops, is characterized by a high affinity for ATP, suggesting a coordinating and structurally stabilizing function.[145]

There are also differences in the tissue-specific localization of the individual members of the ABCC family, which influence the function and substrate spectrum of these transporters (Table 1). For example, an ABCC transporter may be found in the apical membrane in one organ and in the basolateral of another organ. Importantly, specific physiological functions are linked to localization.[146]

This section deals with the function and localization of ABCC transporters of the liver. There are five ABCC proteins that are expressed on protein level in the liver.[113] Besides ABCC7 (CFTR), these are ABCC2, ABCC3, ABCC4, and ABCC6. Additional information on the structure, function, localization and medical applications of ABCC7, which is not discussed here, are described in refs 86, 87, and 147.

We have implemented a short part to the structure of ABCC1, although it is not expressed in a healthy liver. The reason is the recently published structure,[148] which represents probably the closest structural information for the liver ABCC subfamily. ABCC1 shares approximately 50% sequence identity with other liver ABCCs. A cross sequence alignment of the different liver ABCCs are provided in Table 3 and demonstrate the relation within this subfamily. However, we have examples from nature, in which an even higher degree of identity results in physiologically different functions, for example, the 79% amino acid identity between ABCB1 and ABCB4.[149,150] ABCB1 transports hydrophobic compounds often with aromatic properties, whereas ABCB4 transports lipids of the phosphatidylcholine family. Therefore, we would like to point out that molecular differences between the different structures of the liver ABCCs may be crucial and can only be elucidated if structures are determined. However, the structure of ABCC1 may act as a prototype for this subfamily.

In general, the ABCC subfamily transports a variety of substances across the corresponding plasma membranes including drugs, such as cancer therapeutics, but also conjugated metabolites. Characteristic for the ABCC family are substrates that are conjugated with glutathione (GSH), sulfate or glucuronate prior to transport. However, these substrate characteristics are not found to the same extent in all ABCCs. ABCC6 for example shows only a low affinity to GSH conjugated substrates.[151] The varying degree of redundancy of the substrate spectra of liver ABCCs is controlled under physiological conditions via different *in vivo* export kinetics. This means that although two ABCCs may have an overlapping substrate spectrum, in practice only one transporter will export the substrate[152,153] thereby regulating the detoxification pattern. It is therefore possible that the function of defective ABCCs is compensated by other ABCCs. For example, an ABCC2 deficiency can be counteracted by the upregulation of other transporters of the ABCC subfamily such as ABCC3 or ABCC4 as it could be shown in r*Abcc2* deficient rats.[154−156] However, since these ABCC members are localized in different membranes of the hepatocyte, ABCC2 substrates that would normally be exported to bile would now, compensated by ABCC3 or ABCC4, appear in the sinusoidal bloodstream.[157,158]

3.2. General Lessons Learned from Structures of the ABCC Family

First, we have no direct structural information for the ABCC transporters of the liver. Thus, information on the characteristics such as the TMD0 of long ABCC transporters must be derived from other members of the ABCC subfamily.

So far, structures of three members of the ABCC family have been published. These include CFTR, SUR1, and ABCC1.[100,159,160] From a functional as well as mechanistic point of view, the information gained from CFTR and SUR1 for the four liver ABCCs (ABCC2, ABCC3, ABCC4, and ABCC6) are limited, but might provide hints for the role of TMD0. In SUR1 for example, TMD0 interacts with a potassium channel to form a larger macromolecular complex.[98,159,160]

Additional information can be derived from the structure of ABCC1 (PDB entries 5UJ9, 6BHU, 5UJA)[100,161] and CFTR. First, ABCC1 provides structural information for long ABCC transporters, since it is the closest-related structure based on sequence identity (Table 3). TMD0 of ABCC1 as a prototype of long ABCC subfamily members of the liver such as ABCC2, ABCC3, and ABCC6 is highlighted in Figure 5. When the protein core of TMD1 and TMD2 undergoes a conformational

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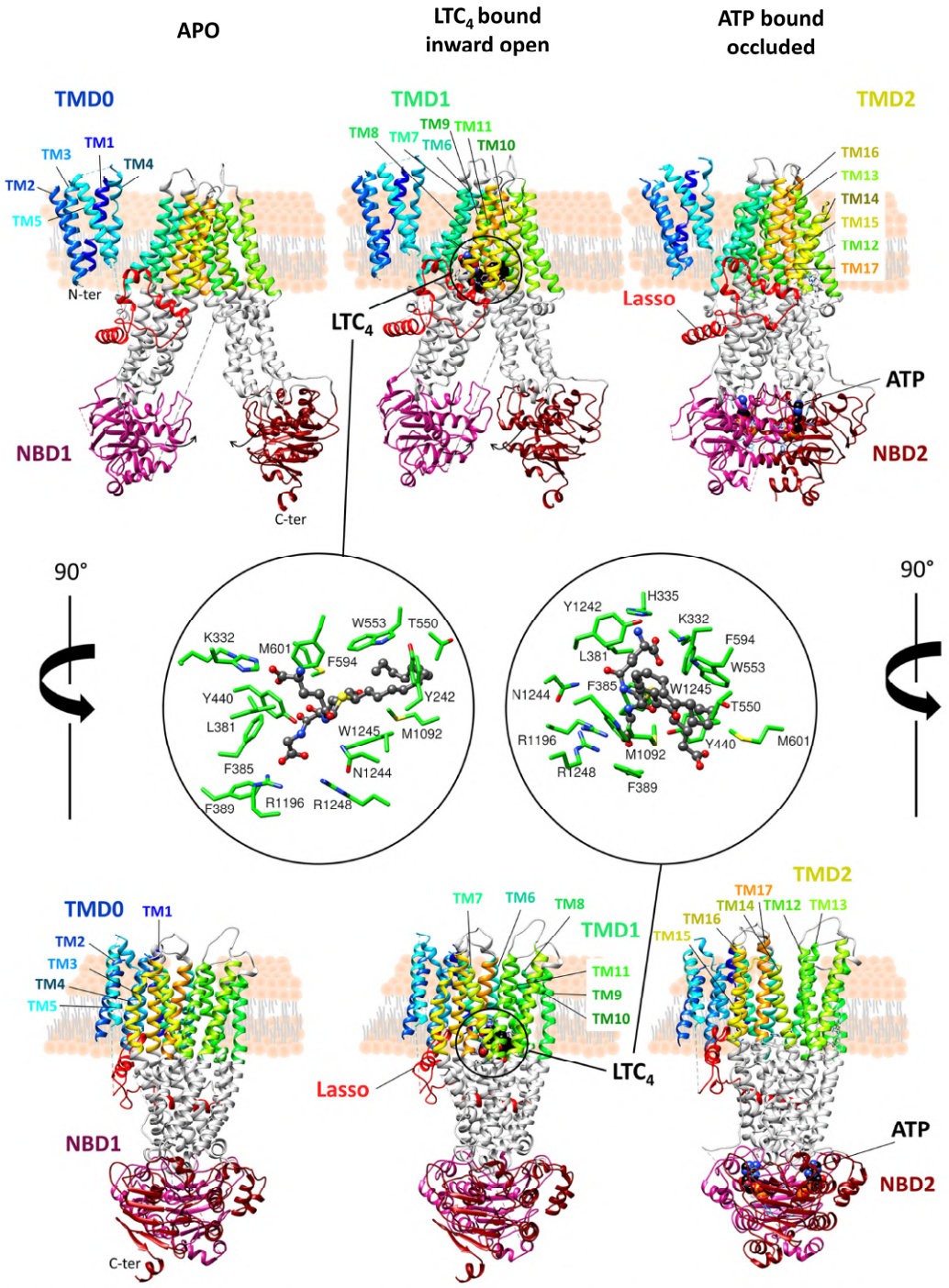

Figure 5. Structures of ABCC1. Apo (bovine, PBD entry 5UJ9), LTC_4-bound inward open (bovine, PDB entry 5UJA), and ATP-bound occluded (bovine, PDB entry 6BHU) conformations of ABCC1. The TMD0 is colored in blue, the TMD1 in turquoise, and the TMD2 in yellow. NBD1 is pink, and NBD2 is dark red. The typical lasso motif of the long ABCCs is highlighted in red. Substrates such as ATP and LTC_4 have a black background. The upper panel offers a frontal view. The lower panel is rotated by 90°. The N- and C-terminus are marked. The dashed lines in the upper left and upper middle picture are flexible regions of the protein with no structural information. Information about flexible areas in ABC transporters may be obtained here.[163]

change due to substrate binding and/or upon ATP binding the NBDs come closer together, it seems as if the conformation of TMD0 is nearly identical within all three structurally described states of ABCC1. This indicates a function prior and

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Chem. Rev. 2021, 121, 5240−5288

independent of substrate transport. Strikingly, deletions of TMD0 in the case of ABCC1, does not impair its export function,[102] which explains the rigid structure of this TMD0. Note that the connection to the core protein is not visible due to missing electron densities. This indicates a highly flexible area and might be explained by the fact that some parts of the protein have to be flexible in order to make up for the rigid TMD0. Taken together this suggests that TMD0 is involved in protein−protein interactions for ABCC members belonging to the long subfamily, but lack an involvement in substrate translocation.

A so-called lasso motif appears to be structurally highly conserved within the ABCC family.[148] Confirmatively, the lasso motif from ABCC1 differs from the analogous lasso motif of ABCC7 only by an rmsd of 1.1 Å over amino acids 1−64 from ABCC7 and 205−268 from ABCC1,[148] even if both proteins provide physiologically diverse functions. The assumption that this motif is also conserved in the more closely related ABCCs found in the liver seems convincing. It is a motif following TMD0 but is more closely positioned to the core protein and is connecting the flexible regions derived from TMD0 with TMD1 (highlighted in red in Figure 5). The function of this lasso motif is essential for the correct folding and trafficking of ABCC members.[87,148] Deletions led to misfolded proteins that exhibited impaired functionality.[162]

The substrate binding pocket, on the other hand, appears to show larger differences between the individual ABCCs of the liver. Substrate binding pockets are known to be localized in the TMD of ABC transporters. The ABCC subfamily is no exception. Again, highlighting the point, that up to now we have no direct structural data of any of the liver ABCC transporters making ABCC1 the closest relative. In the ABCC1 structure two binding pockets are present, the positively charged (P) and the hydrophobic (H) pocket. The prototype binding pocket of LTC_4 bound to ABCC1 and the engaged amino acid residues are depicted in Figure 5. Obviously, the binding pockets of the liver ABCCs must harbor different residues and/or folds in order to explain the substrate diversity of this family. However, there are also overlaps in the substrate spectrum within the liver ABCCs. It seems that ABCC transporters in general have two binding pockets for ligands, first substrates and second modulators.[164−166] Interestingly, potential nutrient-drug interactions in ABCC2 as observed for genistein and sorafenib[167] in the context of hepatocellular carcinoma (HCC) point to a possibility of two binding pockets. Substantially different is the binding pocket of ABCC1 and ABCC2 even if they share common substrates such as leukotriene C4 (LTC_4). Studies, in which the highly conserved W1254 and P1158 of ABCC2 and the homologues residues of ABCC1 and ABCC3 were exchanged, led to changed transport efficiencies of these three substrates.[168−170] Furthermore, particularly charged amino acids in TMD1 and TMD2 seem to be important for the substrate specificity of ABCC2. Estradiol, for example, is a prominent substrate of ABCC2, but it also has a regulatory influence *via* the allosteric binding pocket.[171] To investigate this dual character in more detail, *de novo* structures of ABCC2 or even other liver ABCC transporters are required.

However, the general conformational changes that occur due to substrate binding, followed by ATP hydrolysis, are key characteristics for all ABC transporters (Figure 5); thus, they may also appear in the other liver ABCC transporters as the prototype structure of ABCC1 exemplifies. We therefore show the known conformations of ABCC1 (Figure 5). More details about the liver ABCCs regarding biological function in the liver can be found in the following chapters (ABCC2, 3, 4, and 6).

3.3. ABCC2 (MRP2): The Only ABCC Transporter of the Canalicular (Apical) Membrane

Originally, ABCC2 was referred to as canalicular multidrug resistance-associated protein (cMRP)[172] or canalicular multispecific organic anion transporter (cMOAT),[173] because it was initially found in the canalicular membrane of hepatocytes. However, ABCC2 can also be found in the apical membrane of other polarized cell types.[124,174,175] TMD0 and the cytoplasmic helix 3 of ABCC2 are apparently responsible for the specific apical localization.[176] ABCC2 functions at the final stage of the bioelimination process. Potential toxins or metabolites diffused though the cell, where they were conjugated, are finally eliminated by ABCC2, as indicated by the apical localization in the membrane.[153,177] The substrate spectrum of ABCC2 includes conjugated endogenous or xenobiotic substances, which are exported into the bile duct rather than into the blood system, unlike other multidrug exporters. The substrate spectrum of ABCC2 is defined by its lipophilic properties and contains mainly organic anions. These substrates are often conjugated with glutathione, sulfate or glucuronate. Endogenous metabolites include bilirubin, bile acids[178−180] and LTC_4.[179,181] Additionally, ABCC2 also has a broad spectrum of cancer drugs such as *vinca* alkaloids, tamoxifen, cisplatin, or anthracyclines[182−186] as substrates. Cisplatin, for example, is an anticancer drug with a broad response spectrum and is often used in cancers for which no specific targets are known.[187] Accordingly, overexpression of ABCC2 is a frequently observed problem in antitumor treatment.[188]

ABCC2 deficiency, on the other hand, can lead to insufficient elimination of bilirubin from hepatocytes into the bile, a phenomenon described as Dubin and Johnson disease.[189] It is an autosomal recessive disease and occurs in 0.08% of Iranian Jews[190] and in 0.0003% of the Japanese population.[142,191] The liver appears black in these people, because the dark dye of the elevated bilirubin glucuronides in the hepatocytes cannot be exported.

3.4. ABCC3 (MRP3): The Back-up System for ABCC2 in Liver Cholestasis

ABCC3 (MRP3) is transported to the basolateral membrane in the same way as ABCC1 in polarized cell types, but in contrast to ABCC1 it is also found in the hepatocytes of the liver.[113] Previously, it was thought that ABCC3 is highly expressed in the liver, but immunohistochemistry of frozen sections has revealed that in healthy liver, there are only modest levels of ABCC3, especially in the basolateral membranes of cholangiocytes and hepatocytes surrounding the portal tracts.[192] Nevertheless, ABCC3 and ABCC1 show the highest sequence homology (Table 3). However, the substrate spectrum of ABCC3 differs substantially from that of ABCC1 and is rather limited. Particularly noteworthy is the low affinity to the tripeptide glutathione (GSH), which is often conjugated to other substances for ABCC1 mediated transport.[193] Although ABCC3 has been studied mostly in the context of bile acid transport, there are also other drugs and metabolites that are part of the substrate spectrum. These include for example acetaminophen glucuronide, methotrexate, and sorafenib.[194]

Even if ABCC3 deficiency does not lead to a specific phenotype,[193,195] it was detected that ABCC3 was up-regulated in a cholestatic liver. Currently, it is assumed that

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ABCC3 is sort of "back-up system", especially when transport of toxic metabolites by ABCC2 is impaired. Of pharmacological importance is the observation that in *Abcc3*$^{-/-}$ mice conjugated morphine accumulates in the liver and bile, which suggests a definitive role of ABCC3 in its elimination.[195] Furthermore, different ABCC3 single nucleotide polymorphisms (SNPs) may explain the differences in morphine metabolite elimination.[196] However, these ABCC3 variants were located on intronic regions of chromosome 17 (4871392 and 48744612, spanning 13221 bp) and hence do not alter the protein sequence. The authors speculated about regulatory factors that may alter DNA binding or noncoding regulatory sequences. Additionally, an impairment of liver regeneration could be observed in mice with deleted ABCC3 (*Abcc3*$^{-/-}$) after partial hepatectomy. Here, the authors concluded that ABCC3 plays again a nonredundant role in regulation of bile acid efflux during liver regeneration.[197] Altogether, these findings highlight the role of ABCC3 in injured liver systems to help to recover through the export of toxins and toxic metabolites such as bilirubin.[193]

3.5. ABCC4 (MRP4): The Little Brother of ABCC1 in the Liver

ABCC4 is expressed in several human tissues; high levels have been reported in prostate glandular, and in contrast to ABCC1, ABCC4 is also detected in the liver, but at rather low expression levels.[198,199] Here, ABCC4 exports chemically diverse substrates, signal molecules, as well as xenobiotics *via* the basolateral membrane of the hepatocytes. Beyond the liver, ABCC4 is particularly well-known for being not limited to the basolateral membrane, thus exporting substrates through both membrane types of polarized cells.[200,201]

Although ABCC4 expression is low in hepatocytes, it is enhanced in cholestatic livers.[198,202] In fact, the up-regulation of ABCC4 in cholestatic liver of humans provides an adaptive mechanism to eliminate excess bile acids.[202,203] ABCC4 appears to be a clinically relevant drug transporter, along with ABCC1, that makes cancer treatment more difficult.[146] Moreover, xenobiotics may increase the expression of hepatic ABCC4.[203,204] The substrate profile of ABCC4 includes cAMP and cGMP, leukotrienes (such as LTC_4), sphingosine 1-phosphates (S1P)[205] and pesticides.[206] However, the transport efficiency and affinity to the important signal transduction molecules such as cAMP and cGMP is low, which means that although no direct intracellular regulation by ABCC4 is considered possible, an indirect regulation by local microdomains cannot be excluded.[207]

ABCC4, as many ABC proteins, has more than one substrate binding site to account for its diverse substrate spectrum.[165] This assumption is based on homology modeling.[166] Amino acids involved in substrate binding, namely, F368, F369, E374, R375, E378, W995, and R998 are located at the surface of intramembrane water pore in between TMHs. The aromatic residues can selectivity bind aromatic substrates by $\pi-\pi$ stackings.[164,200,208] Additional theoretical work on ABCC4, also based on homology models generated by molecular dynamic simulations, provided insights in clinically relevant mutations, especially G187W. This SNP affected the tertiary structure of ABCC4 massively and provides an explanation for its transport disrupting effect.[209] Furthermore, it was shown, that certain substrates have been found to act in a concentration-dependent manner.[164]

In conclusion, these findings point to a role of ABCC4 in detoxification and liver damage, and suggests that its modulation could influence hepatocyte repair and regeneration during hepatocellular injury especially in cholestatic liver situations.[210,211]

3.6. ABCC6 - The ABC Transporter That Transports ATP

ABCC6 is mainly expressed in the liver and kidney, and deficiencies are linked to a rare disease of hereditary mineralization disorder, *pseudoxanthoma elasticum* (PXE).[126] ABCC6 is thought to mediate the secretion of ATP into blood, where ATP is rapidly converted into AMP and the calcification inhibitor PP_i.

The absence of ABCC6 leads to a substantial decrease of plasma PP_i.[7,128,130,212] PP_i would act as a counter reagent to ectopic calcification that occurs in PXE patients. This theory of a substrate export via ABCC6 into blood circulation and transport through the whole body is known as metabolic hypothesis.[130,144] However, whether ATP is a direct substrate of ABCC6 remains to be proven. ABCC6 could also be an activator of other ATP export systems of the cell. A problem for the export of ATP by an ABC transporter is the millimolar concentration of ATP in the cells. ABC transporters have a relatively low affinity to this highly abundant molecule in the cell, whose hydrolysis provides the energy for substrate transport.

ABCC6 seems to have evolved from a gene duplication event of ABCC1. Both genes are located on chromosome 16 (*ABCC1* 16p13.11 and *ABCC6* 16p13.12).[213]

Like all ABCC transporters, substrates of the organic anions and GSH derivatives and conjunctions were tested for the substrate spectrum of ABCC6. Although an overlapping substrate spectrum of many ABCC typical substrates such as LTC_4 was found, it is worth to mention that the transport efficiency of ABCC6 is significantly lower.[151,214] A K_m value of 600 nM and a V_{max} value of 50 pmol/mg of membrane protein/min for LTC_4 transport were estimated.[151] In comparison the kinetic parameters of ABCC1 are a K_m of 77 nM for LTC4 and a V_{max} of 291 pmol/mg/min.[215] This clearly supports the theory that ABCC6 is mainly an ATP exporter *in vivo*.

4. THE ABCB SUBFAMILY - STRUCTURALLY CLOSE BUT DIFFERENT SUBSTRATES

4.1. The Multidrug Efflux Pump ABCB1

ABCB1 was first described in 1976 by Ling[216] as a permeabilization glycoprotein (P-gp). Later, the name was linked to its function, and it was called multidrug resistance protein 1 (MDR1). According to the nomenclature of human ABC transporters, MDR1 is now termed ABCB1. ABCB1 is ubiquitously expressed in various tissues[217] and also in organs associated with elimination processes in liver, kidney, or the blood−brain barrier. In the liver, ABCB1 does not yet perform one specific task; rather, it exports a large number of substances from the hepatocyte to bile and contributes considerably to the first defense against drugs.[218] Interestingly, the laboratory of Yin demonstrated in 2018 that ABCB1 can transport bile acids. Here, a model is suggested, in which rapamycin rescues BSEP-deficient phenotypes by activating alternative excretion of bile acids via ABCB1.[219]

Accordingly, ABCB1 is located in hepatocytes in the canalicular membrane and reduces the drug concentration in the cell by continuous export. The substrate spectrum mainly

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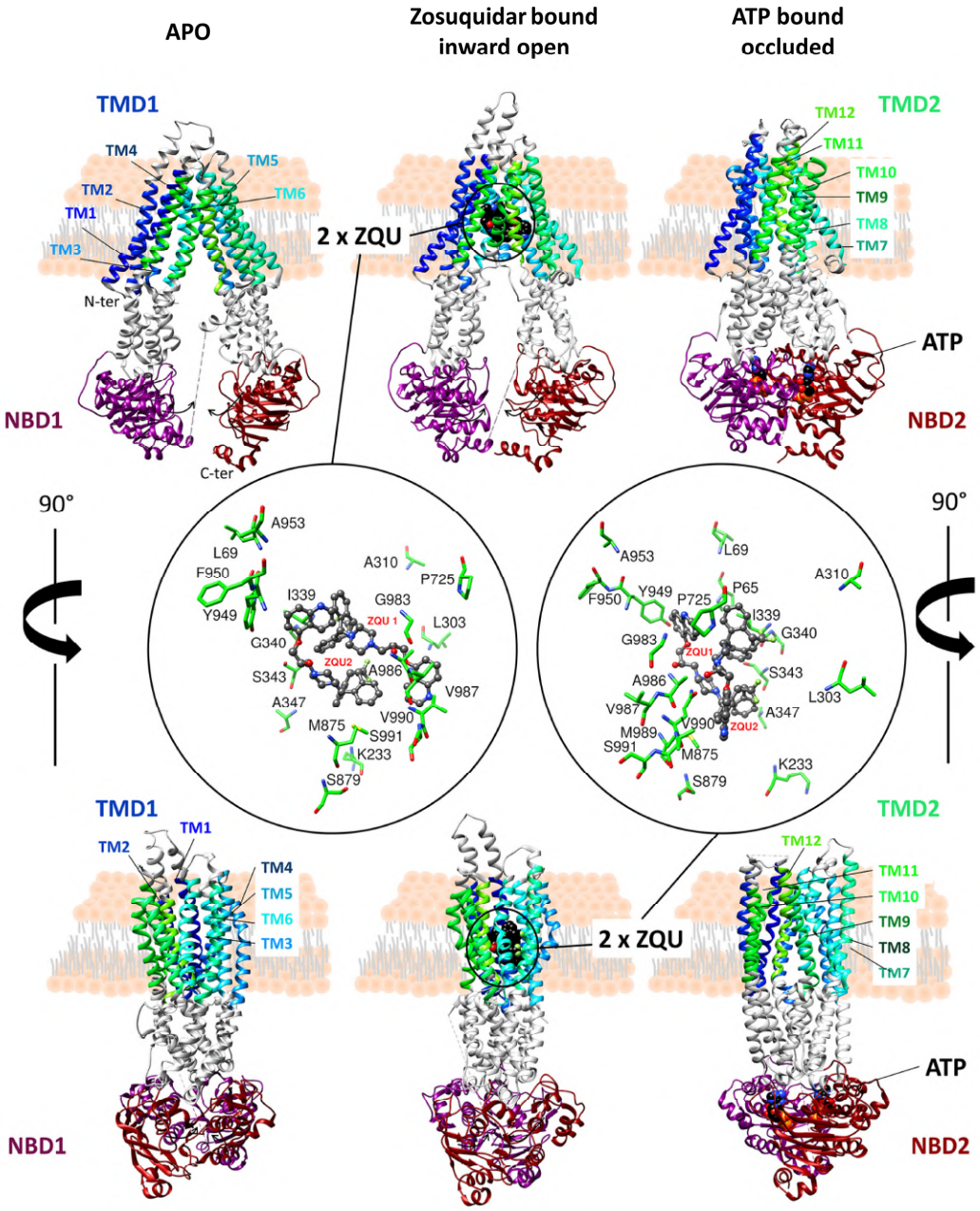

Figure 6. Structures of ABCB1. An apo (*Mus musculus*, PDB entry 4M1M),[233] zosuqidar-bound, inward open (human-mouse chimera, PDB entry 6QEE)[234] and ATP-bound outward facing (human, PDB entry 6C0V)[235] conformations of ABCB1. Blue colors are used for the TMD1, and TMD2 is held in greenish colors. NBD1 is pink and NBD2 is dark red. The binding pocket of the inhibitor bound structure is highlighted. The amino acids interacting with the inhibitor are visible in an enlarged harvester. The upper panel offers a frontal view. The lower panel is rotated by 90°. The N- and C-termini are marked. The dashed lines in the upper left and upper middle picture are flexible regions of the protein with no structural information. Information about flexible areas in ABC transporters may be obtained in ref 163.

comprises hydrophobic substances, some with aromatic ring systems and slightly amphiphilic character.[220−222] ABCB1 is one of the best studied MDR exporters and is often considered as prototype of human ABC transporters[223] and directly associated with cancer.[224] As little is known about a specific task within the liver, as much is known about the over-expression of ABCB1 in cancer cells.[225] For example, in mice that possess two orthologous genes (*Abcb1a* and *Abcb1b*), knockout does not lead to a direct phenotype; however, several drugs did accumulate in these mice.[226] Additionally, investigations indicated that ABCB1 is involved in the export of short chain lipids albeit with low efficiencies.[222] So far, more than 300 substrates, modulators, and inductors have been described that interact with ABCB1. For a summary of these

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compounds, see refs 227−230. Additionally, polymorphisms appear to be medically relevant in ABCB1, as different variants can sometimes lead to altered drug excretion kinetics.[231] This is one reason why personalized medicine as treatment approach might also be an option for ABCB1.

The investigations, which led to structural insights into the transport mechanism of ABC transporters, were pioneering work for the entire family. ABCB1 was not the first ABC transporter, for which structural information became available, but was also next to Sav1866, a prototype of an ABC transporter.[108,232] Besides the structures of ABCC1, the structures of ABCB1 cover most of the individual states of a substrate transport cycle in humans, in the sense of different conformations including bound ligands. These structures comprise besides several structures of the apo state, the structurally more important ATP-bound and substrate-bound structures. To illustrate the different conformations of ABCB1, we chose some exemplary structures in Figure 6 (PDB entries 4M1M, 6QEE, 6C0V).[233−235] Table 1 contains more structures of ABCB1. The compendium of these structures allows to follow the complete transport cycle of an ABC transporter. The various conformations observed in these structures might also serve as a template for other members of the ABCB family. Again, the structure comprises a four-domain structure of two NBDs and two TMDs encoded on one gene in the canonical order of TMD1−NBD1−TMD2−NBD2. In the case of ABCB1, the NBDs harbor all conserved sequences motives in a canonical fashion and both NBS are active with respect to ATP hydrolysis.[236,237] Equally important, the two NBS alternately hydrolyze ATP. This mechanistic harmony is also reflected in the high ATP turnover of ABCB1 in the presence of a substrate.[235]

Unlike the NBDs, the TMDs are much more diverse in the ABC transporter family. They represent the transport pathway for substrates, and this is also where the substrate binding sites are located. In ABCB1, more than 30 amino acids have been identified to interact with substrates, although the exact number strongly depends on the substrate. However, they provide a flexible binding pocket, which ABCB1 requires to accommodate such a broad substrate spectrum.[238] Three-dimensional insights are provided by the two structures of ABCB1, in which one substrate (taxol, PDB entry 6QEX) or two inhibitor molecules (zosquidar, PDB entry 6QEE) are bound[234] (Figure 6, middle panel).

Because of these different structures of ABCB1, a detailed transport cycle can be proposed. In the ground state, the lateral gate between TMH 10 and 11 is flexible and allows substrates of different sizes to bind to the proteins interior.[239] Subsequent binding of ATP allows the transporter to switch to the outward-facing conformation. This conformation possesses a lower affinity to the substrate as in the inward-facing ground state. The parts of the transporter that do not interact with the membrane and point toward the extracellular medium are flexible and allow the final release of the substrate.[234] As a result, the export path is closed from the outside. The two ATP molecules stabilize the dimerized NBDs in the outward facing conformation[235] and ensure the release of the substrate. Subsequent ATP hydrolysis resets the entire system to the inward-facing ground state.

4.2. The PC-Lipid Floppase ABCB4

Although the structure of ABCB4 has been published only in 2019,[240] the function of ABCB4 was investigated since its finding in the late 1980s,[241,242] since ABCB4 is involved in a number of liver diseases (see section The ABC of Liver Diseases). ABCB4 was first identified as a membrane protein highly identical to ABCB1.[241,242] As stated above, both proteins share 76% identity and 86% homology, respectively. The highest degree of differences lies within the N-terminus and the linker region connecting both halves of this full-size ABC transporter. Therefore, it was initially assumed that both proteins perform the same physiological function. An assumption that turned out to be incorrect. While ABCB1 possesses a broad substrate spectrum and represents a classic multidrug resistance protein,[243] ABCB4 specifically but not exclusively, recognizes lipids of the PC family.[150,244] *In vitro* studies demonstrated that substrates of ABCB1 such as aureobasidin A, digoxin, paclitaxel, vinblastine, or ivermectin are recognized by ABCB4 heterologous expressed in yeast or pig cell lines.[245,246] Additionally, the ABCB1 inhibitors verapamil and cyclosporine also inhibited ABCB4 in cell culture.[246] This overlap in substrate spectrum suggests at least an artificial structural resemblance of some features of both proteins. However, *in vivo* studies demonstrated beyond any doubt that ABCB4 localized at the canalicular membrane is essential for the transport of PC lipids into the bile duct.[242,247−249] Especially for mouse ABCB4 (mABCB4, also termed mdr2), many studies investigated the function of (m/r)ABCB4.[250−257] For example, homozygous knockout mice did not contain cholesterol and PC lipids in their bile. Furthermore, bile composition changes in patients suffering from PFIC-3. In the heterozygous mutant mABCB4$^{\mp}$, approximately 60% of wildtype levels of PC lipid and nearly normal cholesterol levels were observed in bile. Subsequently, human ABCB4 was expressed in Abcb4$^{-/-}$ mice, which resulted in even higher phospholipid levels,[258] demonstrating that both perform the same function. It is now well accepted that ABCB4 recognizes PC lipids, i.e. choline headgroup.[150,259] In other words, lipids of different subfamilies bearing a different headgroup are not translocated[253,260] and showed no stimulation of ATPase activity in the case of purified ABCB4.[259] In contrast, a preference for a specific acyl chains are only poorly understood. One study demonstrated that short chain PC lipids are favorized in pig kidney epithelial cells transfected with human ABCB4.[150] In contrast to this study, another study indicated that ABCB4 might be a low affinity transporter and recognizes long chain acyl chains as well as unsaturated lipids.[261] Connecting both views a characterization of the ATPase activity of ABCB4 was performed concluding that every PC lipid productively interacts with ABCB4, while C 18 fatty acid esterification's with unsaturated chains do best.[244]

Is ABCB4 is "just" flopping PC lipids from the inner to the outer leaflet or it is lifting the PC lipid for subsequent extraction into the bile? So far, no experimental data exist that addresses this important question; however, the nearly total absence of PC lipids in *abcb4* knockout mice[255,262] and PFIC3 patients[263] suggests that ABCB4 may do much more than simply flopping PC lipids, but an extraction via bile salts is also plausible.[244] However, further investigations are necessary to understand ABCB4 in detail.

A study, expressing mABCB4 in yeast indicated that glycosylation is not necessary for expression, proper location, and function, respectively.[264] The two glycosylation sites for human ABCB4 are located between TMH1 and TMH2 at position 91 and 97.[241] For mABCB4, three glycosylation sites are known. These are also located in the first extracellular loop,

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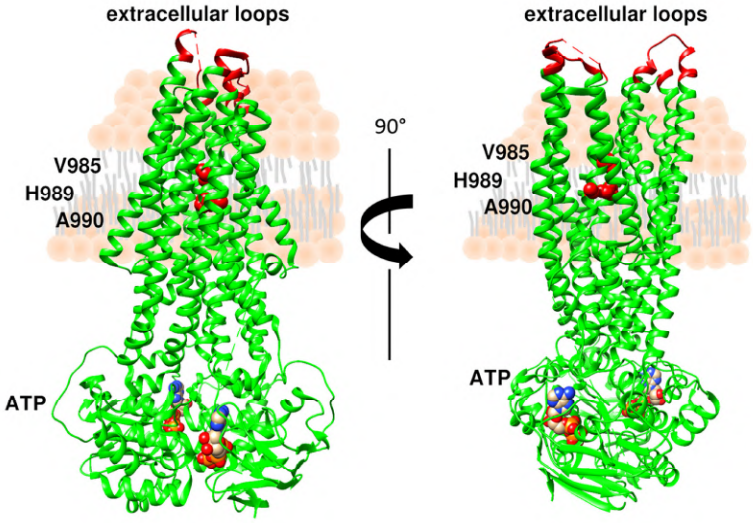

Figure 7. Structure of ABCB4 (human, PDB entry 6S7P,[266]). Left panel: frontal view, right panel: view after rotation by 90° as indicated. The structural areas, whose mutation led to a loss of function are marked in red.

but located at amino acid positions 83, 87 and 90.[251] In contrast to glycosylation, phosphorylation is essential for the function of ABCB4. Mutations with an altered phosphorylation pattern resulted in markedly decreased PC lipid translocation, despite the fact that the protein showed minor defects in maturation and localization.[265]

Recently, the closed conformation of the ABC transporter ABCB4 structure was published (PDB entry 6S7P).[266] ABCB4 has the characteristics of most eukaryotic ABC transporters.[267] The topology comprises a four-domain structure of 2 NBDs and 2 TMDs. The TMDs are defined by six α-helices, each. The typical "domain swap arrangement" is also present in ABCB4. TMH 4 and 5 of TMD1 reach over to the other half of the transporter and *vice versa* (TMH 10 and 11 for TMD2). In the two NBDs, two ATP molecules were observed in the typical head-to-tail dimer of the NBDs. Interestingly, only in the NBS1 an electron density for Mg^{2+} was found. Since Mg^{2+} is obligatory for ATP hydrolysis, the presence of Mg^{2+} may indicate an active NBS1 and a (temporarily) inactive NBS2. This structural asymmetry in the two NBS could not be shown for ABCB1. Whether ABCB4, like ABCB1, has two functional NBS that alternately hydrolyze ATP could not be deduced.[236] However, the structural asymmetry of the NBDs was extended by the H-loop, which differs in the two NBDs of ABCB4. The H-loop was shown to be essential for ATP hydrolysis in the NBD of the bacterial ABC transporters HlyB.[268]

Olsen et al. proposed an "alternating access" mechanism for ABCB4.[109] This requires a nucleotide-bound outward conformation starting from an apo structure (without substrate) and resulting in an inward occluded structure (with substrate). Intermediate stages of ABCB4 could not been captured structurally so far. The ABCB4 structure is an exception in that it varies from the known conformations of ABCB1 as it resembles a closed conformation without bound substrate. Mechanistically, this conformation seems to represent a state immediately after substrate release.

The authors also tried to derive a mechanism from the high degree of relationship between ABCB1 and ABCB4. For this purpose, the substrate binding in ABCB1 was first examined. From the substrate and inhibitor bound conformations of ABCB1, 20 amino acids of the substrate binding pocket were defined. Most of them are also conserved in ABCB4,[234] except three (Figure 7). These residues, V985M, H989Q, and A990V, are located in the hydrophilic core of the protein. A triple mutant of these residue exchanging the three amino acid residues to the ones present in ABCB1 had a strong influence on substrate binding and inactivated the transporter.[266] The authors concluded that the hydrophobic cavity in the core of the proteins in ABCB4, analogous to ABCB1, is the substrate binding site. In another chimera, the extracellular loops of ABCB4 were exchanged with the extracellular loops of ABCB1. This chimeric transporter led to a loss of function as observed in whole cell extrusion assays (Figure 7).[240] Accordingly, the extracellular loops could support a reorientation of the PC lipids.

Mechanistically, however, there are still unresolved questions. How does the lipid from the inner leaflet enters the central cavity and leaves this hydrophobic binding pocket and how it ends up in the extracellular leaflet? A floppy state does not seem to be completely answered by a single conformation of ABCB4. A hydrophilic cavity was also observed between TMH 10 and 11 in the ABCB4 structure. Although sufficiently large for PC lipids, it is located slightly below the membrane interface and thus does not provide an optimal transfer pathway for a PC lipid to the cytoplasmic leaflet. This cavity might represent an allosteric modulation binding pocket for cytoplasmic ligands but is different to the lateral gate of ABCB1. Because of its localization at the cytoplasm-membrane interface, amphipathic substances would be particularly suitable to modulate the activity of ABCB4.

A preselection mechanism to select PC-lipids from the pool of phospholipids of the canalicular membrane has not yet been determined. A selection of lipids, which just takes place in the substrate binding pocket, i.e., in the hydrophobic cavity within the core protein, appears to be thermodynamically disadvantageous and could possibly lead to complications that slow down or even completely block the translocation process. In contrast, a mechanism of selection of phospholipids was reported for a

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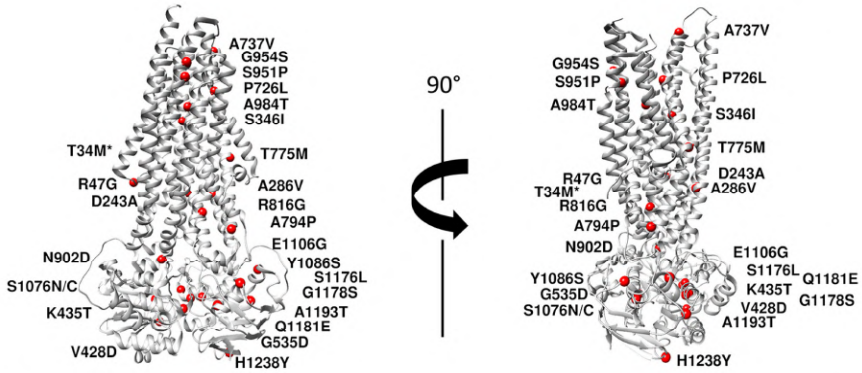

Figure 8. Structure of ABCB4. Disease-causing and potentially disease-causing mutations are highlighted in the structure of ABCB4 (human, PDB entry 6S7P,[266]) with refs 265, 266, 271−282. On the left, a frontal view, on the right the view after a rotation by 90°. Only the C_α atoms of the mutants are shown in red spheres. T34M* is not part of the structure. For the position S1076, two mutations (S1076N or S1076C) are described.[266,271]

P4-type ATPase[269] specific for PS lipids and might represent the starting point for further investigations to unravel how ABCB4 achieves specificity for PC lipids.

In general, however, the structure provides a basis to study functional ABCB4 deficiencies that lead to known clinical pictures associated with ABCB4 deficiency such as LPAC, ICP, and PFIC3.[149,270,271] Therefore, we summarized the known functional impairing mutations in Figure 8 and Table 4.

Originally, the mRNA of ABCB4 was detected especially in the liver hepatocytes (~70%) with low levels in the adrenal gland (~5−10%), lymph node (~7%), spleen (~6%), and heart (~4%), respectively.[283,284] On a protein level, mature ABCB4 is exclusively found in the canalicular membrane of hepatocytes.[285] Intuitively, this makes sense, since *m/rABCB4* expression in rodents is linked to bile acids.[256,286] Later, it was demonstrated that exclusive expression of ABCB4 in hepatocytes is due to the regulation of ABCB4 by the nuclear hormone receptor FXR (farnesoid X receptor).[287] Ligands of this receptor are mainly chenodeoxycholic and cholic acid. While the first one acts as a strong activator, the latter one is only a weak one. This could also answer the question of why ursodeoxycholic acid, a bile used as treatment in ABCB4 related diseases, did not modulate the transcription of ABCB4. In 2003, an FXR response element was identified in the human *ABCB4* gene.[288] In addition to FXR, the expression of ABCB4 is also under the control of a second nuclear hormone receptor, PPAR-α (peroxisome proliferator-active receptor alpha). Two studies demonstrated that fibrates induce the expression of mABCB4 in mice,[254,289] while PPAR-α double knockout mice displayed no response.[290] However, in humans, PPAR-α displays a different activation pattern toward fibrates than in mice. In a clinical trial, no induction of ABCB4 expression was observed in patients treated with bezafibrate (a fibrate known for lowering lipid levels).[291] Another study performed in HepG2 cells supported these results, but on the other hand observed a significant increase in the excretion of NBD-labeled PC into the pseudocanaliculi of these cells.[292] Thus, a hypothesis was put forward proposing the presence of an intracellular pool of ABCB4, which is mobilized to the canalicular membrane in the presence of bezafibrate. This is in line with the intracellular pools of ABCB11 and ABCG5/G8.[293] Interestingly, two studies demonstrated that the parenteral nutrition (TPN) affects the expression of ABCB4.

Table 4. Overview of Disease-Causing and Potentially Disease-Causing (see also Figure 8) Mutations of ABCB4[b]

disease	mutation	ref
drug induced liver injury (DILI)	T34M	265
intrahepatic cholestasis of pregnancy (ICP)	T34M	265
	G535D	273, 282
low phospholipid- associated cholelithiasis (LPAC)	T34M	265
	R47G	265, 277
	A286V	275, 278
	S1176L	282
	G1178S	282
biliary cirrhosis (BC)	T34M	265
	D243A	274, 281
	K435T	276
	G535D	273, 282
progressive familial intrahepatic cholestasis type 3 (PFIC3)	S346I	272, 279
	P726L	279
	A737V	280
	T775M	275, 279
	G954S	272, 275, 279
	S1076N/C	266, 271
	A1193T	275
impaired nucleotide binding[a]	V428D	271
	E1106G	271
impaired TMD-NBD communication[a]	A794P	271
	N902D	271
	Y1086S	271
	Q1181E	271
impaired linker phosphorylation[a]	R816G	271
TMD instability[a]	S951P	271
impaired substrate translocation[a]	A984T	271
impaired catalytic activity[a]	H1238Y	271

[a]Potentially disease-causing mutations. [b]Mutations may appear more than once if mutations are linked to more than one disease. DILI - drug-induced liver injury, ICP - intrahepatic cholestasis of pregnancy, LPAC - low phospholipid associated cholelithiasis, BC - biliary cirrhosis, PFIC3 - progressive familial intrahepatic cholestasis type 3.

In mice, TPN reduced *abcb4* expression, while *abcb11* expression was increased.[294] In contrast, in humans the

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absence of TPN caused a reduction in bile secretion and a particular dysfunction of ABCB4.[295]

A number of interaction partners are known for ABCB4, which are mainly responsible for trafficking.[285] The HS1-associated protein X-1 (HAX-1) is well studied in the case of endocytosis of ABCB11[296] and might perform the same function in the case of ABCB4. Furthermore, the myosin II regulatory light chain (MLC2) was identified as an interaction partner of ABCB4. MLC2 was necessary for proper trafficking of ABCB11,[297] but whether or not it performs the same function in the case of ABCB4 is currently unknown. A third interaction partner, the receptor for activated C-kinase 1 (RACK1), was identified and demonstrated as an essential protein for expression and targeting of ABCB4.[298] Furthermore, a PDZ domain-binding motif was identified at the C-terminus of ABCB4. This motif is known from CFTR and ABCC2 to link these transmembrane proteins to the cytoskeleton.[299−301]

4.3. The Bile Salt Export Pump ABCB11

Hepatocytes not only synthesize bile acids, they also take up bile acids, which underwent enterohepatic circulation.[302,303] This needs a coordinated action of distinct hepatocellular transporters located in both the sinusoidal and canalicular membrane of hepatocytes. Bile acid uptake occurs either in a sodium ion dependent manner mainly by NTCP or to a minor extent in a sodium-independent manner by members of the organic anion transporting polypeptides family.[304−308] In contrast, bile acid secretion across the canalicular membrane is mediated by an ATP-binding cassette (ABC) transporter, ABCB11, or bile salt export pump (BSEP). Additionally, this transporter represents the rate limiting step of hepatocellular bile acid secretion.[309−311]

Mammalian ABCB11 was identified in 1995[312] and functionally described in 1998 as the sister of P-gp,[309] mediating the transport of conjugated bile acids. In the same year, the gene of *ABCB11* was identified as the causative reason for progressive familial intrahepatic cholestasis type 2 (PFIC-2).[313]

Early after the discovery of the *ABCB11* gene, Gerloff et al. demonstrated that this ABC transporter specifically transports bile acids with a preference for taurine conjugations.[309] However, it is also capable of transporting glycine conjugated bile acids, which are the major form of bile acids in humans. Additionally, the absence of certain bile acids in the bile of PFIC2 patients confirmed the function and significance of this transporter.[313,314] Importantly, findings obtained in rodents can be extrapolated to humans since mice, rat, and human ABCB11 possess similar kinetic parameters.[315] However, one always has to keep in mind that rodent and human ABCB11 have different substrate specificity,[316] probably due to different ratios of bile acid conjugations present in both organisms. For example, human ABCB11 prefers taurine conjugated over glycine conjugated bile acids, although glycine is the major form of bile acid conjugation in humans. Furthermore, ABCB11 distinguishes between the primary bile acids: chenodeoxycholic acid containing two hydroxyl groups is preferred over cholic acid containing three hydroxyl groups.[315] Additionally, human ABCB11 transports taurolithocholate, while rat BSEP does not.[316] *In vitro* studies, on the other hand, demonstrated that ABCB11 transports also substances such as taxol,[317] pravastatin,[318] vinblastine, or calcein AM,[319] which are prominent substrates of ABCB1. Transport of sitosterol was however not observed, although it is structurally related to cholesterol and bile acids.[320] Nevertheless, the physiological function of ABCB11 remains the ATP dependent export of bile acids into the bile duct, which is emphasized by the absence of nearly 100% of bile acids in the bile of patients with severe forms of intrahepatic cholestasis due to mutations of the *BSEP* gene.[314] To address the impact of clinically relevant mutations in ABCB11 that affected the transport function, Stindt et al. developed an overexpression protocol in the methylotrophic yeast *Pichia pastoris*.[321] This was subsequently employed to established a purification protocol[322] and determined the membrane interactome of ABCB11. Additionally, the impact of mutations within ABCB11 were analyzed,[323] and a transport rate of at least eight bile acids per second and transporter was determined.[324]

The ATP-dependent excretion of bile acids across the canalicular membrane and the post-translational modification of this ABC transporter is modulated by two different compounds that are not substrates. The first compound is cholesterol.[325] Cholesterol is an integral part of the cellular membrane as well as bile. Additionally, it is the substrate of ABCG5/G8, which is the third ABC transporter within the bile ABC triumvirate. The transport efficiency for human, rat, and mouse ABCB11 were analyzed in vesicles derived from baculovirus-infected Sf9 cells overexpressing ABCB11. The membrane of Sf9 insect cell contain less cholesterol compared to the canalicular membrane of hepatocytes. Overall, the kinetic parameters (v_{max} and K_m) of ABCB11 were determined for four different bile acids at endogenous cholesterol levels of Sf9 plasma membrane vesicles and after the addition of 1 mM cholesterol. v_{max} values for all four bile acids increased in the presence of additional cholesterol, while K_m values remained unchanged, and the general preference toward these four bile acids was preserved for human and mouse ABCB11. Quantitatively, cholesterol showed the largest effect for rat ABCB11 and the smallest effect for human ABCB11,[325] while expression levels were not influenced in the presence of additional cholesterol. Moving one step further, Guyot et al. fine-tuned the concentration of cholesterol in the membrane via depletion with cyclodextrins and also observed a correlation between the concentration of cholesterol and transport efficiency of ABCB11.[326] The second compound, which affects the function of ABCB11, is lipopolysaccharide (LPS).[327] It is a component of the cell wall of Gram-negative bacteria and can cause cholestasis, which is accordingly called endotoxin- or sepsis-induced cholestasis. Normally, LPS activates macrophages by binding to the CD14 receptor. In contrast, Kupffer cells are the primary targets of LPS in the liver. Kupffer cells respond to LPS with the secretion of different proinflammatory cytokines such as TNF-alpha, IL-1beta, IL-6, and IL-10. These cytokines bind to receptors on the sinusoidal membrane of hepatocytes and initiate a complex intracellular signaling pathway. Elferink et al. investigated the effect of LPS on the level of mRNA and correct trafficking of ABCB11, NTCP, and ABCC2 in rats and human, respectively. In humans, mRNA levels of NTCP were downregulated, while this was not the case for ABCB11 and ABCC2. In contrast, none of the three transporters was detected in the membrane of liver slices after LPS treatment, suggesting that LPS effects cellular trafficking of ABCB11 and ABCC2.[327]

Recently, the structure of ABCB11 determined by single particle cryo-EM was published in the inward facing state without any bound nucleotide or substrate (PDB entry

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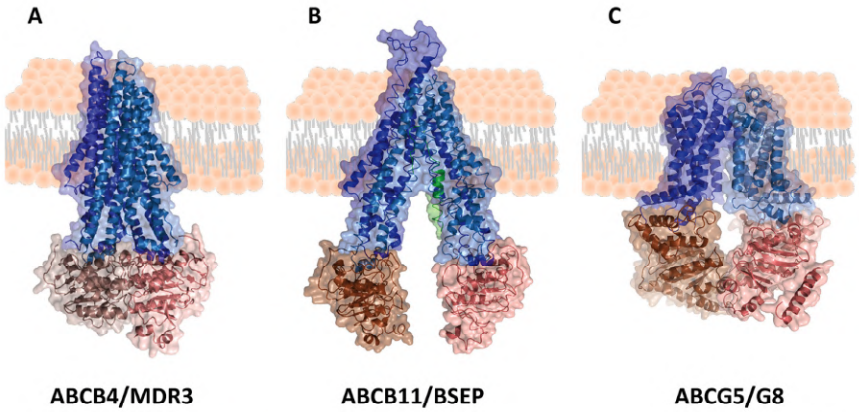

Figure 9. Structures and surface representation of the ABC transporter of the bile triumvirate colored by their domains. (A) The full-size transporter ABCB4/MDR3 (PDB entry 6S7P[266]) in the outward facing conformation with dimerized NBDs. (B) ABCB11/BSEP (PDB entry 6LR0[328]) in the inward facing conformation and totally separated NBDs. Both are colored according to their four domains: TMD1 in blue, NBD1 in brown, TMD2 in sky-blue, and NBD2 in salmon. Additionally, the unusual positioned N-terminus is highlighted in green in ABCB11. (C) AGCG5/G8 (PDB entry 5DO7[111]) are shown in using the following color code: TMD and NBD of G5 in blue and brown, respectively. TMD and NBD of G8 in sky-blue and salmon, respectively.

6LR0)[328] (Figure 9). The topology and overall structure correlate well with structures of other members to the ABCB subfamily. The full-size transporter ABCB11 harbors the four typical domains of ABC transporters, two NBDs and two TMDs. Each TMD is composed of six TMHs. First observed in the crystal structure of Sav1866[108] (Figure 3A) and subsequently also in all structures of members of the ABCB subfamily, the TMDs are split into two parts as already described above for other members of the B subfamily. Another important aspect of the ABCB11 structure concerns the N-terminus, which is different from all known structures of ABCB transporters.[329−331]

The N-terminus (highlighted in green in Figure 9) is extended and locates to the region of the putative substrate binding pocket of ABCB11. This is in line with the ABCB1 homologue from *Caenorhabditis elegans*.[332] Here, the N-terminal region adopts a helical hairpin, which inserts into the translocation cavity. However, and in contrast to ABCB1 from *C. elegans*, the N-terminus of ABCB11 adopts an α-helix followed by a loop that is located at the top of the putative substrate binding cavity. To analyze the function of the N-terminus in more detail, the authors created a truncated ABCB11 version lacking the N-terminus and determined the ATPase activity in the absence and presence of a bile acids for both wildtype and truncated ABCB11. Interestingly, no difference in ATPase activity was observed, but the expression level of ABCB11 was reduced 2-fold for the truncated version. The remaining part of the structure of ABCB11 is highly similar to the canonical structures of the ABCB subfamily. It possesses a large first extracellular loop, and both NBDs harbor the classical signature motives (see section A General Introduction to ABC Transporters in Humans). It is safe to assume that NBS1 is inactive, since the highly conserved glutamate of the Walker B motive is substituted by a methionine. The second NBS contains all catalytically important amino acid residues and is active. Since their structure represents a first snapshot, one has to assume that ABCB11 uses the "alternating access" mechanism[109] to translocate bile acids from one side of the canalicular membrane to the other. To deduce mechanistic insights, more structural snapshots such as a nucleotide-bound conformation or substrate bound structures are required.

Nevertheless, the first structure of human ABCB11 provides important insights into the three-dimensional architecture of ABCB11 and represents an excellent starting point for further structure−function analysis aimed to understand the mechanism of bile acid translocation.

The *ABCB11* gene is located on the chromosomes 2q24−31[313] and encodes for a glycosylated protein of 1321 amino acids,[309] which is spread on 28 exons.[315,333] Its expression is regulated by a complex network of stimuli acting directly or indirectly on the promoter of ABCB11. The predominant regulator is the bile acid sensor FXR.[334−336] FXR regulates not only the expression of ABCB11, but also the expression of NTCP and the bile acid biosynthesis system.[335−337] FXR regulates the expression of target genes by binding as a heterodimer together with the retinoid X receptor alpha (RXRα). RXRα is activated by the vitamin A derivative 9-cis retinoic acid. In a recent study, cholate feeding of mice with vitamin A deficiency resulted in a marked induction of mABCB11 expression at the protein and mRNA level.[338] In other studies, it was observed that vitamin D3 repressed mABCB11 expression,[339] and in contrast ursodeoxycholic acid upregulated mABCB11 expression as it functions as an agonist for FXR.[340,341] Finally, the canalicular P-type ATPase ATP8B1 (FIC1) activates the promoter of BSEP in parallel with an upregulation of FXR.[342]

However, mature ABCB11 is localized in the canalicular membrane of hepatocytes in humans. It either derives from the Golgi, which is regulated by the p38 MAP kinase,[343] or for short-term regulation from rapid inserting transporter-containing vesicles.[344] Interestingly, ABCB11 was also detected in the testis on mRNA level in humans,[345] but Northern blotting of ABCB11 in pig,[312] rat,[309] and mouse[319,346] revealed a predominant expression in the liver.[345]

Recent studies have presented evidence for the presence of distinct microdomains in the canalicular membrane of rat hepatocytes.[347] These microdomains are characterized by a high cholesterol and sphingolipid content. ABC transporters including ABCB11 were found to partition into caveolin-1

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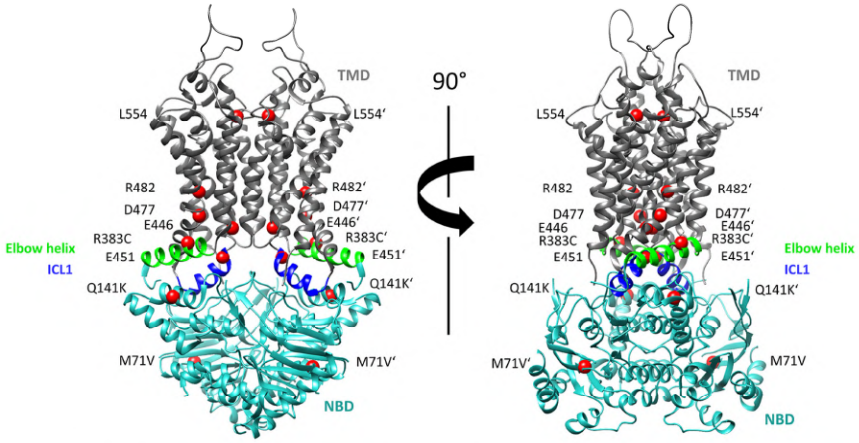

Figure 10. Structure of ABCG2 (PDB entry 6HZM)[359] Highlighted are crucial amino acids necessary for proper function (R482, E451, E446, and D477), prominent disease-causing mutations (M71V, Q141K, R383C) and the "plug" leucine L554 which separates the two cavities. The right panel is the same functional homodimer of ABCG2 after a 90° rotation. Amino acids with primes indicate the opposing monomers amino acids.

enriched microdomains.[348] There is also evidence for an intracellular pool of ABCB11 that can be mobilized if higher secretion rates of bile acids are required.[293]

5. THE ABCG SUBFAMILY - THE REVERSE TOPOLOGY HALF-SIZE TRANSPORTER SUBFAMILY

5.1. ABCG2 - Brother in Arms with ABCB1

ABCG2, also known as breast cancer resistance protein (BCRP) or methotrexate resistance protein, was discovered about 20 years ago.[349,350] It is a half-size ABC transporter that requires homodimerization to form a functional unit[351] and is part of the body's chemo immunity defense system.[352−356] It is not only expressed in tissues with detoxifying function such as the liver, but also in stem cells, fulfilling a protective role.[357] In addition to ABCB1 and the MRPs, ABCG2 is the major multidrug exporter. Accordingly, it is not surprising that ABCG2 is on the US Food and Drug Administration list for drug−drug interactions, as a transporter to be monitored.[2] Especially stem cells, but also cancer cells, overexpress these drug exporters, whereby the apparent drug concentration within the cell is reduced by continuous export. As a consequence, many compounds are exported before they reach their destination, the cytoplasm, or other cellular organelles.

On the basis of studies with stem cells, ABCG2 was considered as a stem cell marker, which led to the development of a diagnostically used antibody (5D3)[358] for rapid identification of stem cells. However, only with the elucidation of the structure of ABCG2,[359] it was clarified how the 5D3 antibody affects protein activity: the 5D3 antibody binds to one extracellular loop (ECL3) of each monomer.[359] However, the binding of one monomer is sufficient to limit the conformational-switch from the inward-facing conformation to the outward-facing conformation, thus arresting ABCG2 in the inward-facing conformation.

Independent of its role in stem cells, ABCG2 plays a central role in the detoxification function of the human body. ABCG2 is complemented in the liver by the functional relatives, ABCB1 and MRP2, as mentioned above.[352−356] ABCG2 is localized to the canalicular membrane of hepatocytes.[360] Here, it was shown in primary human hepatocytes that exposure to certain nuclear receptor ligands results in increased levels of ABCG2 mRNA similar to many other hepatobiliary transporters.[361,362] Especially, the constitutive androstane receptor (CAR) seems to transactivate ABCG2 expression.[363]

Furthermore, ABCG2 has an even broader substrate spectrum than ABCB1. The spectrum of substrates of ABCG2 covers hydrophobic, partially water-soluble, but also charged substances. Physiologically, it is now assumed that ABCG2 plays a minor role in uric acid metabolism.[364,365] Genome studies have shown that ABCG2 variants that lead to ABCG2 deficiency are associated with gout.[366,367] Gout is characterized by the crystallization of uric acid in joints, which can lead to joint hardening. On the other side, hormone derivatives such as estradiol sulfate or estradiol glucuronides are very poor substrates of ABCG2.[368] Cholesterol, a precursor of these hormones, seems to be a decisive modulator of the transporter. Depletion of cholesterol or the reconstitution of ABCG2 in artificial membrane systems without cholesterol led to a reduced ATPase and transport activity.[369−371] Experimental evidence that ABCG2 is indeed a sterol transporter including cholesterol and estradiol stems from the observation of a biphasic activation pattern of the ATPase activity of ABCG2 similar to substrates interacting with ABCB1 and the identification of a functional estrogen response element in the human *BCRP* promoter.[372,373] However, cholesterol, bile acids, or detergents such as CHAPS may also just modulate the activity of ABCG2 without being true substrates.[371,374] Further contradictory observations were made, fluorescence-labeled bile salts accumulated in cells expressing ABCG2,[375] but this observation was not confirmed in studies using radioactive-labeled bile acids.[374] Nevertheless, the detergent effect of bile salts may separate the homodimer into monomers, and this can already have a detrimental effect on the function of ABCG2.[371,374]

Moreover, ABCG2 belongs to the class of type II exporters (Figure 10). In contrast to the ABCB subfamily, its fold is characterized by the absence of a domain swap.[359,376] Another striking feature is the proximity of the two NBDs in the apo state, which is likely due to the shortened dimensions of the TMDs compared to type I exporters.[359,376]

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In summary, the ABCG family in general is characterized by a more compact form (exemplary ABCG2 in Figure 10), which is more reminiscent of bacterial import systems than its human functional relatives such as ABCB1 as the prototype of the B subfamily.[377,378]

The cryo-EM structure (PDB entry 6HZM) provided strong evidence that ABCG2 substrates can already access the transporter from within the membrane.[359,376] This model was supported by mutational and functional studies of the transporter.[364] Binding of substrates directly from the membrane was structurally confirmed by the observation of a central cavity (central cavity 1), which is accessible from the cytoplasm as well as from the inner leaflet of the membrane.[359,376] It is still a matter of debate whether substrates are more likely to be flopped, i.e., leave the protein on the extracellular half of the membrane, or whether the extracellular loops allow direct export into the extracellular space.[379] In addition to cavity 1, a second cavity closer to the extracellular space was identified in the structure. Here, an undefined electron density was observed, which was interpreted as two cholesterol molecules.[359] The two cavities are separated by leucine residues from opposing monomers (L554, Figure 10), which serve as a plug.[359] This second cavity is located directly beneath ECL3 and is not accessible from the extracellular site. It appears to be a cavity with lower substrate affinity, which becomes accessible by a conformational change and allows substrate release. Finally, cavity 2 is thought to be a shared feature of the ABCG subfamily.[359]

ABCG2 is stabilized by intra- and intermolecular disulfide bridges are present, intramolecular between C592−C608 within one monomer, and intermolecular C603−C603′ of opposing monomers.[359] Other stabilizing elements are the salt bridges between E451-K473 and E458-R383 in the so-called elbow helix.[376] Mutations in this region led to an almost complete loss of function.[376] The amphipathic elbow helix anchors the protein in the membrane and brings the NBD in close proximity to the water−membrane interface.[376] ICL1 can be seen as the equivalent of the classical coupling helix because it plays a crucial role in the regulation of NBD dimer formation.[376] Important for the function are the amino acids R482, E451, E446, and D477 (Figure 10).[376] R482 mutations alter the substrate specificity, but presumably by allosteric interactions, because it is located about 9 Å away from the binding pocket. E451 can be mutated to E451D and thus show increased ATPase activity, but loses transport efficiency. This mutation may be a first indication of decoupled transport; i.e., ATPase activity independent of substrate presence may build a functional bridge from ABCG2 to its close relative Pdr5 from *S. cerevisiae*, where decoupled ATPase activity occurs.[376,380] Indeed, the authors classified ABCG2 as an uncoupled transporter.[359,376]

Mutations of E446 and D477 also lead to an impaired ATPase and transport activity. While the amino acids discussed so far were artificial, the mutations discussed next have been identified in patients. We have made a selection of medically relevant ABCG2 variants and refer the interested reader to a recent publication for a more comprehensive collection of ABCG2 mutations.[381] The most well-known ABCG2 variant is Q141K, which is associated with hyperuricaemia.[382] This mutant leads to decreased ABCG2 expression, misfolding, and subsequent degradation by endoplasmic reticulum-associated proteins. Although the variant is still functional *in vitro*, a repulsion of the positive charge of lysine and TMH1 of the opposing monomer leads to misfolding, and the quality control of the cell degrades this variant.[376,383] Interestingly, the deficiency of this variant led to the discovery of the physiological substrate uric acid in a genome-wide study. Less relevant is the M71V variant, which is also characterized by misfolding and trafficking problems, which again led to degradation.[384] Another group of variants results in truncations that lead to a complete absence of ABCG2 from the plasma membrane. Examples for this effect are Q126X[385] in Asian populations or Q236X and R385C. Here, X implies a translation termination codon, which leads to a truncated and unfunctional polypeptide. This lack of ABCG2 in the plasma membrane of red blood cells leads to the rare blood type Jr^{-}.[386]

5.2. ABCG5/G8-Cholesterol Efflux into the Bile

Cholesterol is a key component of mammalian cell membranes and makes up to ~40% of the total lipid content of the plasma membrane.[387] Additionally, cholesterol serves as the precursor molecule for steroid hormones that modulate gene regulation and for bile acids. Since only a few cells have the capacity to metabolize cholesterol, elimination through biliary and intestinal secretion is essential to maintain homeostasis. Sterols (especially cholesterol) in peripheral tissues are mobilized to high density lipoproteins (HDL) and delivered to the liver for elimination by the reverse cholesterol transport (RCT) pathway as described in detail by Tall et al.[388] Therefore, the elimination of cholesterol is essential for proper function of liver. ABC transporters are major sterol exporters responsible for both cholesterol efflux from peripheral cells and the elimination of excess cholesterol and dietary sterols.[242,389] The human ABC transporter subfamily G is mostly responsible for sterol (especially cholesterol) transport. ABCG1 and ABCG4 mediate cholesterol trafficking in the plasma membrane and endosomes[390,391] and are believed to regulate cholesterol homeostasis in the brain and the macrophage-rich tissues. ABCG5 and ABCG8 function as a heterodimer and are mainly responsible for cholesterol transport across the canalicular membrane of hepatocytes. Nevertheless, there is evidence for expression of ABCG5/G8 in the intestine and gallbladder in addition to its expression in the liver.[104,392,393]

ABCG5/G8 was discovered in the context of sitosterolemia, a disease in which plant sterols accumulate, while cholesterol levels remain normal (see section The ABC of Liver Diseases). In 1998, the so-called sitosterolemia gene locus (STSL) was localized.[392,394] Subsequently, two teams simultaneously identified not one but two genes located in the STSL locus.[104−106] These genes encoded for the two highly homologous half-size ABC transporters, ABCG5 and ABCG8.

Similar to ABCB4 and ABCB11, mouse models were used to pinpoint the function of this heterodimeric ABC transporter. mABCG5- or mABCG8-deficient mice showed the same phenotype as human patients.[393,395] Loss of (m)ABCG8 in mice and men resulted in increased intestinal absorption of noncholesterol sterols and cholesterol. In addition, knockout of either m*Abcg8* or both genes led to a failure to secrete these sterols into bile. It was unclear at that time whether or not mABCG5 or mABCG8 might have independent function. Overexpression of human ABCG5 and ABCG8 in mice led to supersaturation of cholesterol in bile, with excretion of large quantities of neutral sterols, reduced plasma plant sterol levels, and a compensatory up-regulation of the cholesterol synthesis rates.[396] Furthermore, in these animals lower levels of HDL

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plasma cholesterol and lower cholesterol absorption rates were observed, probably due to an increased bile efflux by human ABCG5/G8. Subsequently, a study demonstrated that overexpression of both genes in hepatocytes was associated with a reduction in the production of apoB-lipoproteins and atherosclerosis.[397] Surprisingly, overexpression of ABCG5/G8 did not lead to artificial gall stone disease, although supersaturation of cholesterol was measured in the bile of these mice. Therefore, it was concluded that the heterodimer of both half-size transporters functions as a sterol efflux of both cholesterol and xenosterols, in liver and the small intestine. Unfortunately, the exact mechanism of how these transporters provide efflux is yet not completely understood. However, it is known that bile acids play a major role. Bile acid-containing micelles were found to be an acceptor of these sterols.[398−400] Furthermore, bile acids are essential for the function of ABCG5/G8 in the hepatobiliary system.[401] In the case of the purified transporter, ATP hydrolysis was only observed if bile acids were present.[402] This suggested that bile acids directly interact with ABCG5/G8 or that the transporter somehow recognizes that cholesterol acceptors are present. The latter hypothesis was proposed by Small, who postulated that ABCG5/ABCG8 may act as "extruder", exposing sterols to the outer leaflet of the membrane for facilitated extraction into the lumen of sterol acceptors such as bile acid: phospholipid complexes.[403] Others have suggested that ABCG5/G8 acts as a floppase similar to ABCB4, just flipping cholesterol from the inner to the outer leaflet of canalicular membranes.[404,405] To support or refute one of these models, a study was conducted, which demonstrated that forced biliary secretion of very hydrophobic bile acids failed to increase biliary cholesterol secretion in *Abcg8*$^{-/-}$ mice since it was not possible to extract cholesterol from the rigid canalicular membrane.[406] Although these data support the "flop hypothesis", a subsequent lifting of cholesterol from the outer leaflet is not disproven by this study.

The crystal structure of heterodimeric ABCG5/G8 was published in 2016 at a resolution of 3.9 Å (PDB entry 5DO7, see Figures 3 and 12).[407] Surprisingly and as already elaborated in section A General Introduction to ABC Transporters in Humans, the expected domain swap arrangement was not present. Rather, the structure resembled the fold of type I ABC importers.[408] Each TMD composed of six TMH resembled a more or less isolated entity with the putative substrate-binding pocket at the interfaces. This moderate degree of amino acid conservation is contrasted by a high degree of structural conservation reflected by a root mean squared deviation (rmsd) of only 2.0 Å. Interestingly, ABCG5/G8 harbors more α-helices in its extracellular loops, which again reflect the higher similarity to type I ABC importer systems. The lack of electron density for nucleotides and the separation between opposing NBDs indicates that the ABCG5/G8 structure represents a nucleotide-free state in the inward facing conformation, which is similar to other nucleotide-free structures of ABC exporters[79,409,410] and importers.[145,411] Nonetheless, the two NBDs contact each other at the extreme cytoplasmic end to interact through a pair of NPXDF motifs. These motifs are conserved in the ABCG family and are required for cholesterol efflux by ABCG1.[412] Another feature of ABCG5/G8 is the asymmetry of the NBDs. As stated in section A General Introduction to ABC Transporters in Humans, each transporter comprises two NBS, in which the Walker A motif of one NBD with the ABC signature motif of the other NBD[413,414] come together to interact with ATP. ABCG8 contains a degenerate Walker A motif (GSSGCGRAS, see Table 2). Additionally, ABCG5 possesses a degenerated ABC signature motif (ISTGE, see Table 2). As a consequence, one NBS is degenerated, while the other is canonical, i.e., actively hydrolyzing ATP. This catalytic asymmetry is supported by functional studies using recombinant ABCG5/G8 mutants in ABCG5/G8-deficient mice.[415] In cases in which the Walker A or Walker B motif in ABCG5 was mutated, biliary sterol secretion was abolished. On the other hand, mutations of the corresponding residues in ABCG8 showed no effect on the biliary sterol secretion. For the ABC signature motif, the opposite effect was observed. Mutations on the ABC signature motif of ABCG5 had no effect on the biliary sterol secretion, while mutations of the corresponding residues in ABCG8 abolished sterol secretion.[416]

As elaborated above, this functional asymmetry is mirrored by a structural asymmetry. In brief, the degenerated signature motif of ABCG5[416] is located next to the CnH/CpH/E-helix bundle. In contrast, the canonical signature motif of ABCG8 is positioned near the three-helix bundle of G8. This arrangement suggested that the stable three-helix bundle of ABCG5 acts as an inflexible body, whereas the three-helix bundle of ABCG8 exhibits greater flexibility. With the proximity of G8-CpH to the catalytically active NBD, these conformational changes could allosterically link ATP hydrolysis to sterol transport.[407]

The structure of ABCG5/G8 harbors additional electron density, which might represent cholesterol.[407] Assuming that this electron density represents indeed cholesterol, an efflux mechanism was proposed. According to this proposal, the binding pocket of ABCG5/G8 is located at two symmetrical "vestibules" on opposing faces of the TMD dimer. Additionally, a lateral gate toward the lipid bilayer, which extends to the center of the dimer interface is present. One of the two vestibules is flanked by TMH1 and TMH2 of ABCG5 as well as TMH4, TMH5, and TMH6 of ABCG8. The other vestibule is formed by the inverse arrangement (TMH1 and TMH2 of ABCG8 as well as TMH4, TMH5, and TMH6 of ABCG5). Additionally, a kind of "ceiling" is formed in each vestibule by an α-helix from an extracellular loop pointing into the membrane. Several residues of the vestibule are highly conserved and may line an entry site for sterols to access the core of the heterodimer interface. To prove this proposal, a conserved amino acid of ABCG5 was mutated (Y432A). Although no complete absence of cholesterol transport was observed in mice, it was drastically decreased compared to the wild type transporter.

On the basis of a molecular dynamics (MD) simulation of ABCG5/G8, an upward movement of the TMDs and an inward movement of the NBDs occur simultaneously.[407] This result was supported by an evolutionary analysis. Here, several coevolved pairs of amino acids were identified, which are conserved but more than 8 Å apart in the apo structure. During a transport cycle, these residues might come in contact. However, several studies of ABCG5/G8 indicated an asymmetric use of the two NBDs,[415,416] the requirement of bile acids for sterol efflux[400,401] and ATP hydrolysis.[402] Thus, more structural information is required for this cholesterol transporter to understand the transport cycle on the molecular level.

The genes of ABCG5 and ABCG8 are in close proximity (only approximately 370 bp apart) and arranged in a head-to-tail orientation.[104] Both indicate that they share a common

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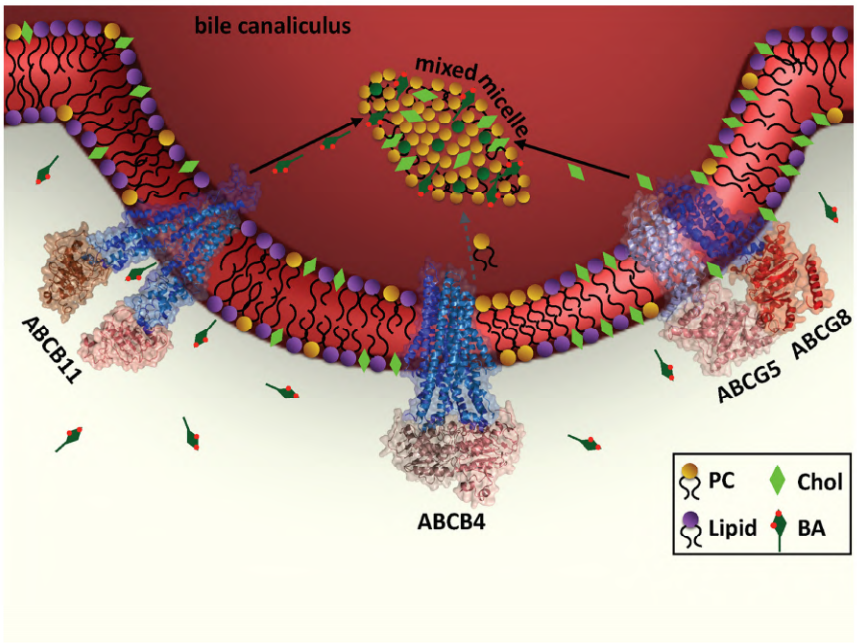

Figure 11. Bile triumvirate of the canalicular membrane. The ABC transporter ABCB11 and ABCG5/G8 are involved in the secretion (black arrows) of the bile compounds bile acids (BA) and cholesterol (Chol) across the canalicular membrane (violet lipid can represent any other kind than PC), respectively. In concert with PC-lipids (PC), which are flopped by ABCB4 from the inner to the outer leaflet, these three compounds from mixed micelles in the bile canaliculus. This prevents bile acids from solubilizing cell membranes and cholesterol from crystallization. However, it is not yet known how PC-lipids are taken up into the mixed micelles (gray dashed arrow). The full-size transporter ABCB4 (PDB entry 6S7P) and ABCB11 (PDB entry 6LR0[328]) are colored by their four domains: TMD1 in blue, NBD1 in brown, TMD2 in sky-blue and NBD2 in salmon, respectively. AGCG5 and G8 (PDB entry 5DO7) are shown as dimer in corresponding colors: TMD and NBD of G5 in blue and brown, TMD and NBD of G8 in sky-blue and salmon, respectively.

promotor and regulatory elements. The latter have been identified within the intergenic region, including the nuclear transcription factor hepatocyte nuclear factor 4 alpha (HNF4α), GATA4, GATA6,[417] and liver receptor homologue-1 (LRH-1).[418] HNF4 α seems to be the major reason for tissue specific expression of ABCG5/G8 and can be partially explained by epigenetic regulation. In tissues that do not express ABCG5/G8, chromatin in the regulatory region is methylated and histones are acetylated.[419] Although no specific response elements have been identified for FXR, FXR plays a prominent role in transcriptional control of ABCG5/G8 via bile acid signaling.[420] Additionally, administration of cholic acid in mice enhances ABCG5/G8 transcription. One study demonstrated that FXR may be partially mediated by fibroblast growth factor 15/19 (FGF15/19), while the other observed an inhibition of nuclear factor kappa-light-chain-enhancer of active B cells (NF-κB).[421] Two additional liver receptor response elements have been identified in the *ABCG5/G8* genes. Interestingly, one study demonstrated that insulin signaling can regulate expression of ABCG5/G8 via forkhead box protein O1 (FOXO1).[422]

Recombinant expression revealed that both transporter halves (ABCG5 and ABCG8) are glycosylated, depend on calnexin and calreticulin chaperones proteins for folding, and require dimerization to exit the endoplasmic reticulum.[423,424] Trafficking of the complex to the canalicular membrane is poorly understood, but there is evidence that bile acids, sterols,[395,425] and cAMP[426] are important factors. Similar to ABCB11, an intracellular pool of ABCG5/G8 transporters might exist for cases in which cholesterol secretion has to be increased. In the case of the latter, these transporters can be mobilized to increase cholesterol efflux as it was suggested by Guyot and Stieger.[293]

5.3. The Bile Triumvirate ABCB4, B11, and ABCG5/G8

Primary bile mainly consists of bile acids, PC lipids, cholesterol, which form mixed micelles, bilirubin glucuronides, and organic anions. Analysis of bile from healthy humans revealed that the bile in the gallbladder consists of bile acids (~70%), PC lipids (~20%), and cholesterol (~8−9%).[427] Each of these substances is transported by an individual ABC transporter into the canaliculus. Bile acids by ABCB11, PC-lipids by ABCB4, and cholesterol by ABCG5/G8 (Figure 11).

In the previous chapters, we provided detailed information about these liver ABC transporters. Here, we will briefly summarize details about the interplay of these three ABC transporters. Already in 2003, Small concluded that these three ABC transporters interplay in a specific order to ensure the proper formation of bile.[403] Bile acids in the canaliculi promote the release of PC lipids from the membrane to form mixed micelles, which are necessary for solubilization of cholesterol to prevent its crystallization.[403] It was reported that the reduction of PC lipids in primary bile leads to an increase of the toxic activity of bile acids against the ducts, which can result in cholestatic liver diseases.[428] This study emphasized how important a balanced ratio of PC lipid to bile acid and cholesterol is. For ABCG5/G8, one study demonstrated that *in vitro* ATPase activity is absent without any bile acids present.[402] This suggests that ABCG5/G8 sense the substrate

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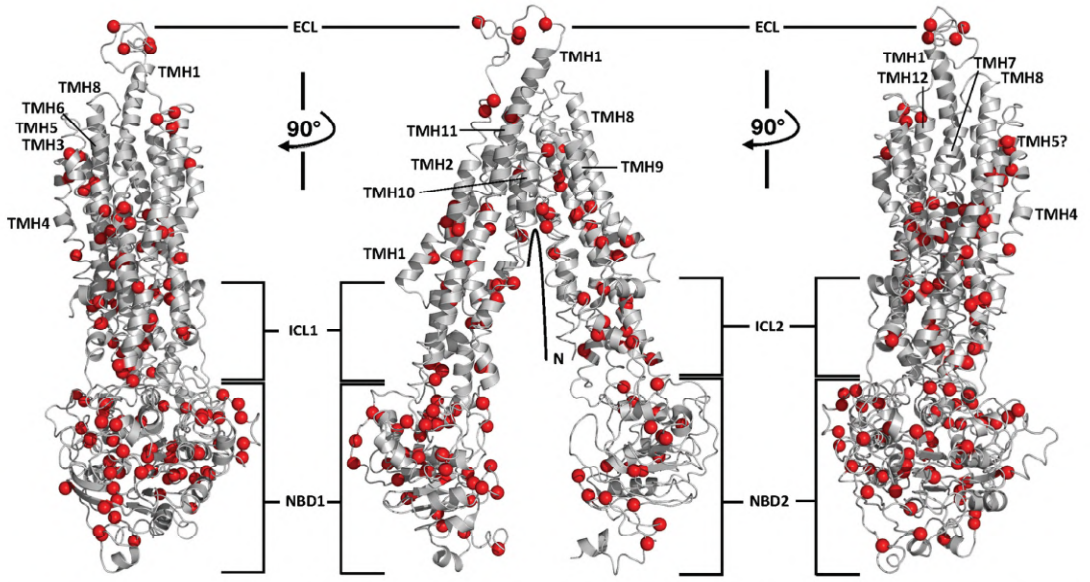

Figure 12. ABCB11 structure with highlighted mutations. The red spheres highlight the positions of disease-causing mutations in ABCB11, which impair function. Because of the large number of identified mutations in ABCB11, mutations, which affect expression, degradation, splicing, and trafficking were not taken into account. This lasso resulted, for reasons of clarity, that not every position was labeled with the corresponding mutation. Rather only the TMHs, ICDs, and NBDs were labeled. According to this scheme, a complete list of the mutations is provided in Table 6. 1: these amino acids are in a flexible region (the loop connecting NBD1 with TMD2) and therefore not resolved in the single particle cryo-EM structure (PDB 6LR0[328]). Left panel: the transporter was rotated by 90° with respect to the left panel for better visualization of all mutants.

of ABCB11 and becomes transport competent. Following the same line, ABCB11 responds to the concentration of cholesterol in the membrane.[325,326] Elegant studies by the Stieger laboratory demonstrated that administration of taurocholate increases the rate of PC secretion in canalicular vesicles derived from rats.[309] These examples clearly suggest a crosstalk between the transporters of the ABC triumvirate. However, we still lack molecular insights into a concrete crosstalk, and many more details are completely unknown.

6. THE ABC OF LIVER DISEASES

6.1. Diseases Related to ABCB11

Over 300 mutations and polymorphisms in the ABCB11 gene have been identified so far, which cause hereditary liver diseases. Fortunately, an up-to-date database provides the available information on mutant variants of ABC transporters including ABCB11 (see http://abcmutations.hegelab.org/).[429] Additionally, a number of reviews focused on ABCB11 mutations and provide huge lists of the known disease-causing ABCB11 variants or tried to map these in different models.[271,430−438] Although all regions of ABCB11 are affected, an apparent hot spot for mutations are the NBDs and cytoplasmic loops (Figure 12 and Table 5).[271,436]

Progressive familial intrahepatic cholestasis (PFIC) represents a number of inherited cholestatic liver diseases, which typically reveal during early childhood[434,439] and likely lead to liver cirrhosis, often resulting in the need of liver transplantation (LTX).[440] As stated above, PFIC2 is based on mutations in the ABCB11 transporter (see section Progressive Familial Intrahepatic Cholestasis (PFIC2) and Benign Recurrent Intrahepatic Cholestasis Type 2 (BRIC2)). Interestingly, it was shown in 2009 that children who are transplanted because of PFIC-2 have a special risk for disease recurrence. The reversion of the symptoms is caused by the *de novo* appearance of anti-BSEP antibodies.[441,442] The risk of disease recurrence after LTX depends on the cause of liver failure. In the case of hepatitis C infection, liver damage always recurs, although the severity can be altered due to the host immune system, the virulence of the virus, or the regimen of immunosuppression.[443,444]

About 10 years ago, a recurrence of PFIC2 phenotypes after LTX was described.[441,442] After a patient underwent a second LTX, a cholestatic liver situation again developed after one year, although no evidence of rejection or other causes of cholestasis were detected. Finally, analysis of the posttransplant serum revealed the presence of antibodies directed against ABCB11, which were absent in the pretransplant serum. Obviously, *de novo* anti-ABCB11 antibodies developed after LTX,[445] which impair the efflux of bile acids by binding to the first extracellular loop of ABCB11.[441,446] As a therapy, reduction of serum antibodies by plasmapheresis and anti-CD20 antibodies (rituximab), which temporarily alleviated cholestasis,[441] was achieved. Subsequently, further cases of anti-BSEP antibodies were reported,[447−450] and it is estimated that up to 8% of transplanted PFIC-2 patients develop anti-BSEP antibodies.[448]

These findings raised the question of why no autoimmune disease for ATP8B1 and ABCB4 (recurrence of PFIC1 or PFIC3) has been detected so far. One hypothesis is that, due to the low abundance of ABCB11 in PFIC2 patients,[434] they do not achieve autotolerance. After LTX, however, the massive availability of ABCB11 may stimulate BSEP-reactive T lymphocytes, which lead to clonal expansion of anti-BSEP antibody-producing B cells. Consequently, the absence of ABCB11 in the patients is a requirement or indication to develop an autoimmune disease against ABCB11.[441,451]

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Table 5. Diseases Caused by ABCB11 Mutations in Correlation with Figure 12[a]

disease	localization	mutations	refs
PFIC2	N-terminus	G19R, L50S	271, 310, 313, 314, 432−436, 438, 442, 551−579
	TMH1	M62K, C68Y	
	ECL	C107R, I112T, W114R	
		Q121L	
	TMH2	Y157C, A167V, A167T	
	ICL1	I182K, M183V, M183T	
		G188W, V284L, E297G	
		R303G, R303K, Q312H	
		R313S, A390P	
	TMH3	M217R, R223C, S226L	
		G238V	
	TMH4	T242I, A257G	
	TMH5	G327E, W330R, C336S	
		Y337H, W342G	
	TMH6	A382G, R387H	
	NBD1	G410D, L413W, I420T	
		D440E, G455E, K461E	
		T463I, R470Q, V481E	
		R487H, R487P, N490D	
		I498T, I512T, N515T	
		R517H, R517T, F540L	
		I541L, I541T, F548Y	
		D549V, G556R, A570T	
		L581F, A588V, S593R	
		I627T, E636G	
	Connecting Loop[1]	R698C, R698H, S699P	
		E709K	
	TMH7	G758R, G766R	
	TMH8	Y818F	
	ICL2	R832C, R832H, T859R	
		A865V, Q869R, R948C	
		A1044P, L1055P	
	TMH9	G877R	
	TMH10	S901R	
	TMH11	N979D, G1004D	
	TMH12	T1029K, G1032R	
	NBD2	C1083Y, A1110E, S1114R	
		G1116E, G1116F, G1116R	
		S1120N, R1128C, S1144R	
		S1154P, N1173D, N1211D	
		V1212F, L1242I, D1243G	
		A1283V, G1292V, G1298R	
BRIC	TMH2	E135L, E137L	271, 310, 430−435, 438, 451, 454, 553, 557, 558, 560−563, 565, 566, 570, 571, 576, 578, 580−582
	ICL1	R181I, E186G, L198P	
		A277E, E297G	
	TMH6	G374S	
	NBD1	R432T, V444A, I498T	
		A570T, T586I, G648V	
		T655I	
	TMH10	T923P	
	ICL2	A926P, R948C, R1050C	
	TMH11	G1004D	
	NBD2	G1116R, R1128H, L1197G	
		R1231Q	
ICP	ICL1	V284D	271, 274, 430, 432, 433, 435, 438, 485, 563, 572, 581−585
	NBD1	R415Q, V444A, N591S	
		T655I	
hypothyroidism (elevated TSH)	TMH1	L71H	553
pediatric hepatocellular carcinoma	ICL1	A389P	454, 586

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Table 5. continued

disease	localization	mutations	refs
	ICL2	R1050C	
DILI	NBD1	V444A, N591S	271, 430, 433, 571

[a]Listed are only these mutations, which impair protein function and sorted according to the disease that they cause. Mutations, which affect expression, degradation, splicing, and trafficking were not taken into account.

6.2. Progressive Familial Intrahepatic Cholestasis Type 2 (PFIC2) and Benign Recurrent Intrahepatic Cholestasis Type 2 (BRIC2)

These two diseases have in common that they are hereditary and caused by loss of function of ABCB11. In both diseases, ABCB11 fails to export bile acids due to a complete lack or insufficient amount of functional ABCB11 in the canalicular membrane of hepatocytes. This leads to intrahepatic cholestasis characterized by reduced bile synthesis, less bile flow, and accumulation of bile acids within the hepatocyte.[452] Several mutations in ABCB11 are the causative reason for PFIC2 and/or BRIC2. These include the relatively frequent E297G and D482G variants, which are present in 58% of European PFIC2 patients.[434]

PFIC is an early onset disease with a rapid progression leading to end stage liver damage before adulthood. Defective ABCB11 can lead to impaired bile acid secretion, accumulation of bile acids in hepatocytes, and subsequent hepatocellular injury, apoptosis, or necrosis. Missense mutations cause less severe disease forms, whereas the risk of hepatobiliary malignancy is increased in the case of mutations leading to truncations of ABCB11.[453] PFIC patients often develop pediatric hepatocellular carcinoma and subsequently cholangiocarcinoma.[454,455] BRIC, in contrast, is the milder form of PFIC and comes with recurrent episodes of cholestasis and consequently pruritus without substantial liver damage in most cases.[431]

The clinical features of the patients usually include cholestatic jaundice in the neonatal period. Pruritus is usually the dominant feature. Clinical features and disease progression may be more severe in PFIC2 than in PFIC1. Other features may include growth failure and deficiency of fat-soluble vitamins. These patients are at considerable risk of hepatobiliary malignancy, hepatocellular carcinoma, or cholangiocarcinoma, and thus close surveillance is important for these patients.[434] The diagnosis is based on elevated bile acids, normal serum GGT, electron microscopy showing amorphous, or filamentous bile. Light microscopy shows nonspecific giant cell hepatitis. Immunohistochemical staining for canalicular BSEP is negative in majority of patients.[453] In general, these patients have poor response to ursodeoxycholic acid. Partial external biliary diversion (PBED), which interrupts the enterohepatic circulation by partially diverting bile from the gallbladder through a loop of jejunum connecting the gallbladder to abdominal skin, may be useful and if performed early enough may delay or interrupt hepatic injury. Liver transplantation is usually successful in this group of patients, although recurrence after transplantation has been reported.[442]

6.3. Acquired BSEP Deficiency Syndrome (Intrahepatic Cholestasis)

Inhibition of ABCB11 by endogenous metabolites such as aberrant bile acids[456] or drugs (see section Drug Induced Liver Injury (DILI)) leads to the so-called acquired BSEP deficiency syndrome. People with genetic or acquired defects in the biosynthesis of bile acids may suffer from cholestasis. In patients with a deficiency of steroid dehydrogenase/isomerase, aberrant bile acids are observed in urine and bile. Other non-natural bile acids are detected in the urine of patients with a deficiency of SP-reductase. It was observed that aberrant bile acids can inhibit the apical, ATP-dependent transport system for cholyltaurine in a competitive manner. These unusual bile acids act as cholestatic agents by inhibiting the canalicular ATP-dependent transport system for bile acids, which constitutes the rate-limiting step in the overall process of bile acid transport across hepatocytes and thereby leads to intrahepatic cholestasis[456]

6.4. Drug Induced Liver Injury (DILI)

Drug induced liver injury is different to most of the other liver diseases, in which ABC transporters are involved. While in the other cases the transporter is mutated or expression, proper trafficking, or membrane insertion is reduced, it is commonly accepted that DILI affects the wildtype situation. Additionally, patients who suffer from DILI normally also suffer from a more prominent disease, for which a drug is administered. This drug, especially if it is given orally and thereby taken up in the duodenum, perfuses the liver as the first organ. If this drug is taken up into the hepatocytes, it can potentially act as an inhibitor for the ABC transporter in hepatocytes. Especially if one of the ABC transporters involved in bile formation is affected, the delicate equilibrium between bile acids, PC lipids, and cholesterol is changed. This can result in symptoms as if the patient suffers from a liver disease related to one of these ABC transporters. That is the reason why a lot of drugs and treatments are tested for their effect on different ABC transporter, for example, ABCB11. For a more detailed investigation to DILI, we refer the interested reader to two recent reviews.[457,458]

6.5. ABCB4 - A Transporter with Trending Relevance for Hepatobiliary Diseases

In a sequence analysis of an Icelandic population, ABCB4 was the only hepatobiliary transporter correlating to an increased risk for a liver disease if deficient.[459] In general, diseases linked to ABCB4 deficiency can be sorted based on altered expression levels, misfolding, stability, or activity.[282] Here, the reduction in PC lipid presence in the bile correlates with the severity of the underlying ABCB4 mutation. As a result, free bile salts that would be otherwise safely packed into mixed micelles may lead to bile duct injuries, a principle that is known as the "toxic bile concept".[460] Mild forms of cholestasis related to ABCB4 deficiency are indicated by elevated serum levels of γ-GT.[270] Intermediate forms of cholestasis, which involve ABCB4 deficiencies are intrahepatic cholestasis of pregnancy (ICP) and low phospholipid associated cholelithiasis (LPAC). Even though the genetic background may be diverse (see next chapter - Intrahepatic Cholestasis of Pregnancy (ICP)) common fetal as well as maternal symptoms like pruritus can occur.[461,462] The fetus may be at risk of preterm birth or even fetal death. Even though ICP is reversible, patients may be at higher risk for subsequent hepatobiliary diseases.[463] Symptoms

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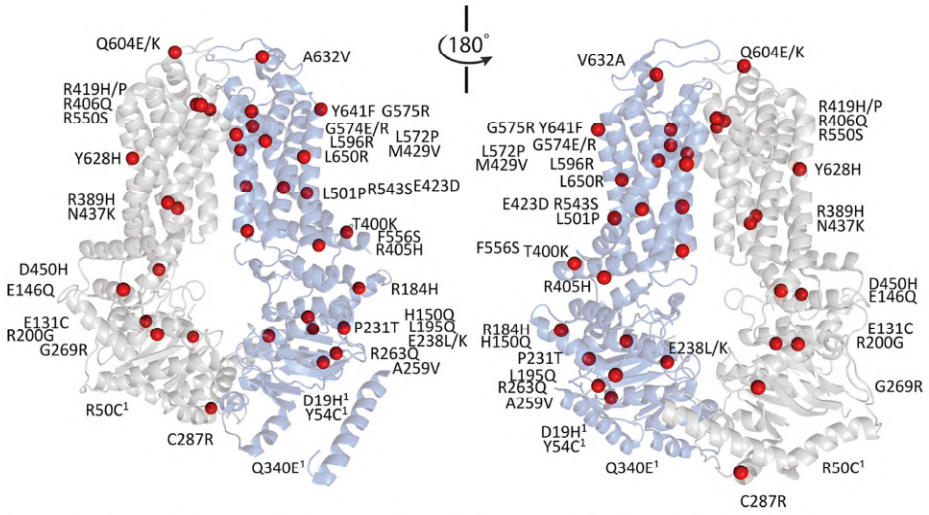

Figure 13. ABCG5/G8 structure with highlighted mutations. Left panel of the central structure of the ABCG5/G8 transporter is the monomeric ABCG5 structure (gray), in the right panel is the structure of monomeric ABCG8 (sky-blue). Red spheres correspond to positions of disease-causing mutations. 1: these amino acids are in flexible regions such as the two termini or loops and therefore not resolved in the X-ray structure (PDB 5DO7[111]). The transporter was rotated by 180° for better visualization of all mutants.

for LPAC are intrahepatic hyperechogenic foci, sludge, or microlithiasis probably via an increased biliary-cholesterol to phospholipid ratio resulting in cholesterol crystallization.[464] A disease of modern pharmaceutic treatment could lead to drug-induced liver injuries (DILI), in which drugs administered to cure even liver-unrelated diseases negatively interact with hepatobiliary transporters such as ABCB4.[465] Last but not least, progressive familial intrahepatic cholestasis type 3 (PFIC-3) is the most severe form of ABCB4 deficiency. It is described by a complete absence of biliary phospholipids and injured bile canaliculi. Symptoms are jaundice, discolored stool, hepatomegaly, splenomegaly, and pruritus.[466]

In fact, the severity of ABCB4 deficiency is not only based on the mutation itself, but also on the status of zygosity. Admittedly, heterozygous mutations are less severe than homozygous ones. One key to fight genetic diseases is therefore a comprehensive summary of mutants linked to empirical data of patients or even better to their molecular basis. A genetic investigation for example connected homozygous, compound heterozygous, and heterozygous mutations of ABCB4 with molecular reasons based on MD simulation.[271] In recent years, several drugs were investigated to cure ABCB4 deficiency; the success however is strongly linked to the underlying severity of the mutation.[279,282,462,467,468]

6.6. Intrahepatic Cholestasis of Pregnancy (ICP)

Liver dysfunction during pregnancy represents a unique challenge to health care providers and is detected in approximately 3% of all pregnancies.[469] However, severe liver injury is encountered in only a small subset of these patients.[470] Early recognition and appropriate management are warranted to avoid potentially serious consequences for both the mother and the fetus. Other major considerations are the teratogenic effect of certain medications and the risk that pregnancy may impose on any underlying liver condition. Symptoms may vary according to the severity of liver injury from abdominal pain, nausea, and vomiting to signs of acute liver failure. Common physical examination findings in pregnancy are palmar erythema and spider angiomas, and their presence may not indicate an underlying chronic liver disease. Normal changes seen in pregnancy should be considered in the interpretation of liver function test (LFT).[469] Alkaline phosphatase can be elevated in the third trimester due to placental production. Alpha fetoprotein (AFP) is not routinely obtained in this group of patients; however, it is produced by the fetal liver and commonly elevated.[471] Differential diagnosis include diseases unique to pregnancy, pregnancy in the setting of underlying chronic liver diseases, and coincidental conditions to pregnancy such as viral hepatitis, and Budd-Chiari.[472] Gallstones are commonly seen during pregnancy and liver ultrasound remains the best initial diagnostic imaging modality, followed by magnetic resonance imaging without gadolinium[473] and liver biopsy.[474,475]

ICP is the most common liver disease to appear during pregnancy and typically arise during the fourth to six week of pregnancy.[476] The incidence of ICP is higher among pregnant women infected with hepatitis C virus, which raises the concern for the need to screen all patients with ICP for HCV.[477,478] The other risk factors for ICP include personal or family history, multiple pregnancies, advanced maternal age, and cholelithiasis. Estrogen and progesterone, genetic mutations in bile acid transport proteins (such as ABCB11), and environmental factors play a role in the pathogenesis of ICP.[479−484] In the case of ABCB11, the well-known V444A polymorphism is a predisposition for ICP.[485] Additionally, mutations in ABCB4 are often observed in ICP.[486,487]

A study from 2015 demonstrated that ICP (due to an *Abcb11* deficiency) is associated with adverse neonatal survival and is estimated to impact between 0.4 and 5% of pregnancies worldwide. The neonatal death occurs within 24 h due to atelectasis-producing pulmonary hypoxia, which recapitulates the neonatal respiratory distress of human ICP. In neonates of Abcb11-deficient mothers elevated pulmonary bile acids could be obtained, which altered the pulmonary surfactant structure. These findings have important implications for neonatal

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respiratory failure, especially when maternal bile acids are elevated during pregnancy.[488]

As treatment so far only the application of ursodeoxycholic acid (UDCA) is successful. It consistently reduces the symptoms and improves LFTs.[489,490] The mechanism of action is not completely understood, but studies have demonstrated that after treatment there is a reduction in total serum bile acids in both, maternal and umbilical cord serum, and a qualitative change in the serum bile acid pool.[341,491,492] For more detailed information we recommend excellent reviews such as refs 469, 484, 493−496.

6.7. Diseases Related to ABCG5/G8

Abnormal elevations in plasma cholesterol contributes to hyperlipidemia, a critical factor leading to cardiovascular diseases and other metabolic disorders.[497] Twenty-four variants are known for ABCG5, which are related mostly to sitosterolemia. In ABCG8, 40 mutations have been identified so far. Again, the majority of these variants is related to sitosterolemia. The review by Tada et al. provides a recent and excellent overview of the localization of ABCG5/G8 mutations and polymorphism on DNA level[498] and we mapped these mutations on the crystal structure of ABCG5/G8 (Figure 13).

For ABCG5, it is obvious that there are three hot spots next to the NBD (Table 6). The first one covers amino acid residues E146 and D450 in the coupling helices. Since the coupling helix of ABCG5 are supposed to be inflexible due to the asymmetry in the NBDs (see section "Structure of ABCG5/G8") it can hardly suggest how these mutations affect the transporter. The second hot spot (R389 and N437) is positioned at the lower end of the TMD. But they are in the polar relay of ABCG5 and conserved polar residues that form the TMD polar relay proximal to CpH and CnH.[111] The third one comprises the three arginines R406, 419, 550, and glutamate Q604, which are located at the extracellular part of the TMD and the external loops. They might be important for cholesterol release. In the case of ABCG8 (Table 7), the majority of the mutations, which are not located in the NBD, are spread throughout the entire TMD. Nevertheless, they can be divided into four groups. First, there are amino acids of the coupling helices (H150 and R184), which possibly abolish the communication between NBD and TMD. Other amino acids (E423, L501, and R543) are located at the polar relay of ABCG8.[111] The third hot spot is located at the upper end of the TMHs facing toward the possible gate and ABCG5. It is compromised of the amino acids M429, L572, G574, and G575, G574, and G575 are part of the heterodimer interface and abolish dimerization, if mutated. The last group of mutations are located in the TMD facing the lipid bilayer. It includes the amino acids T400, Y641, and L650. The T400K mutation sits on the tip of the CnH helix and is a risk factor for gallstone disease.[499,500] Interestingly, only one mutation (V632) occurs in the external loops of ABCG8, but is localized at the same height and position as Q604 in ABCG5. Mutation in both lead to gall stone disease.[500−502] Nevertheless, it was shown that only Q604 is associated with cholesterol kinetics.[503]

Table 6. Diseases Caused by ABCG5 Mutations in Correlation with Figure 13[a]

disease	mutations	refs
sitosterolemia	E146Q, R419H	105, 106, 502, 503, 587−594
	G269R, N437K	
	C287R, D450H	
	R389H, R550S	
	R406Q, Q604E	
	R419P, Q604K	
familial hypercholesterolemia[b]	Y131C	595
	R200G	
	Y628H	
	E146Q	
gallstone disease	R50C	105, 502, 503, 593, 594
	Q604E	
	Q604K	
low HDL-C level	Q604E	594

[a]Mutations may appear more than once if mutations are linked to more than one disease. [b]Only the pathogenic are listed.

Table 7. Diseases Caused by ABCG8 Mutations in Correlation with Figure 13[a]

disease	mutations	refs
sitosterolemia	R184H, L501P	104, 106, 498, 591, 593, 594, 596−603
	L195Q, R543S	
	P231T, L572P	
	E238L, G574E	
	E238K, G574R	
	A259V, G575R	
	R263Q, L596R	
	Q340E, Y641F	
	R405H, L650R	
	E423D	
familial hypercholesterolemia[b]	H150Q, R543S	595
	E238L, F556S	
	R263Q, G574E	
	R405H, G575R	
	E423D, G575R	
gallstone disease	D19H, M429V	498−503, 591, 594, 602, 604−606
	Y54C, V632A	
	T400K	
high LDL-C levels	D19H	502, 503, 604, 498, 499, 501, 591, 594
	T400K	
	V632A	
renal disease in type 2 diabetic patients	T400K	607

[a]Mutations may appear more than once if mutations are linked to more than one disease. [b]Only the pathogenic mutations are listed.

6.8. Sitosterolemia

Sitosterolemia is a rare, monogenic, autosomal recessive disease characterized by the presence of plant sterols (the major type of xenosterols) and premature coronary artery disease in the face of relatively normal plasma cholesterol level.[504] When plant sterols are ingested, they compete with the bulk cholesterol for solubilization, thereby reducing dietary absorption of cholesterol and lowering plasma cholesterol.[505] However, the majority of xenosterols that enter enterocytes are immediately excreted via ABCG5/G8 back into the intestinal lumen. Human subjects that fail to actively prevent xenosterol absorption develop sitosterolemia named after the most

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Chem. Rev. 2021, 121, 5240−5288

abundant dietary xenosterol, sitosterol. The disease was first described by Bhattacharyya and Connor.[506] In addition, sitosterolemia is also characterized by hypercholesterolemia, premature cardiovascular disease, hematologic manifestations, arthritis and in rare cases hepatic failure. Hypercholesterolemia-induced premature cardiovascular disease can manifest as premature coronary heart disease or sudden cardiac death.[506] Hemolytic anemia, splenomegaly, bleeding disorders, and macrothrombocytopenia can result from the accumulation of plant sterols in platelet membranes, producing hypertrophic and hyperplasic dysfunctional platelets.[392,394,507,508] Recent studies in tissue-specific ABCG5/G8-knockout mice revealed that activity in either organ is sufficient to protect from sitosterolemia; however, cholesterol elimination is partially compromised.[509] ABCG5/G8 is the primary sterol transporter responsible for the elimination of dietary neutral sterols through transintestinal cholesterol efflux.[510] What remains unclear is whether ABCG5/G8 possesses a selectivity between cholesterol and xenosterols.

6.9. Gallstone Disease

Gallstones have a prevalence of 10−15% in adults[511] in the United States and Europe. However, about 75% of these adult patients have no symptoms. Nevertheless, gallstone disease generates major economic and social burdens if symptoms or complications occur.[511−513] In Western countries, gallstones are comprised mainly of cholesterol in 75−80% of the cases, and are often associated with systemic abnormalities.[514] There are five primary defects, which play a critical role in the pathogenesis of cholesterol gallstones:[513] (1) predisposing genetic factors, (2) hepatic hypersecretion of cholesterol (resulting in supersaturated gallbladder bile), (3) rapid phase transitions of cholesterol in bile, with the precipitation of solid cholesterol crystals, (4) impaired gallbladder motility with hypersecretion and accumulation of mucin gel in the gallbladder lumen and immune-mediated gallbladder inflammation, and (5) intestinal factors involving absorption of cholesterol, slow intestinal motility, and altered gut microbiota

Lifestyle and dietary factors influence the pathogenesis of gallstone disease because the mean energy intake may be higher in gallstone patients than in control subjects.[515] Thus, diet and lifestyle have a potential role in primary prevention of cholesterol gallstones. The European Society for the Study of the Liver panel concludes that healthy lifestyle and food, regular physical activity, and maintenance of an ideal body weight might prevent cholesterol stones and symptomatic gallstones.

7. CONCLUDING REMARKS

The liver is, beyond any doubt, the central organ for glucose and lipid metabolism, *de novo* bile acid synthesis, and detoxification mechanisms. This of course requires an intense communication with the "surrounding cells, tissues, and organs". Thus, membrane transport proteins perform a pivotal role. Interestingly, hepatobiliary transporters of the ABC superfamily are concentrated in the canalicular membrane (Figure 1). Here, they perform the expected export function, e.g., drug export (ABCB1 and ABCG2), the bile triumvirate (ABCB4, ABCB11, and ABCG5/G8), or transport of metabolites (by the members of the ABCC subfamily).

Tremendous progress was made over the last few decades in our molecular understanding of the structure and function of hepatobiliary ABC transporters. This includes among many other things, the expression levels, environmental clues regulating these levels, the precise role of these transporters in many diseases of the liver (Table 1), their substrates or spectrum of substrates, and in particular even in the last five years their three-dimensional structure (Figure 14). We have

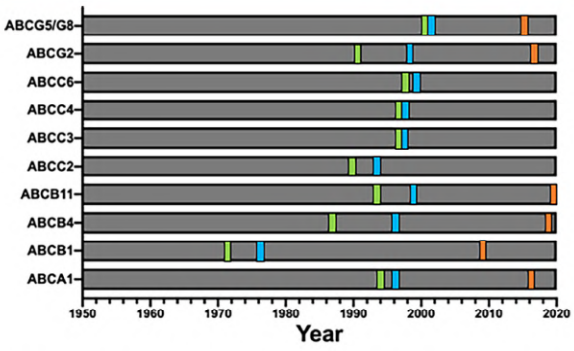

Figure 14. Timeline of important insights into the structure and function of hepatobiliary ABC transporters. The identification of the genes is highlighted in green, while the disease caused by mutations in the ABC transporter genes is given in blue. The year, in which the first structure of a hepatobiliary ABC transporters was determined, is highlighted in orange. Please note that the *x* axis is on year scale.

tried in this review to summarize this knowledge. However, we are fully aware that such an endeavor cannot cover every aspect and every detail. We thus apologize to all our colleagues whose work was not cited appropriately.

Obviously, structural information is a prerequisite for a molecular understanding of the entire transport cycle (Figure 14). Especially, the developments in single particle cryo-EM have had an important impact on membrane protein structure determination. Thus, it does not come as a surprise that most of the structures of hepatobiliary ABC transporters have been determined in the last five years. However, with the exception of ABCB1, we do not have sufficient structures to define an entire transport cycle. It is mandatory to capture all conformations of the different states of a membrane transporter at appropriate resolution. Only than a molecular picture will emerge that defines the changes in space that underlie transport of a compound across a biological membrane. Here, homology models will not necessarily be helpful since many of the transporters do have significant sequence identities, which would make these systems ideal candidates for homology modeling. However, the function is often very different as exemplified by ABCB1 and ABCB4 (76% sequence identity), and it is likely that such models will provide a correct overall picture, but fail to answer the important functional questions.

Structural data however have to be complemented with knowledge about the molecular transitions in time. How fast is a substrate recognized? How fast does occur translocation? How is the hydrolysis of ATP in the NBD coupled to substrate transport in the TMDs? These answers require *in vitro* systems, in which biophysical techniques such as single molecule Förster resonance energy transfer (FRET) or electron paramagnetic resonance (EPR) can be applied. And these approaches should be complemented by molecular dynamics simulations. Examples of such approaches exist for ABC transporters,[516−518] but have to be applied to the ABC transporter present in liver. But also, classic biochemical

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Chem. Rev. 2021, 121, 5240−5288

transport assays are needed to provide quantitative data for these systems. To complicate the situation even further, the elegant studies generating and using double and/or triple transfected cell line have demonstrated that hepatobiliary ABC transporters are not isolated entities. Rather, molecular communication and networks exist that fine-tune their function to the physiological demands of the liver. Only if we obtain a molecular understanding covering space, time, and networks, we will be able to efficiently fight liver diseases and promote liver regeneration when necessary. In summary, we have witnessed an impressive increase of knowledge on hepatobiliary ABC transporters, but the future will still hold many surprises as well as exciting but also unexpected insights.

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Sander Smits obtained his Ph.D. from the Heinrich Heine University in 2008. Since 2008, he has been a senior scientist at the Institute of Biochemistry and initiated and founded the Center for Structural Studies in 2018 of which he is the managing director.

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ABBREVIATIONS

ABC	ATP-binding cassette
ATP	adenosine triphosphate
BCRP	breast cancer resistance protein
bp	base pairs
BRIC-2	benign recurrent intrahepatic cholestasis type 2
BSEP	bile salt export pump
cAMP	cyclic adenosine monophosphate
CAR	constitutive androstane receptor
CFTR	cystic fibrosis transmembrane conductance regulator
cGMP	cyclic guanosine monophosphate
CH	coupling helix
cMOAT	canalicular multispecific organic anion transporter
cMRP	canalicular multidrug resistance-associated protein
CnH	connecting helix
DILI	drug induced liver injury
ECL	extracellular loop
EPR	electron paramagnetic resonance
FGF15/19	fibroblast growth factor 15/19
FOXO1	forkhead box protein O1
FRET	Förster resonance energy transfer
FXR	farnesoid X receptor
GATA4/6	GATA binding protein 4/6
GSH	gluthathione
HAX-1	HS1-associated protein X-1
HCC	hepatocellular carcinoma
HCC	hepatocellular carcinoma
HDL	high density lipoproteins
HepG2	hepatoma G2 cell line, human carcinoma cell line
HlyB	hemolysin B
HNF4α	hepatocyte nuclear factor 4 alpha
HSC	hepatic stellate cells
ICL	intracellular loop
ICP	intrahepatic cholestasis of pregnancy
IF	inward facing
KC	Kupffer cells
LPAC	low phospholipid-associated cholelithiasis
LPS	lipopolysaccharide
LRH-1	liver receptor homologue-1
LSEC	liver sinusoidal

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LTC4	leukotriene C4
LTX	liver transplantation
MATE	multidrug and toxin extrusion
MaVEC	macrovascular
MD simulation	molecular dynamics simulation
MDR	multidrug resistance protein
MLC2	myosin II regulatory light chain
MRP	multidrug resistance-associated proteins
NBD	nucleotide binding domain
NBS	nucleotide binding site
NF-κB	nuclear factor kappa-light-chain-enhancer of active B cells
NTCP	sodium taurocholate cotransporting polypeptide
OATP	organic anion transporter peptides
OF	outward facing
PC lipids	phosphatidyl choline lipids
PDZ	combining the first letters of the first three proteins: PSD95, Dlg1, zo-1
PDZ	combining the first letters of the first three proteins: PSD95, Dlg1, zo-1
PFIC1/2/3	progressive familial intrahepatic cholestasis
PPAR-α	peroxisome proliferator-active receptor alpha
PP_i	pyrophosphate
PS lipid	phosphatidylserine lipid
PXE	pseudoxanthoma elasticum
RACK1	receptor for activated C-kinase 1
RCT	reverse cholesterol transport
rmsd	root-mean-square deviation
RXRα	retinoid X receptor alpha
SLC	solute carrier
STSL	sitosterolemia gene locus
SUR1/2	sulfonylurea receptor1
TAP	transporter associated with antigen processing
TMD	transmembrane domain
TMH	transmembrane helix
TPN	the parenteral nutrition
VLDL	very-low-density lipoprotein

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2 Aims

ABC transporters are often considered as single entities. One contradiction are the ABC transporters of the bile triumvirate, namely ABCB4, ABCB11 and the heterodimer ABCG5/G8. Hence, the idea of an orchestrated interplay of these transporters with their substrates was suggested.[1] More precisely, this would mean, ACB4 as the lipid translocator should react on the presence of conjugated bile acids (the substrates of ABCB1), cholesterol (the substrate of ABCG5/G8) and vice-versa. There exists a sensitive equilibrium of these specific substrates. If an imbalance occurs due to the reduced functioning of one of these three transporters, cells are harmed. Different diseases are related to these three ABC-transporter, for example PFIC-2/3 in case of ABCB11 or ABCB4, or gallstone disease as in case of ABCG5/G8. Subsequently, an interplay of these transporters and their corresponding substrates is suitable to assume. Kis *et al.* [2] demonstrated that for ABCB11, the enrichment of cholesterol in the membrane increases the maximal transport velocity of bile acids by ABCB11. Johnson *et al.* [3] demonstrated that for detergent-solubilized and purified ABCG5/G8, the presence of bile acids is required to activate the transporter, suggesting a chaperon-like mechanism of bile acids. However, so far for ABCB4, only the enrichment of PC lipids in the extracellular medium in the presence of bile acids was used as an evidence for an interplay between ABCB4 and bile acids. However, in recent years, the theory of bile formation has subsequently changed based on a theoretical model [4] and a finding that the bile triumvirate transporters can only be found in PC lipid-enriched microdomains of the outer leaflet [5]. Based on these studies, a formation of PC-enriched microdomains and piles by ABCB4 is assumed. Subsequently, the bile acids enter these PC-rich microdomains and rim off patches due to their harsh detergent action (formation of mixed micelles). Hence, the transport of PC lipids and extraction of these lipids by bile acids were seen as two different steps, with their individual parameters. Hence, bile acid extraction studies do not provide the application platform to investigate the direct effect on ABCB4.

Thus, a system to investigate isolated ABCB4 *in vitro* is crucial. In previous works by Ellinger *et al.*[6] and Kluth *et al.*[7] such a system was established. They managed to overexpress ABCB4 in the yeast *Pichia pastoris* and designed a tandem affinity

chromatography to successfully purify human ABCB4. Based on the above-mentioned research, the overall aim of this thesis was to characterize the modulation of ATP hydrolysis of ABCB4 by bile acids and cholesterol and address the following four major questions and subsequently, employ the findings into a biologically relevant context:

1. Is ABCB4 ATPase activity modulated by human bile acids?
   If yes, at which concentration do bile acids affect ABCB4 and is there a correlation between the different chemical or physical properties of bile acids? Furthermore, is the modulation of ABCB4 by two different bile acids cooperative?
2. Can the medically relevant but non-human versions of UDCA modulate ABCB4 and how does the effect differ in comparison to human bile acid?
3. Is cholesterol able to modulate ABCB4?
   If yes, at which concentration and is bile acid modulation still possible in the presence of cholesterol?
4. Does the modulation of ABCB4 activity by bile acids or cholesterol persists or changes in presence of PC lipids?

3 Results - Publications

3.1 Chapter 1 – ABCB4 in health and disease

Title:	**ABCB4 in heath and disease – at the crossroads of biochemistry**
Authors:	Martin Prescher, Tim Kroll, Lutz Schmitt
Published in:	Biological Chemistry
Impact factor:	4.238
Proportionate work:	40%
On this manuscript:	Contributions to the content of this review is displayed in tables below. In general, all authors red and corrected each chapter.

Chapter	Contributed by:
Abstract	**All listed authors**
Introduction. ABC transporters in general	**All listed authors**
The human ABC transporter ABCB4 (MDR3)	
Structure and function of MDR3	Martin Prescher, **Tim Kroll**
Bile formation	**Tim Kroll**
Phosphatidylcholine – from biosynthesis to protection	Martin Prescher
What we can learn from structurally-related proteins such as ABCB1	Martin Prescher
ABCB4 in disease	
Liver diseases related to ABCB4 deficiency - PFIC3, ICP, LPAC, DILI	Martin Prescher, **Tim Kroll**
Possible treatment before liver transplantation is necessary	Tim Kroll
How molecular mutations can cause dysfunction in MDR3	Martin Prescher, **Tim Kroll**
Challenges in characterization of ABCB4 and its variants	**All listed authors**
The future of ABCB4 research	**All listed authors**
Acknowledgements	**All listed authors**
References	**All listed authors**

Figures	Contributed by:
Figure 1: Homology model of ABCB4 in the inward and outward conformation.	**Tim Kroll**
Figure 2: Spectrum of ABCB4-related diseases.	Martin Prescher

Tables	Contributed by:
Table 1: ABCB4 related liver diseases.	Martin Prescher
Table 2: Drug treatment and treatment options of ABCB4-related liver diseases	**Tim Kroll**

DE GRUYTER Biol. Chem. 2019; 400(10): 1245–1259

Review

Martin Prescher, Tim Kroll and Lutz Schmitt*

ABCB4/MDR3 in health and disease – at the crossroads of biochemistry and medicine

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Abstract: Several ABC transporters of the human liver are responsible for the secretion of bile salts, lipids and cholesterol. Their interplay protects the biliary tree from the harsh detergent activity of bile salts. Among these transporters, ABCB4 is essential for the translocation of phosphatidylcholine (PC) lipids from the inner to the outer leaflet of the canalicular membrane of hepatocytes. ABCB4 deficiency can result in altered PC to bile salt ratios, which led to intrahepatic cholestasis of pregnancy, low phospholipid associated cholelithiasis, drug induced liver injury or even progressive familial intrahepatic cholestasis type 3. Although PC lipids only account for 30–40% of the lipids in the canalicular membrane, 95% of all phospholipids in bile are PC lipids. We discuss this discrepancy in the light of PC synthesis and bile salts favoring certain lipids. Nevertheless, the *in vivo* extraction of PC lipids from the outer leaflet of the canalicular membrane by bile salts should be considered as a separate step in bile formation. Therefore, methods to characterize disease causing ABCB4 mutations should be considered carefully, but such an analysis represents a crucial point in understanding the currently unknown transport mechanism of this ABC transporter.

Keywords: ABC transporter; ABCB4 deficiency; bile acids; liver disease; phosphatidylcholine.

Introduction: ABC transporters in general

The human genome contains 48 genes encoding for ABC transporters (Dean et al., 2001) and several of these are expressed in human hepatocytes. ABC transporters are by definition ATP hydrolyzing, primary transporters, which possess two copies of an ATP binding cassette, known as the nucleotide binding domain (NBD), and two copies of a transmembrane spanning segment, known as the transmembrane domain (TMD; Figure 1). Furthermore, the NBDs of ABC transporters harbor conserved sequence motifs (Figure 1B, D), which form the foundation of this superfamily of membrane transport proteins: the Walker A (consensus sequence: GxxGxGKT/S, where x can be any amino acid), the Walker B (consensus sequence: $\phi\phi\phi\phi$D, where ϕ represents any hydrophobic amino acid), the C-loop (consensus sequence: LSGGQ), the Q-loop and the D-loop (consensus sequence: SALD) (Schmitt and Tampe, 2002). The C-loop and the D-loop are the hallmarks of the protein family, with specific locations between the Walker A and B motifs (C-loop) and downstream of the Walker B motif (D-loop). These five motifs serve as a diagnostic tool to identify new ABC proteins. The Q-loop and a highly conserved histidine (~30 amino acids downstream of the D-loop, also called the H-loop) are essential for the ATP hydrolysis (Zaitseva et al., 2005a). Additionally, a Pro-loop connects the two subunits of NBDs (Schmitt et al., 2003).

The TMDs form the translocation pathway across a biological membrane. In the case of eukaryotic exporters each TMD in general consists of six membrane spanning α-helices. Two TMDs form the functional unit that is required together with the energy, which is provided by ATP binding and/or hydrolysis within the NBDs to allow substrate transport against a concentration gradient (Schmitt and Tampe, 2002). However, there are cases where the number of domains of single ABC transporters vary from the canonical number of six. In humans, for example, ABCA4 has two additional extra cellular domains (Illing et al., 1997; Bungert et al., 2001). Additionally, a number of transporters of the ABCC subfamily harbor a third TMD, which is generally termed TMD0 (Kruh and Belinsky, 2003).

ABC transporters can be divided into importers and exporters. Prokaryotes and plants have both kinds of ABC transporters, while all other eukaryotes mainly harbor exporters, with the often-discussed exception of ABCA4

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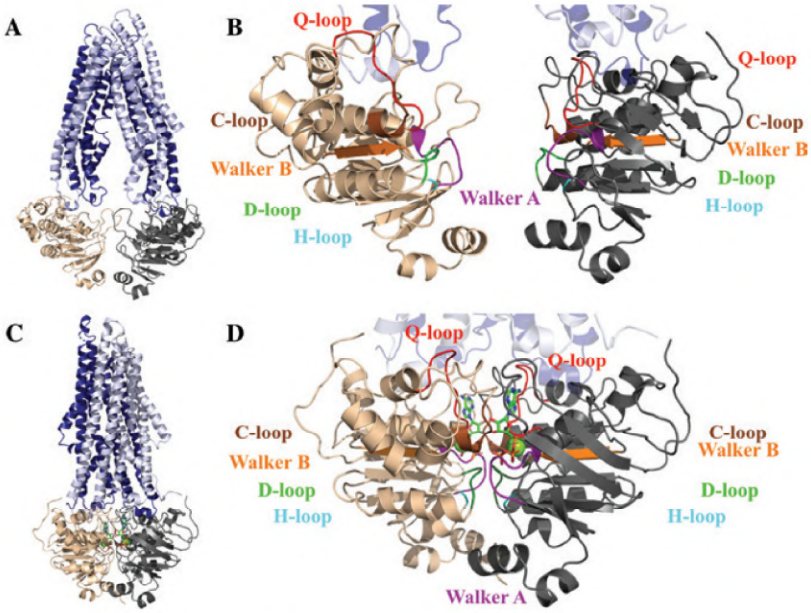

Figure 1: Homology model of ABCB4 in the inward and outward facing conformation.
(A) The inward facing conformation of the ABCB4 homology model was calculated by the bioinformatic tool Phyre2 (Kelley et al., 2015). The two TMDs are highlighted in blue and light blue, while the two NBDs of ABCB4 are displayed in gray and light brown. (B) Zoom-in into the NBDs of the inward facing conformation with the five motifs and the conserved sequence motifs highlighted. (C) The outward facing structure of the ABCB4 model is based on the single particle cryo-EM structure of human ABCB1 in the ATP bound state [PDB # 6C0V (Mi Sun Jin et al., 2012)]. Color-coding corresponds to panel (A). (D) Zoom-in into the NBDs in their head-to-tail orientation of the ATP-induced dimeric state. The two bound ATP molecules are depicted in balls-and-spheres representation and Mg^{2+}-ions as a green sphere.

(Beharry et al., 2004; Molday, 2007). Additionally, eukaryotic ABC transporters can be divided in full-size and half-size transporters. A gene of the former encodes all four modules, while a gene coding for a half size transporter harbors only one copy of the TMD and NBD. They can form homodimers (two copies of the same gene) or heterodimers (two different copies containing one of each domain) (Hyde et al., 1990).

To get insights in the mode of action, it is essential to obtain structural information in combination with biochemical analysis. This has led to an understanding of the mechanism of ATP hydrolysis and several models of transport. All structurally analyzed NBDs are L-shaped and can be divided into a catalytic and a helical subunit (Chen et al., 2016; Li et al., 2016; Locher, 2016). The first one contains both Walker motifs, while the latter one harbors the C-loop. Analysis of isolated NBDs in the presence of ATP demonstrated that the two NBDs form a dimer: the adenosine and the phosphate moiety of one ATP molecule are coordinated by the Walker A and B motifs, while the γ-phosphate interacts with the C-loop of the opposing NBD and *vice versa* for the second ATP. Here, ATP acts as a molecular glue inducing the formation of a head-to-tail dimer of the NBDs (Smith et al., 2002; Chen et al., 2003; Zaitseva et al., 2005b).

The knowledge of ATP-induced dimerization derived from structures of isolated NBDs and subsequently supported by structures of full-length ABC transporters led to one of the most favorized models of substrate transport for ABC exporters: the continuous ATP-switch model (Higgins and Linton, 2004; Locher, 2016). For ABC exporters in the resting or ground state, the so-called inward-facing conformation, the substrate binding site in the TMDs is accessible from the cytosol. The NBDs are far apart in an apparent 'monomeric state'. ATP binding induces dimerization of the NBDs, which results in a conformational change of the TMDs. This exposes the substrate binding site to the extra cellular space (the so-called outward-facing conformation). Additionally, the substrate affinity is reduced, and the substrate can be released. After ATP hydrolysis, the transporter switches back to the ground state (inward-facing conformation) (Higgins and Linton, 2004). Nevertheless, some conformations of the transport cycle of ABC transporters are still unknown. Furthermore, the coordination and timing of substrate and ATP binding as well as the control of futile ATP hydrolysis are still open questions.

The human ABC transporter ABCB4 (MDR3)

Structure and function of MDR3

The complete cDNA sequence of a gene highly homologous to human *ABCB1* [also called multidrug resistance protein 1 (MDR1) or P-glycoprotein (P-gp)] was published in 1987 (Van der Bliek et al., 1987) and subsequently termed ABCB4 (or MDR3). Sequence analysis of this gene identified two halves of an ABC transporter consisting of six putative transmembrane helices (TMHs) and one NBD, in each half of the gene (van der Bliek et al., 1988). Additionally, the sequences of the NBDs as well as the TMDs were highly identical to human ABCB1. Both proteins share an overall 76% identity and 86% homology, respectively. The highest discrepancy is within the N-terminus and the linker region connecting both halves (Van der Bliek et al., 1987).

ABCB4 mRNA is mainly transcribed in the liver especially in hepatocytes (68–69%) with low mRNA levels also found in the adrenal gland (5–10%), heart (4%), lymph node (7%) and spleen (4–7%) (Nishimura and Naito, 2005; Fagerberg et al., 2014). In the hepatocytes, ABCB4 is exclusively localized in the apical (canalicular) membrane. The intracellular trafficking of canalicular ABC transporters was discussed in detail by Kipp and Arias (2002). Briefly summarized, three interaction partners of ABCB4 are known, which ensure intracellular trafficking. Pulldown assays revealed the HS1-associated protein X-1 (HAX-1), the myosin II regulatory light chain (MLC2) and the receptor for activated C-kinase 1 (RACK1) as interaction partners for ABCB4. HAX-1 is known to be involved in the apical endocytosis of ABCB11 and might also regulate ABCB4 endocytosis (Ortiz et al., 2004). It has to be further verified whether MLC2 is required for ABCB4 trafficking similar to ABCB11 (Chan et al., 2005). On the other hand, RACK1 is essential for the expression and localization of ABCB4 (Ikebuchi et al., 2009). Additionally, ABCB4 contains a C-terminal motif that resembles the PDZ domain-binding motif. This PDZ motif might interact with scaffolding proteins as it could be shown in the cystic fibrosis transmembrane conductance regulator (CFTR) (Swiatecka-Urban et al., 2002) and the multidrug-regulated protein 2 (MRP2) (Kocher et al., 1999; Hegedus et al., 2003) in order to link transmembrane proteins to the cytoskeleton.

ABCB4 contains characteristic posttranslational modifications. Two N-glycosylation sites, which are located between TMH1 and TMH2 at position 91 and 97 (van der Bliek et al., 1988) as well as several phosphorylation sites. In the mouse homologue, Mdr2, of human ABCB4 three N-glycosylation sites have been identified, which are located in the first extracellular loop at positions 83, 87 and 90, respectively (Devault and Gros, 1990). Even though, the heterologous expression of mouse Mdr2 in the yeast *Pichia pastoris* did not contain N-glycosylations (Urbatsch et al., 2001), it had no effect on expression, nucleotide binding or catalytic characteristics indicating that glycosylation is not necessary for function. On the other hand, phosphorylation sites of ABCB4 do have an influence. Mutations with an altered phosphorylation pattern resulted in markedly decreased phosphatidylcholines (PC)-secretion, despite the fact that the protein showed minor defects in maturation and targeting to the apical membrane (Gautherot et al., 2014).

First indications for a function of the protein were published in 1993 in a study, in which *Mdr2* (the mouse homologue of *ABCB4*) was knocked out. The homozygous knock-out mice did not contain cholesterol and PC lipids in their bile. In contrast, in the heterozygous mutant $Mdr2^{-/+}$ 40% of wildtype PC lipid and nearly normal cholesterol levels in the bile were observed (Smit et al., 1993). Furthermore, complementation of mouse Mdr2 by human ABCB4 demonstrated that both ABC transporters mediated the same function. Here, $ABCB4^{+/+}$ mice possessed even higher phospholipid levels (Smith et al., 1998). Most importantly, the use of [^{3}H] choline-labeled PC proved that human ABCB4 expressed at the surface of transgenic mice fibroblasts acted as a PC flippase (Smith et al., 1994). Additionally, van Helvoort et al. demonstrated the specific translocation of a short-chain PC lipid in polarized pig kidney epithelial cells transfected with human *ABCB4* (van Helvoort et al., 1996).

The high amino acid identity of ABCB1 and ABCB4 initially led to speculations that both ABC transporters are multidrug resistance proteins and to the misjudgment classifying ABCB4 as a transporter providing multidrug resistance. Even today, there are data in favor of and against this scenario. For example, ABCB4 heterologously expressed in yeast resulted in resistance against the antifungal aureobasidin A (Kino et al., 1996). In another study, transport of several ABCB1 substrates (digoxin, paclitaxel, vinblastine or ivermectine) was demonstrated in polarized pig kidney monolayers transfected with the cDNA of *ABCB4*. Additionally, verapamil and cyclosporine, well-known ABCB1 inhibitors, inhibited the ABCB4 mediated transport of digoxin (Smith et al., 2000). In contrast to these studies, experiments with *ABCB4* or *Mdr2* transfected cells showed no drug resistance (Van der Bliek et al., 1987; Buschman and Gros, 1991, 1994; Gros and Buschman, 1993). Additionally, ABCB4 has not been detected in MDR cell lines (Raymond et al., 1990).

In summary, it is now well documented and accepted that ABCB4 specifically translocate lipids of the PC family from the inner to the outer leaflet of the canalicular membrane of hepatocytes and has at the most an artificial function as a multidrug resistance protein.

Bile formation

Bile is synthesized and secreted by hepatocytes and plays a key role in digestion and absorption of fat and fat-soluble vitamins. Primary bile mainly consists of mixed micelles formed by PC lipids, bile salts and cholesterol, bilirubin glucuronides and organic anions. Different ABC transporters are involved in this process, for example, bilirubin glucuronides are excreted into bile by ABCC2 (MRP2) (Kamisako et al., 2000) and transport of glutathione, the most common organic anion, is facilitated by ABCC1 (MRP1) (Cole and Deeley, 2006), while bile salts are secreted by ABCB11 [bile salt export pump (BSEP)] (Gerloff et al., 1998b), PC lipids are translocated by ABCB4 (Smit et al., 1993) and cholesterol is a substrate of the heterodimeric ABC transporter ABCG5/G8 (Graf et al., 2003). In 2003, Small concluded that the latter three ABC transporters interplay in a specific order to ensure the proper formation of bile. Bile salts in the canaliculi promote the release of PC lipids from the membrane to form mixed micelles, which are necessary for solubilization of cholesterol to prevent its crystallization (Small, 2003). It was reported that the reduction of PC lipids in primary bile leads to an increase of the toxic activity of the bile salts against the ducts, which can result in cholestatic liver diseases (Trauner et al., 2008). This study pointed out how important a healthy ratio of PC lipid to bile salt and cholesterol concentration is. Variations in ABCB11 (Carlton et al., 1995; Bull et al., 1998) and ABCB4 (Deleuze et al., 1996; de Vree et al., 1998) can lead to altered ratios and therefore to different kinds of liver diseases. Since then, the interest to understand these proteins and their role in liver diseases has increased dramatically.

Phosphatidylcholine – from biosynthesis to protection

Most biological membranes such as the plasma membrane are asymmetric, i.e. they consist of different lipid compositions in the cytosolic (inner) and the exoplasmic (outer) leaflet. Lipids in eukaryotic plasma membranes are distributed with a majority of sphingomyelin (SM) and PC in the outer leaflet. On the other hand, amino phospholipids such as phosphatidylethanolamine (PE) and phosphatidylserine (PS) are the major lipid components of the inner leaflet, respectively (Devaux, 1991). However, passive transbilayer lipid movement especially against a concentration gradient is a relatively slow event, which occurs on the time scale of minutes to hours and depends on charge, shape and length of the lipid. In general, the more amphipathic and bulky a lipid becomes the more unfavorable is the passive flip-flop. An unassisted flip-flop of a single 1,2-dioleyl-sn-glycero-3-phosphocholine (DOPC) lipid in a DOPC bilayer occurs, for example, once per 25 h (McConnell and Kornberg, 2002). Thus, a protein mediated lipid flip-flop is required to build up this asymmetry and the superfamily of ABC transporters is involved in such transbilayer movement of lipids. In contrast to scramblases, the transport of ABC transporters is unidirectional and can be further subdivided into inward (flip) or outward (flop) transport (Borst et al., 2000).

Many ABC transporters [e.g. ABCA1 (Oram and Lawn, 2001), ABCB1 (Higgins and Gottesman, 1992), ABCB4 (Smit et al., 1993), ABCC1 (Deeley and Cole, 2006), ABCG1 (Klucken et al., 2000) and ABCG2 (Doyle and Ross, 2003)] have been described to be involved in lipid homeostasis. ABCB4 is maintaining this asymmetry in the canalicular membrane of hepatocytes with PC lipids.

PC can be synthesized by either the cytidine diphosphate (CDP)-choline pathway or the PE methylation pathway (PEMP). The CDP-choline pathway uses choline as the initial substrate, while the PEMP methylates PE in three sequential steps to PC.

Although PC is mainly synthesized by the CDP-choline pathway (Kent, 1995), PEMP is highly active in hepatocytes (Yao and Vance, 1988). Two-thirds of PC originates from the CDP-choline pathway, while one-third is synthesized via the PEMP. Both choline synthesis pathways have characteristic acyl-chain preferences. The CDP-choline uses mainly medium-chain, saturated acyl-chains, for example, 16:0, 18:0 variants, whereas the PEMP uses more diverse, longer and poly-unsaturated acyl-chains, for example, 18:1, 18:2, 20:4 (DeLong et al., 1999). PC products derived from PEMP seem to be more metabolically active and involved in cellular regulations [e.g. arachidonic acid (20:4) (Needleman et al., 1986)] compared to the CDP-choline synthesis products.

The PC proportion of the canalicular membrane is only about 30–40% (Yousef and Fisher, 1976; Meier et al., 1984), but lipids of the PC family build up the majority (95%) of all phospholipids in bile (Boyer, 1986; Puglielli et al., 1994) with hardly any other phospholipid detectable. This raises the question of how this discrepancy can

be explained. It is unlikely that an unknown protein is involved in mediating the 'lipid exposure' process (Pohl et al., 2005) of PC lipids, however, there is evidence for bile acids actively extracting phospholipids from the canalicular membrane (Elferink et al., 1997). 'Lipid exposure' explains the uptake of lipids out of the membrane into or through a specific acceptor. In the case of ABCB4 the acceptor seems to be the mixed micelles, while bile salts act as extractors. Considering the detergent nature of bile salts and the fact that nearly exclusively it is PC lipids that are solubilized from the extracellular leaflet in the bile duct, it is surprising to see that bile salts discriminate between lipids of different acyl chains (Wustner et al., 2000), as shorter and more hydrophilic lipids are more favorably taken up into mixed micelles independently of their headgroup (Nibbering et al., 2001). Studies investigating the characteristics of the acyl-residues of biliary lipids found that mainly PC lipid variants with 16:0–18:2 (50%) and 16:0-20:4 acyl-chains (20%) (Boyer, 1986; Puglielli et al., 1994; Nibbering et al., 2001) were secreted. These findings are supported by the fact that SM variants found in bile are mainly the more hydrophilic, short-chain ones [e.g. 16:0 acyl-chain lipids (Carey and Carey, 1998)] and membranes with a high sphingomyelin proportion make membranes highly detergent resistant (Guyot and Stieger, 2011).

The puzzling enrichment of PC as the major lipid component in bile can be explained with (i) the asymmetry of the canalicular membrane and (ii) with the insertion capabilities of bile salts, which can just integrate into the outer leaflet of the canalicular membrane. It is important to point out that for ABCB4 the substrate selection is not characterized. Indications are present, in which the choline head group seems to be the part of the lipid, which is recognized (Smith et al., 1994; van Helvoort et al., 1996; Kluth et al., 2015). However, a complete substrate specificity of ABCB4 with insights on preferences for the nature of the acyl chains has so far not been investigated. Therefore, the 'lipid exposure' step which follows the lipid flop from the inner to the outer leaflet of the canalicular membrane may also be part of the ABCB4 translocation process.

However, enrichment of PC lipids in bile may also be explained by the regulation of PC synthesis and/or the discrimination between specific medium-chain lipids by acceptors like bile salts. The fact that PC lipids are highly abundant in the outer leaflet of hepatocytes due to the ABCB4-mediated PC lipid flop may not be omitted. This could be the reason why the hepatocytes utilize PC lipids derived from the CDP-choline synthesis pathway as well as the ATP-consuming translocation by ABCB4 to protect itself from solubilization by bile salts. This is supported by the fact that ABCB4 is suggested to be a low affinity transporter (Ishigami et al., 2013), as ABCB4 also recognizes PC-variants of the PEMP, i.e. longer acyl-chains and poly unsaturated lipids, the integrity of the membrane remains intact even in the presence of bile acids reflected by the relatively high activity of the PEMP in hepatocytes.

'Lipid exposure' is generally a crucial step in lipid translocation. However, it is often not included in mechanistic considerations and little is known about the implications on ABCB4. ABCB4 might not have to expose lipids out of the outer leaflet itself. Rather, the transporter creates a high abundance of various PC lipid variants without the discrimination by the choice of its substrates. Bile acids, on the other hand, discriminate and extract due to a higher affinity the abundant 16:0–18:1 PC-variants, which were derived from the CDP-choline pathway. Therefore, ABCB4 protects the biliary tree from the harsh detergent activity of bile salts, while bile salts preferably solubilize PC-variants with a characteristic nature. ABCB4 may do more than merely flop PC lipids. If lipid extraction would only be based on bile salt selectivity at least some phospholipids should remain in the bile of patients with ABCB4 deficiency, probably with a changed lipid composition away from the PC majority. Supported by the observations that the bile salt to phospholipid and cholesterol to phospholipid ratio in PFIC-3 patients is five times higher (Davit-Spraul et al., 2010). These findings together with experiments of *Mdr2* knock-out mice, which show nearly no remaining phospholipids in bile (Smit et al., 1993; Crawford et al., 1997), point towards a crucial role of ABCB4 itself in the uptake of PC lipids into bile. On the contary, it could be observed that bile acids largely release PC lipids from isolated canalicular vesicles, demonstrating the need for further investigations (Gerloff et al., 1998a).

It remains unclear which part contributes the most to the enrichment and uptake of PC in bile. It might be (i) *de novo* PC synthesis via CDP-choline or PEMP, (ii) the accumulation or even lateral tension in the canalicular leaflet mediated by ABCB4 translocation, (iii) the statistical abundance of PC in the outer leaflet of the canalicular membrane by the active flop of PC by ABCB4, (iv) ABCB4 selection of certain PC lipids or (v) the bile acid composition and its concentration.

What we can learn from structurally-related proteins such as ABCB1

The amino acid sequence of the ABC transporter ABCB4 is highly homologous to other transporters of the ABC subfamily B especially ABCB1. Their substantial sequence

identity covers not only the conserved NBDs, but also the TMDs. ABCB1, probably one of the most investigated ABC transporter, allows extracting information regarding the key features of ABCB4, as, for example, the three-dimensional structure through homology modeling (Figure 1). Even though ABCB4 and ABCB1 share up to 76% identity (86% homology), they transport structurally, physically and physiologically unrelated substrates. The difference between these two transporters likely hides in the molecular mechanisms of substrate recognition. However, homology models of ABCB4 derived from the known structures [inward-facing conformation, (Li et al., 2014)] or Kim et al., [outward-facing conformation (Kim and Chen, 2018)], obviously remain structural models of ABCB4. This is, for example, emphasized by the fact that for ABCB1 at least 40 residues are known to interact with substrates (Loo and Clarke, 2001; Vahedi et al., 2017), while key residues in ABCB4 involved in substrate binding and translocation remain unknown.

ABCB4 in disease

Liver diseases related to ABCB4 deficiency – PFIC3, ICP, LPAC, DILI

Liver diseases related to dysfunctional ABCB4 are very diverse (Figure 2). They include progressive familial intrahepatic cholestasis type 3 (PFIC3), intrahepatic cholestasis of pregnancy (ICP), low phospholipid associated cholelithiasis (LPAC), drug-induced liver injury (DILI) and some forms of chronic cholangiopathy with biliary fibrosis (for further details see Table 1). They can be divided into inherited (PFIC3, ICP, LPAC) and acquired (DILI) diseases (recently reviewed by Reichert and Lammert, 2018). The interested reader can obtain further information about jaundice as a symptom of liver diseases in a recent review from Chen and colleagues (Chen et al., 2018).

PFIC is caused by variations, for example, in hepatocanalicular transporters disease-causing mutations (DCM) in ATP8B1 result in PFIC-1, DCM in ABCB11 result in PFIC-2 and DCM in ABCB4 result in PFIC-3 and is the most severe form of all 'ABCB4-related' liver diseases. ABCB4 deficiency can be sorted into ABCB4 variants causing altered expression levels, folding, stability or activity (Delaunay et al., 2017). All cases therefore reduce or abolish net PC lipid translocation. Not only the mutation itself, but also the status of zygosity can cause different severities of diseases like ICP, LPAC or even PFIC-3 (Figure 2). The accumulation of free bile salts not safely stored in mixed micelles may lead to bile duct injuries, which is known as the 'toxic bile concept' (Trauner et al., 2007).

Mild forms of cholestasis are indicated by elevated serum cholestasis marker such as alkaline phosphatase (ALP), alanine transaminase (ALT) and aspartate transaminase (AST). An indicator for liver diseases which are related to ABCB4 deficiency, is an elevated γ-GT serum level (Gudbjartsson et al., 2015).

Intermediate forms of severity of liver diseases are ICP and DILI. ICP typically occurs during the third trimester of pregnancy and is the most common primary liver disease in pregnancy (Lammert et al., 2000; Williamson

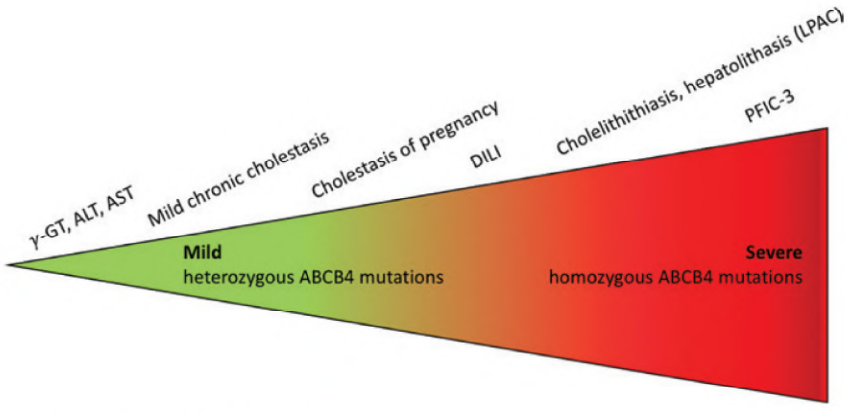

Figure 2: Spectrum of ABCB4-related diseases.
From left to right increasing severity of ABCB4 related diseases (from mild forms to severe forms). γ-GT, γ-glutamyl transpeptidase; ALT, alanine transaminase; AST, aspartate transaminase; DILI, drug induced liver injury; PFIC-3, progressive familial intrahepatic cholestasis type 3. Adopted from Reichert and Lammert (2018).

Table 1: ABCB4-related liver diseases.

Disease	Symptoms	Physiopathology
PFIC-3	Jaundice, discolored stool, hepatomegaly, splenomegaly, pruritus	Absence of biliary phospholipids, injury of bile canaliculi and epithelium, high serum γ-GT activity
LPAC	Intrahepatic hyperechogenic foci, sludge or microlithiasis	Increased biliary cholesterol to phospholipid ratio, lithogenicity of bile with crystallization of cholesterol
ICP3	Slightly elevated serum γ-GT activity in contrast to 'classic ICP' with no elevated serum γ-GT	Likely heterozygous state for an ABCB4 defect, associated with nongenetic factors, such as female sex hormones and metabolites during pregnancy, cholestasis derives from the toxicity of bile/reduced biliary phospholipids
DILI	Cholestasis with high serum γ-GT activity caused by oral contraceptive pill treatment	Xenobiotic inhibits one or a number of hepatic transporters

The table summarizes the symptoms and physiopathology for the four most common ABCB4-related liver diseases: progressive familial intrahepatic cholestasis (PFIC-3), low phospholipid associated cholelithiasis (LPAC) intrahepatic cholestasis of pregnancy type 3 (ICP-3) and drug induced liver injury (DILI), respectively (Davit-Spraul et al., 2010). γ-GT, γ-glutamyl transpeptidase.

and Geenes, 2014). Fetal as well as maternal symptoms can occur. Typical symptoms for pregnant women are pruritus and increased bile acid concentrations in the serum. Fetuses may be at risk for fetal distress, preterm birth or even fetal death, if the bile salt concentration in serum exceeds 40 µmol/l (Chappell et al., 2012; Bacq et al., 2017). ICP is reversible and ends after pregnancy, nevertheless, ICP patients may be at higher risk for subsequent hepatobiliary diseases (Wikstrom Shemer et al., 2015).

DILI can result from a variety of drugs due to the inhibition of hepatobiliary transporters, for example, ABCB4, ABCB11 or ATP8B1 and is an acquired liver disease (Stieger et al., 2000; Schuster et al., 2005; Smith and Schmid, 2006; Bleibel et al., 2007; Stieger, 2010; Bhamidimarri and Schiff, 2013). Next to ABCB11 little attention was paid to ABCB4. Nevertheless, both are listed in the Food and Drug Administration (FDA) list of candidate transporters for drug-induced inhibition (http://bts.ucsf.edu/fdatransportal). Inhibition of BSEP, for example, can trigger elevations in serum bile salt concentration because of increased basolateral transporters mediating the efflux of bile acids into the serum (Rodrigues et al., 2014; Aleo et al., 2017).

LPAC is already classified as a severe liver disease. It occurs in young adults and can be diagnosed with findings of cholesterol crystals in the hepatobiliary duct system. The low concentration of PC in bile is attributed to ABCB4 deficiency (Poupon et al., 2013). LPAC has an overlapping molecular background to PFIC-3, heterozygous ABCB4 variants tend to manifest in LPAC, whereas homozygous or compound heterozygous variants are more likely to confer PFIC-3 (Poupon et al., 2010).

PFIC occurs in most patients in the first year of life (Droge et al., 2017). It rapidly progresses to liver cirrhosis in childhood. The main symptoms are jaundice, pruritus, gallstones, and in case of PFIC-3 elevated γ-GT levels are measurable together with reduced PC-levels in bile. It is caused by both alleles and typically results in the need for liver transplantation.

Possible treatment before liver transplantation is necessary

Drugs applied to ABCB4 deficiency can be classified based on their mode of action. Drugs may stimulate ABCB4 expression, its intracellular transport from the Golgi apparatus to the canalicular membrane, the ATPase activity or they may stimulate the translocation process in general (Table 2).

Ursodeoxycholic acid (UDCA) has been the only treatment in patients with ABCB4-related diseases and provided benefit in patients with ICP or LPAC syndrome. UDCA, a natural bile acid found in bears is used to recover liver function with the aim of preventing liver cirrhosis. The success of the therapy depends on the severity of ABCB4 deficiency (Gordo-Gilart et al., 2015). Indeed, UDCA treatment unfortunately fails in approximately 50% of the patients with PFIC-3. Non-responders often lack ABCB4 in the canalicular membrane due to truncated variants of the protein and subsequent degradation (Jacquemin et al., 2001). Patients with at least one third of functional ABCB4 are favorable to respond to UDCA treatment (Gordo-Gilart et al., 2015).

The UDCA derivative NorUDCA is a shorter, C_{23} version of UDCA (C_{24}). It undergoes cholehepatic shunting and resembles bile acids to lower the harsh detergent activity, making it a promising drug against primary sclerosing cholangitis (Halilbasic et al., 2017).

Patients with known genotypes can be treated with more specific drugs, for example, drugs that stimulate

Table 2: Drug treatment and treatment options of ABCB4-related diseases.

Drug	Clinical trials[a]	Physiological function	Mode of action	Reference
UDCA	In use	Bile salt	Reduction of the harshness of bile, stimulates secretion of bile salts	(Gordo-Gilart et al., 2015; van der Woerd et al., 2017)
Rifampicin	In use	Semisynthetic antibiotic	Upregulation of detoxification enzymes and export pumps	(van Dijk et al., 2015; van der Woerd et al., 2017)
Nor-UDCA	Phase II study completed 2016	Half synthetic bile salt	Reduction of the harshness of bile	(Trauner et al., 2015; Halilbasic et al., 2017)
Obiticholic acid	Phase I study completed 2015	FXR agonist	Stimulating the ABCB4 expression	(Hirschfield et al., 2015)
Bezafibrate	Phase III study completed 2012 (Bezafibrate + UDCA)	PPAR alpha agonist	Down regulating the bile acid synthesis	(Honda et al., 2013)
Ivacaftor	In use for CFTR Phase I completed for moderate hepatic impairment	Potentiator	Recover weakened ATP binding	(Delaunay et al., 2017)
Cyclosporin A Cyclosporin C	No clinical study for ABCB4 several studies after liver transplantation	Chemical chaperone	Rescuing impaired ABCB4 trafficking	(Gautherot et al., 2012; Delaunay et al., 2016; Andress et al., 2017)
4-phenylbutric acid	No studies found	Chemical chaperone	Rescuing impaired ABCB4 trafficking	
Curcumin	No clinical study for ABCB4 Two studies for NAFLD	Chemical chaperone	Rescuing impaired ABCB4 trafficking	

The table briefly compiles the information about the different drugs: their current status in clinical phase ([a]taken from clinicaltrails.gov), mode of action and physiological function.

the mRNA expression level in cases where the presence of ABCB4 is marginal. *ABCB4* is under transcriptional regulation of the farnesoid X receptor (FXR), peroxisome proliferator-activated receptor α (PPARα) and presumably the liver receptor homolog 1 (LRH1) (Marrapodi and Chiang, 2000; Hochrath et al., 2012). Transcriptional receptors may be promising drug targets for patients with low ABCB4 expression levels, with the idea of finding agonists stimulating ABCB4 mRNA expression. FXR induces ABCB4 mRNA expression due to binding and therefore activation in the promotor region of ABCB4 (Huang et al., 2003). PPARα transactivates a range of additional mechanisms including cholesterol homeostasis, by down-regulation the bile acid synthesis through cytochrome P450 (CYP) enzyme 7A1. Studies in clinical phase I have shown that targeting the nuclear receptors with the FXR agonist obeticholic acid (Hirschfield et al., 2015) or with the peroxisome proliferator-activator receptor-α agonist bezafibrate (Honda et al., 2013) are promising in the treatment of chronic liver diseases with poor response to ursodeoxycholic acid.

ABCB4 variants with mutations in the NBD may be targeted differently. First experimental evidence observed for ivacaftor. Ivacaftor is a potentiator, which is therapeutically approved in patients suffering from cystic fibrosis with defective gating mutations. In ABCB4 it should recover deficiencies attributed to weakened ATP binding (Delaunay et al., 2017).

Chemical chaperones such as cyclosporin A, C, 4-phenylbutyric acid or curcumin, which lack specificity, but are able to rescue impaired ABCB4 trafficking in an *in vitro* set up, provide proof of principle that protection of misfolded proteins and/or prevention of degradation is a feasible approach (Gautherot et al., 2012; Delaunay et al., 2016; Andress et al., 2017). These examples lay the path for highly specific and tailor-made pharmaceutics.

How molecular mutations can cause dysfunction in MDR3

Databases for ABCB4 [e.g. Genome Aggregation Database (gnomAD; dbSNP Short genetic variation NCBI, 11.2018)] list approximately 250 synonymous, 550 missense and 30 loss of function variants. About 300 of these are distinct disease-causing ABCB4 variants (Pauli-Magnus et al., 2004; Gotthardt et al., 2008; Meier et al., 2008; Ziol et al., 2008; Dixon et al., 2009; Davit-Spraul et al., 2010; Krawczyk et al., 2010; Kubitz et al., 2011; Lammert and Hochrath, 2015; Dixon and Williamson, 2016; Keitel et al., 2016; Delaunay et al., 2017; Droge et al., 2017). However, most ABCB4 variants are family-derived and therefore very likely unique

to this specific genetic background. Additionally, none of these databases combine all the sequencing data and databases are growing every day, making it challenging to track precise statistics. The variants in ABCB4 are distributed largely over the entire *ABCB4* gene, making the identification of the molecular basis of the disease ambitious. The impact of synonymous variants is also challenging, as they do not result in an altered amino acid sequence. However, they may potentially modulate mRNA processing, affect splicing or modulate protein folding as demonstrated for ABCB1 (Kimchi-Sarfaty et al., 2007). Disease causing mutations of ABCB4 can be categorized in mutations which lead to the total absence of ABCB4, protein trafficking or altered function. Nonsense mutations can result in truncated protein variants where stop codons occur within the gene (de Vree et al., 1998; Wendum et al., 2012; Vij et al., 2018). Other variations may not lead to the total absence of ABCB4 but can cause mis-trafficking or intracellular accumulation of ABCB4 (Andress et al., 2014; Gordo-Gilart et al., 2015). For example, the ABCB4 G68H variant remained in the endoplasmic reticulum (Gordo-Gilart et al., 2015). Mutations which do not affect expression or trafficking can affect the functionality of ABCB4. Some of these mutations which can be pinpointed to a conserved motifs of ABC transporters (see Introduction) often result in an inactive ABCB4 variant, as these motifs are necessary to bind and hydrolyze ATP (Dzagania et al., 2012; Kluth et al., 2015; Droge et al., 2017). One example would be a substitution in the Walker A motif (K435T) (Andress et al., 2017). Mutations located in the TMDs are more difficult to interpret. For example, the ABCB4 T715I (TMH7) variant had still 80% of its activity (Park et al., 2016), while ABCB4 T201M (TMH3) showed ~30% activity compared to the wild type (Gordo-Gilart et al., 2015). With an unknown lipid translocation mechanism, the underlying significance of certain ABCB4 variants especially in the case of mutations of non-conserved areas in the protein are more challenging to interpret therefore it becomes inevitable to characterize these ABCB4 variants in adequate *in vivo* or *in vitro* methods.

Challenges in characterization of ABCB4 and its variants

ABCB4 variants with changes in their functionality, expression or trafficking have different molecular origins. Nevertheless, the characterization demands various *in silico*, *in vivo* and *in vitro* methods. We want to highlight crucial challenges which cannot be easily addressed by a single method.

A crucial pitfall in ABCB4 research is the interpretation of data collected from different species. Data derived from animal models, cell culture models or from humans need to be combined, for example, the bile salt pool composition differs between the species with consequences for the pathogenesis (Kararli, 1995; Takahashi et al., 2016). This raises a fundamental question of the different phenotypes occurring after homozygous knockout of *Abcb4* in mice and hereditary dysfunction of ABCB4 in humans (Jacquemin et al., 2001). However, ABCB4 similarities of different species allowed more than a basic understanding (van Helvoort et al., 1996).

Due to *in silico* databases with a focus on conserved amino acid sequences between different species, the importance of ABCB4 variants can be assessed (Droge et al., 2017). Tools from the Berkeley Drosophila Genome Project (Reese et al., 1997) and NetGene2 Server (http://www.cbs.dtu.dk/services/NetGene2/) help to predict altered splicing-sites. However, limits of this *in silico* analysis are obvious for especially non-conserved mutations localized in the TMD.

In vitro studies allow the characterization of ABCB4 variants of all three categories (see the previous section). Absence and mistrafficking can be studied by either immunostaining or expression of GFP fusion proteins (Delaunay et al., 2009; Ellinger et al., 2013; Andress et al., 2014; Gordo-Gilart et al., 2015). Naturally polarized cell lines like MDCK-II or HepG2 should be considered for studying ABCB4 and other biliary transporters. However, PC efflux or secretion assays are not suitable for all of these cell lines. An alternative to investigate the effect of drugs on ABCB4 and ABCB11 would be the use of Transwell plates to generate a basal and apical membrane (Mahdi et al., 2016).

Studying PC efflux or secretion bring other challenges. It is undisputed that ABCB4 translocates lipids of the PC family, however, the molecular basis of this translocation is unknown. To address this problem many transport assays use stable transfectants of common cell culture models to measure ABCB4 translocation (Roulier et al., 1990; van Helvoort et al., 1996; Hojjati and Jiang, 2006; Gautherot et al., 2014). However, it appears to be challenging to monitor a translocation event of certain PC lipids in a membrane full of other lipids. Labels in the form of fluorescence or radioactivity are therefore required. However, labels should not interfere with the substrate recognition machinery (e.g. currently unknown binding site) of ABCB4. Therefore, the use of NBD-labeled PC lipids is controversially discussed, as ABCB1 is also able to flop NBD-labeled PC (van Helvoort et al., 1996), but an *in vivo* proof for this function is still missing. Nevertheless, the fact that,

ABCB4 translocation of NBD-PC lipids can be stimulated by adding taurocholate (TC) in the extra cellular media (Ruetz and Gros, 1995) or that NBD-PC lipids can be found in the pseudocanaliculi of HepG2 cells only if transfected with *ABCB4* (Shoda et al., 2004), suggest using these lipids may be valid. This problem can be circumvented by radioactive assays. A more indirect method is the extraction of total lipids, where PC can be distinguished between others via cleavage by the phospholipase D (Gautherot et al., 2014; Delaunay et al., 2016; Khabou et al., 2017).

Mostly unaddressed is the fact that the flop of PC lipids from the inner to the outer leaflet of a membrane (either native or synthetic) should be distinguished from the extraction process performed by an acceptor. Many transport assays measure the PC concentration in the medium and directly assign this concentration to ABCB4 translocation without considering the discrimination of acceptors for certain lipids or address the velocity of this process (see section 'Phosphatidyl choline – from biosynthesis to protection'). *In vitro* acceptors may be albumin, together with certain bile acids like TC or liposomes and all of them may act differently (Ruetz and Gros, 1994, 1995; van Helvoort et al., 1996). For example, without the acceptor TC no PC translocation by active ABCB4 can be measured (Morita et al., 2007).

One way to overcome this issue is the measurement of the ATPase activity as an indirect evidence for transport, which in general is used for ABC transporters. However, the measurement of ATPase activity in native membranes is challenging due to background activity of other ATP hydrolyzing proteins. Without knowledge of specific inhibitors, the effect of the mutation in ABCB4 can only refer to the difference of their stimulation (Park et al., 2016). A detergent solubilized and purified ABCB4 offers a solution. The detergent solubilized and purified ABCB4 permits direct measurements of kinetic parameters (Ellinger et al., 2013; Kluth et al., 2015). It is feasible to conclude that ATPase stimulation by a certain substrate is linked to its transport, because most substrates do indeed stimulate its ATPase activity as it was shown for ABCB1 (Chufan et al., 2013).

In conclusion an assay is required with a substrate which is easily identified but does not alter the recognition or binding features of ABCB4.

The future of ABCB4 research

Here, we have tried to summarize the current knowledge of the ABCB4 structure, function and its part in bile formation, but also its contribution to different liver diseases. We also described methods that are mainly used to analyze ABCB4 variants. So far, the mechanism of lipid translocation and uptake into bile canaliculi remains unclear or is controversially discussed. Structural information on ABCB4 as well as functional studies on a molecular level are required to understand ABCB4 mechanistically. A molecular mechanism would provide more accuracy for *in silico* tools to predict the effect of mutations in ABCB4. However, many more studies are required to understand the function of ABCB4 on a molecular level.

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3.2 Chapter 2 – Bile acids modulate ABCB4

Title:	**Monomeric bile acids modulate the ATPase activity of detergent-solubilized ABCB4/MDR3**
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JLR RESEARCH ARTICLE

Monomeric bile acids modulate the ATPase activity of detergent-solubilized ABCB4/MDR3

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Abstract ABCB4, also called multidrug-resistant protein 3 (MDR3), is an ATP binding cassette transporter located in the canalicular membrane of hepatocytes that specifically translocates phosphatidylcholine (PC) lipids from the cytoplasmic to the extracellular leaflet. Due to the harsh detergent effect of bile acids, PC lipids provided by ABCB4 are extracted into the bile. While it is well known that bile acids are the major extractor of PC lipids from the membrane into bile, it is unknown whether only PC lipid extraction is improved or whether bile acids also have a direct effect on ABCB4. Using in vitro experiments, we investigated the modulation of ATP hydrolysis of ABC by different bile acids commonly present in humans. We demonstrated that all tested bile acids stimulated ATPase activity except for taurolithocholic acid, which inhibited ATPase activity due to its hydrophobic nature. Additionally, we observed a nearly linear correlation between the critical micelle concentration and maximal stimulation by each bile acid, and that this modulation was maintained in the presence of PC lipids. This study revealed a large effect of 24-nor-ursodeoxycholic acid, suggesting a distinct mode of regulation of ATPase activity compared with other bile acids. In addition, it sheds light on the molecular cross talk of canalicular ABC transporters of the human liver.

Supplementary key words ABC transporter • ABCB4/MDR3 • bile acids • cholesterol • ATPase activity • TLCA • Nor-UDCA • critical micelle concentration

Human bile, which is formed at the canalicular membrane of hepatocytes, consists mainly of mixed micelles formed by predominately phosphatidylcholine (PC) lipids, bile acids, and cholesterol as well as to a smaller extent bilirubin, glucuronides, and organic anions. All these molecules are transported into the bile canaliculi by different ABC transporters. Bile salts are secreted by ABCB11 (bile salt export pump [BSEP]) (1), PC lipids are translocated by ABCB4 (2) and cholesterol is the substrate of the heterodimeric ABC transporter ABCG5/G8 (3). Furthermore, ABCC2 (MRP2) excretes bilirubin (4) and glucuronidated metabolites (5, 6). Further information concerning human hepatobiliary ABC transporter is summarized in (7). In general, the major component of bile is bile acids, which make up approximately 70% of human gallbladder bile (8). In humans, four different bile acids are present (**Fig. 1**A–D), which can be divided into primary and secondary bile acids. Primary bile acids are derived from cholesterol and are synthesized in hepatocytes by a two-step pathway resulting in either the trihydroxy cholic acid (CA) (Fig. 1A) or the two hydroxy chenodeoxycholic acid (CDCA) (Fig. 1B) (9–13). For higher solubility both are conjugated with either glycine or taurine. In humans, the major conjugation is glycine (8, 11, 12). In rodents (e.g., mouse or rats), however, taurine is the main conjugate. These conjugated primary bile acids are then secreted via ABCB11 into bile, stored in mixed micelles, until bile is secreted into the intestine. There, bile acids are required to solubilize hydrophobic compounds, e.g., vitamins or fatty acids. However, the bile acids themselves remain not unmodified in the intestine. Intestinal bacteria modify the conjugated primary bile acids by deconjugation and dehydroxylation specifically at position 7 (14). This results in so-called secondary bile aids. The two hydroxy deoxycholic acid (DCA) (Fig. 1C) is derived from CA, while the monohydroxy lithocholic acid (LCA) (Fig. 1D) is derived from CDCA. Through these modifications, the bile pool becomes more hydrophobic. Next reabsorption (active or passive) results in uptake of nearly 95% of the bile acids from the ileal segment into the blood (15), where it is transported back in to the liver. Here, the sodium taurocholate transporting peptide (NTCP) takes up primary and secondary bile acids (16). This circulation of bile acids is called "enterohepatic circulation." For detailed information, we recommend the review of Martinez-Augustin (17). Furthermore, next to the four bile acids described above, two nonhuman bile acids are relevant in medical treatments. Ursodeoxycholic acid (UDCA) (Fig. 1E) is part of the Chinese black bear bile pool and possesses the highest similarity to

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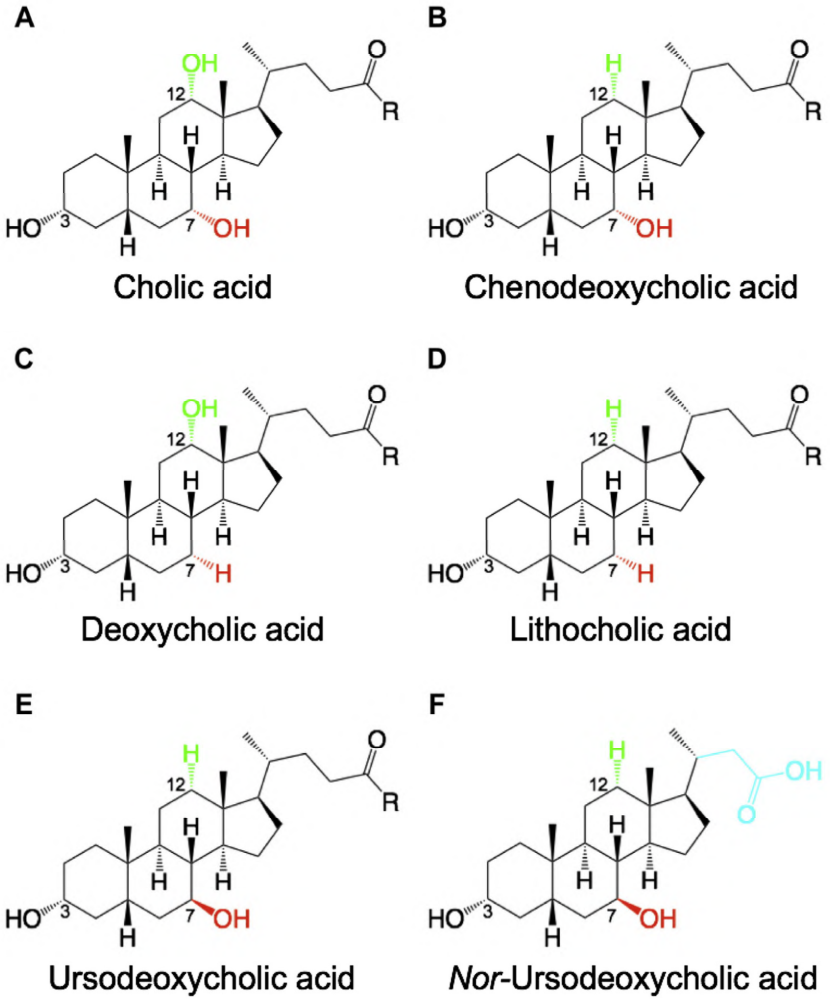

Fig. 1. Chemical structure of relevant bile acids. Bile acids are unconjugated (R equals a hydroxyl group) after synthesis, but get conjugated with either glycine or taurine (R equals glycine or taurine) prior to transport. The primary bile acids cholic (A) and chenodeoxycholic (B) acid are synthesized by two different pathways resulting in either a hydroxylation at position 12 for cholic acid or no hydroxylation at position 12 for chenodeoxycholic acid. Secondary bile acids are derived from the primary ones by dehydroxylation at position 7 by bacteria in the ilium. While deoxycholic acid (C) derives from cholic acid (A) lithocholic acid (D) is the result of dehydroxylation of chenodexycholic acid (B). Ursodeoxycholic acid (E) can be found in small amounts in human, but is one of the major bile acids in Chinese black bear (*Ursus thibetanus*). *Nor*-Ursodeoxycholic acid (F) in comparison to UDCA lacks a methylene group in its side chain (highlighted in violet). Therefore, it is not conjugated like the other bile acids.

CDCA. The only but important difference is the stereochemistry of the hydroxy group at position 7. While the hydroxy group at position 7 (if present) in all human bile acids is in the alpha position, the hydroxy group of UDCA is in the beta position (18). UDCA is a common drug in the treatment of cholestatic liver diseases (19), such as primary biliary cirrhosis (20–22), intrahepatic cholestasis of pregnancy (23), or progressive familial intrahepatic cholestasis (24–26). Since UDCA is such a powerful and widely distributed drug, it was also the target of synthetic modifications. These investigations resulted in a side-chain shortened derivate, 24-*nor*-ursodeoxycholic acid (Nor-UDCA). In comparison to UDCA, it lacks a methylene unit of its side chain (Fig. 1F). This minimal chemical modification results in resistance to amidation with taurine or glycine compared with UDCA (27). Additionally, Nor-UDCA does not undergo the entire enterohepatic circulation, instead it undergoes cholehepatic shunting, which represents the reabsorption by cholangiocytes (28). Nor-UDCA is seen as a novel approach in cholestatic and metabolic liver diseases (29, 30). In the case of ABCB4 knockout mice, Nor-UDCA was superior to UDCA in the treatment of sclerosing cholangitis (31, 32).

ABCB4 belongs to the superfamily of ATP binding cassette (ABC) transporters. The membrane proteins within this superfamily are present in all three kingdoms of life and share a common blueprint (33, 34). In the human genome, 48 genes coding for ABC transporter have been identified, which are divided into seven subfamilies (35). ABCB4 is part of the subfamily B and consists of two transmembrane domains (TMDs) and two nucleotide binding domains (NBDs) encoded on one single gene. Thus, ABCB4 represents a so-called full-size transporter (36), the structure of which was determined in 2019 (37). Due to the high identity (76%) and similarity (86%) of ABCB4 to the P-glycoprotein (P-gp, ABCB1), also termed multidrug resistance protein 1 (MDR1), ABCB4 was termed MDR3. In contrast to the ubiquitous expressed P-gp, ABCB4 is only expressed in hepatocytes (38, 39) and specifically targeted to the apical (canalicular) membrane. Additionally, ABCB4 possesses a different function as P-gp. First indications were derived from the murine homologue of ABCB4, Mdr2. Homozygous *mdr2*$^{-/-}$ knockout mice lacked cholesterol and PC lipids in their bile (2). Complementation of mice *mdr2*$^{-/-}$ by human ABCB4 demonstrated that human ABCB4 carried out the same function as Mdr2 (40). Finally, it was shown that ABCB4 specifically recognizes PC lipids (41–43). Nevertheless, data demonstrated that ABCB4 recognized certain P-gp substrates and inhibitors (44, 45). Based on these findings, it is now generally accepted that in vivo ABCB4 specifically translocates lipids of the PC family from the inner to the outer leaflet of the canalicular membrane of hepatocytes and therefore is part of the bile triumvirate. This bile triumvirate is composed of ABCB4, the bile acid export pump ABCB11 (or BSEP), and the cholesterol heterodimeric transporter ABCG5/G8. Since the substrates of this bile triumvirate are the major compounds of primary bile and form mixed micelles, one might speculate that these three ABC exporters act in concert to ensure proper formation of bile and balance of their compounds (46). For ABCB11, it was demonstrated that the amount of membrane cholesterol has an effect on its activity (47). Furthermore, it was shown for detergent-purified ABCG5/G8 that bile acids stimulate ATPase activity in a concentration-dependent manner (48). For ABCB4, it is known that the presence of a bile acid such as taurocholic acid (TCA) can increase the PC lipid and cholesterol content in the extracellular medium (49). For example, studies with murine ABCB4 demonstrated an explicit higher PC lipid content in mouse bile in the presence of TCA compared with the situation in the absence of TCA (50). In line with these findings is a preferential release of PC lipids from rat liver canalicular vesicles in the presence of TCA (51). For human ABCB4 expressed in HEK cells, it was demonstrated that adding TCA to the extracellular medium results in an increased amount of PC lipid and cholesterol in the extracellular medium after 24 h (49). Furthermore, in a model cell line (LLC-PK$_1$) expressing all three ABC transporters involved in bile formation, higher NBD-labeled PC lipid concentrations in the medium were observed, if cells were treated with albumin or TCA (52). These assays however cannot distinguish between a direct effect of the bile acid on ABCB4 or whether only PC lipid extraction is improved (37).

In this study, we aimed to address this question by measuring the ATPase activity of ABCB4 under defined conditions. The rationale behind this is the coupling between ATP hydrolysis and PC translocation, i.e., higher ATPase activity is the prerequisite for more efficient PC transport. Therefore, we used the previously established heterologous expression system in *Picheria pastoris*, which allows the purification of human ABCB4 (53), which enabled the determination of the kinetic parameters of the basal ATPase activity of ABCB4 wild type (54). In the same study, we were able to demonstrate that lipids of the PC family such as 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) stimulated ATPase activity of detergent-purified ABCB4, while such a stimulation did not occur for non-PC lipids (54). This clearly reflects the in vivo situation (40–42, 55, 56). In this study, we now used this established system to investigate the effect of conjugated or unconjugated human bile acids on the ATPase activity of human ABCB4 in vitro. Additionally, we analyzed conjugated and unconjugated UDCA and Nor-UDCA due to their clinical relevance as well as cholesterol as natural part of the canalicular membrane and substrate of ABCG5/G8. To evaluate our results quantitatively, we also determined the critical micelle concentration (cmc) of the bile acids used in our study under the conditions of our assay. Additionally, the observed modulation of ATPase activity of ABCB4 by bile acids and cholesterol was put in the context of a possible competition between bile acids and PC lipids. Here, we demonstrate that bile acids below their cmc actually modulate the ATPase activity of ABCB4 in a concentration-dependent manner. This effect occurred also in the presence of a natural substrate, DOPC, or cholesterol. This clearly demonstrates that all three substrates of all three bile triumvirate ABC transporters effect the function of ABCB4.

MATERIALS AND METHODS

Chemicals

Fos-choline 16 (FC-16) was obtained from Anatrace and DOPC from Avanti Polar Lipids. All bile acids were purchased from Merck except the conjugated versions of UDCA, Nor-UDCA, which was provided by Prof. Dr Dieter Häussinger, University Hospital Düsseldorf.

Routine procedures

Protein concentration was detected by the Bradford Coomassie Plus Assay (Pierce) or by measuring the absorbance at

280 nm using a NanoDrop™ 1000 Spectrometer (Thermo Fisher Scientific). The monoclonal anti-P-gp C219 antibody (Merck) combined with an anti-mouse IgG-HRP conjugate (Dianova) was employed for immune detection of ABCB4.

Expression and purification of human wild-type ABCB4

Expression, solubilization, and purification of human ABCB4 were performed as described in Kluth *et al.* (54) with the modifications outlined below. Detailed information about cloning and transformation was described in Stindt *et al.* (57) and Ellinger *et al.* (53). For higher cell density during fermentation, the glycerol-fed batch phase was increased to 5–6 h, therefore the expression (methanol-fed batch phase) could be reduced to 24 h as stated in (43). Solubilization was performed at 18°C (43) instead of 4°C (54) to obtain higher amounts of solubilized protein.

BODIPY® FL maleimide labeling of purified ABCB4

ABCB4 was specifically inhibited by labeling the unique cystine of the Walker A motif as described for ABCB1 (58) and ABCB4 (54). A sample of purified ABCB4 was incubated with 10-fold molar excess of BODIPY® FL maleimide (BODIPY® FL N-(2- aminoethyl)maleimide, Molecular Probes) at 22°C for 20 min. The reaction was quenched after 20 min by addition of a 20-fold molar excess of dithiothreitol (DTT) at 22°C for 10 min. Qualitative labeling efficiency was analyzed by visualizing a sample after SDS-PAGE by UV excitation.

Lipid, cholesterol, and bile acid preparation

DOPC was dissolved in chloroform, the solvent was evaporated, and the dried lipid sample was redissolved in ATP hydrolysis reaction buffer (50 mM Tris HCl, pH 7.4 at 37°C, 100 mM NaCl) at a concentration of 25.4 mM following the protocol of Geertsma *et al.* (59). Prior to the addition of the DOPC sample to the ATP hydrolysis assay, it was diluted to 5 mM, sonicated until a clear solution was obtained, and subsequently used at a final concentration of 300 µM. Since chloroform is not suitable for the later ATPase assay and cholesterol at higher concentration is insoluble in water and nearly all solvents, a molar mixture of cholesterol and DOPC was used. This mixture was obtained by adding the required molar ratio of cholesterol to always the same amount of dried DOPC. Afterward, the mixture was treated identical to DOPC only as described above resulting in a final concentration (f.c.) of 300 µM DOPC with 0–1,000 µM cholesterol in the ATPase assay.

The sodium salts of conjugated and unconjugated bile acids were dissolved in double-distilled water (ddH_2O) or 100% DMSO at a concentration of 100 mM. While glyco-/tauro-/cholic acid (G/T/CA), glyco-/tauro-/chenodeoxycholic acid (G/T/CDCA) were dissolved in ddH_2O water, glyco-/tauro-/deoxycholic acid (G/T/DCA), glyco/-tauro-/ursodeoxycholic acid (G/T/UDCA) as wells as Nor-UDCA and taurolithocholic acid (TLCA) were dissolved in DMSO. Glyco- and unconjugated lithocholic acid could not be dissolved in DMSO in the appropriate concentrations and therefore excluded. DMSO concentrations, if necessary, were set equal within one setup and did not exceed 1% for G/T/Nor-/UDCA or 5% for TLCA. Modulation of the ATP hydrolysis by bile acid was determined in a concentration-dependent manner from 0 to 1,000 µM bile acids.

ATP hydrolysis activity measurement of purified ABCB4

Analysis of the ATP activity of ABCB4 was performed by determining the amount of released, free inorganic orthophosphate by the malachite green assay (53, 54, 60) with minor modifications. The assay was performed in a total volume of 100 µl consisting of reaction buffer (50 mM Tris HCl, pH 7.4 at 37°C, 100 mM NaCl) supplemented with the 2.5 × cmc of FC-16 and 10 mM $MgCl_2$. For determining the effect of DOPC, different bile acids or combinations thereof, 1–5 µL of the corresponding stock solutions (see lipid and bile acid preparation) were added. Five micrograms of ABCB4 or the BODIPY®-labeled sample was used in every reaction sample. Reaction was started by addition of ATP (5 mM f.c.) and performed for 40 min at 37°C. After 0 and 40 min, the reaction was stopped by transferring 25 µl of the reaction mixture into a 96-well plate containing 175 µl of 20 mM ice-cold sulfuric acid. Additionally, a phosphate standard with concentrations raging from 0 to 500 µM was used. Subsequently, inorganic phosphate was stained by adding 50 µl of dye (0.096% (w/v) malachite green, 1.48% (w/v) ammonium molybdate, 0.173% (w/v) Tween-20, and 2.36 mM sulfuric acid). After incubation for 15 min, the absorption at 595 nm was measured (iMark™ Microplate Reader, BioRad), and the concentrations of phosphate release were calculated based on the slope of the line calculated based on the phosphate standards.

Basal ATPase activity of ABCB4 was set to 0% and the effect of supplements was calculated as % stimulation or % inhibition of the basal activity, respectively. For determination of kinetic parameters, bile acid concentration was varied and analyzed according to an allosteric sigmoidal fit (Equation 1):

$$v = \frac{v_{max} * [S]^h}{EC_{50}^h + [S]^h} \quad (1)$$

Here, v describes the stimulation (%), v_{max} is the maximal stimulation (%), EC_{50} represents the half maximal effective concentration, S is the substrate concentration, and h is the Hill coefficient

In case of inhibition, the reduction (%) was plotted against the bile acid concentration and data were analyzed using Equation 2:

$$y = \frac{y_{min} + (y_{max} - y_{min})}{1 + 10^{((logIC_{50} - x) * slope)}} \quad (2)$$

Here, y_{max} is the smallest, and y_{min} is the highest amount of inhibition, respectively, and x represents the concentration of the inhibitor. The IC_{50} value is defined as the concentration necessary to obtain 50% inhibition.

In case of both substrate stimulations, followed by substrate inhibition after maximal stimulation was reached, the modulation (%) was plotted against the substrate concertation and data was analyzed using by two fits: First part (stimulation) was as usually fitted using Equation 1. The second part (substrate inhibition) was analyzed by Equation 3 starting at the last point of the plateau:

$$y = \frac{y_{min} + (y_{min} - y_{max})}{\left(1 + \frac{x}{IC_{50}}\right)} \quad (3)$$

Here, y_{max} is the highest value of the plateau after stimulation is reached, while y_{min} is the smallest value of y after

maximal stimulation was reached. The IC_{50} value is defined as the concentration necessary to obtain 50% reduction. Mathematical analysis was performed using Prism (version 8, GraphPad). All experiments were performed as at least triplicates, if not otherwise stated.

Determination of the critical micelle concentration

CMC values were determined as described (61). Here, the fluorescence of Hoechst 33342 is measured in a concentration-dependent manner. 96-well plates suitable for fluorescence spectroscopy (Greiner Bio-One, FIA plate, black, flat bottom, medium binding) were used. Final concentration of Hoechst 33342 was set to 7 µM. CMCs of the bile acids were determined in ATPase reaction buffer (50 mM Tris-HCl, pH 7.5 (at 37°C), 100 mM NaCl) containing no FC-16, $MgCl_2$, or ATP at 37°C using a Tecan M200 plate reader (Atlantic lab equipment). Bile acids were dissolved either in ddH_2O or in 100% DMSO (as stated in "Lipid, cholesterol and bile acid preparation") at a concentration of 100 mM. Different amounts of the stock solution were added into each well containing already the reaction buffer and Hoechst 33342. In case of hydrophobic bile acids, which were dissolved in DMSO, DMSO concentration was adapted to 1% in all wells. Emission spectra were recorded with filters set to λ_{ex} = 355 ± 10 nm, λ_{em} = 460 ± 80 nm. After background correction, fluorescence data were analyzed using Prism (version 8, GraphPad) with Equation 4:

$$F = \frac{F_{max} * (c[BA] - cmc)}{(K_{0.5} - cmc) + (c[BA] - cmc)} \quad (4)$$

The measured fluorescence at each bile acid concentration (c[BA]) is represented by F, while F_{max} is the maximal fluorescence, $K_{0.5}$ is the midpoint of the function, and cmc is the critical micelle concentration of the bile acid.

RESULTS

Expression and purification of ABCB4 wild type by tandem affinity purification

Previously, the expression of chromosomally integrated wild-type ABCB4 in *P. pastoris* was described (43, 53, 54). Fermentation led to an average yield of 1.5 kg wet cell weight. Additionally, FC-16 is suitable to solubilize ABCB4 in large quantities (53). ABCB4 was solubilized in 1% FC-16, and the supernatant was applied to a tandem affinity chromatography procedure after a sequential centrifugation step (43, 53, 54). Purification was analyzed by Colloidal Coomassie Brilliant blue–stained SDS PAGE gels (**Fig. 2**A) and immunoblotting using the monoclonal P-gp C219 antibody, which also recognizes ABCB4 (Fig. 2B). On average, a yield of 5–6 mg of protein out of 100 g wet cell weight was obtained with a homogeneity of approximately 80%–85%.

ATP hydrolysis of purified ABCB4 and BODIPY® FL maleimide-labeled ABCB4

Previously, it was demonstrated that cross-linking the cysteine of the Walker A motif in ABCB1 (P-gp) with maleimide derivates resulted in inhibition of ATP hydrolysis of ABCB1 (58) and was used to study the function of ABCB1 (62, 63). Since ABCB4 also contains such a cysteine in the Walker A motif, BODIPY® FL maleimide (further referred as Bodipy) was used to inhibit specifically ABCB4. Hence, kinetic parameters of ATP hydrolysis of ABCB4 wild type, the EQ-double mutant, and Bodipy-labeled wild-type protein in the presence and absence of 300 µM DOPC, respectively, have been already determined (54). Under UV excitation, an SDS PAGE gel highlights that signal for only the labeled sample at the height of the 130 kDa marker band was observed (Fig. 2C) indicating successful labeling.

As a proof of concept, ATP hydrolysis of ABCB4 and its BODIPY-labeled form was determined in the absence and presence of DOPC (Fig. 2D), respectively. Red bars represent the ATPase activity of ABCB4-Bodipy in the absence and presence of DOPC, which was identical within experimental error (206.8 ± 8.2 nmol min^{-1} mg^{-1} without DOPC and 212.8 ± 19.7 nmol min^{-1} $mg^{-1}$with DOPC). This indicates that the non-ABCB4-mediated ATPase activity is not influenced by the addition of DOPC. Nonlabeled protein displayed an ATPase activity of 338.8 ± 21.3 nmol min^{-1} mg^{-1} and 488.6 ± 44.5 nmol min^{-1} mg^{-1} in the absence and presence of 300 µM DOPC, respectively. These values are in good agreement with published data (54). The specific ATPase activity of ABCB4 was calculated by subtracting the activity of Bodipy-labeled ABCB4 from the activity of the nonlabeled membrane protein. Thus, ABCB4 possessed a basal activity of 132.1 ± 13.0 nmol min^{-1} mg^{-1} and was stimulated twofold in the presence of DOPC (275.8 ± 24.8 nmol min^{-1} mg^{-1}). Additionally, only ABCB4 was stimulated by DOPC since the activity of the Bodipy-labeled protein remained the same in the presence or absence of DOPC.

ATPase activity of purified ABCB4 is modulated by conjugated bile acids

This in vitro setup was used to address the question whether or not conjugated bile acids have an direct effect on the ATPase activity of ABCB4. We investigated both glycine and taurine-conjugated versions of human bile acids, because the majority of bile acids are conjugated with either glycine or taurine in the natural environment of ABCB4. Indeed, a modulation of ATPase activity by different bile acids was observed (**Fig. 3**). Since bile acids are detergents and modulate the activity of other ATPases, we again employed labeling with Bodipy® and subtracted this activity as control. No bile-acid-modulated ATPase activity of such a sample was detected. As described in Materials and Methods, conjugated bile acids were used in the range of 0–1,000 µM. However, due to batch-to-batch variations of detergent-solubilized and purified ABCB4, we decided to present all data as normalized

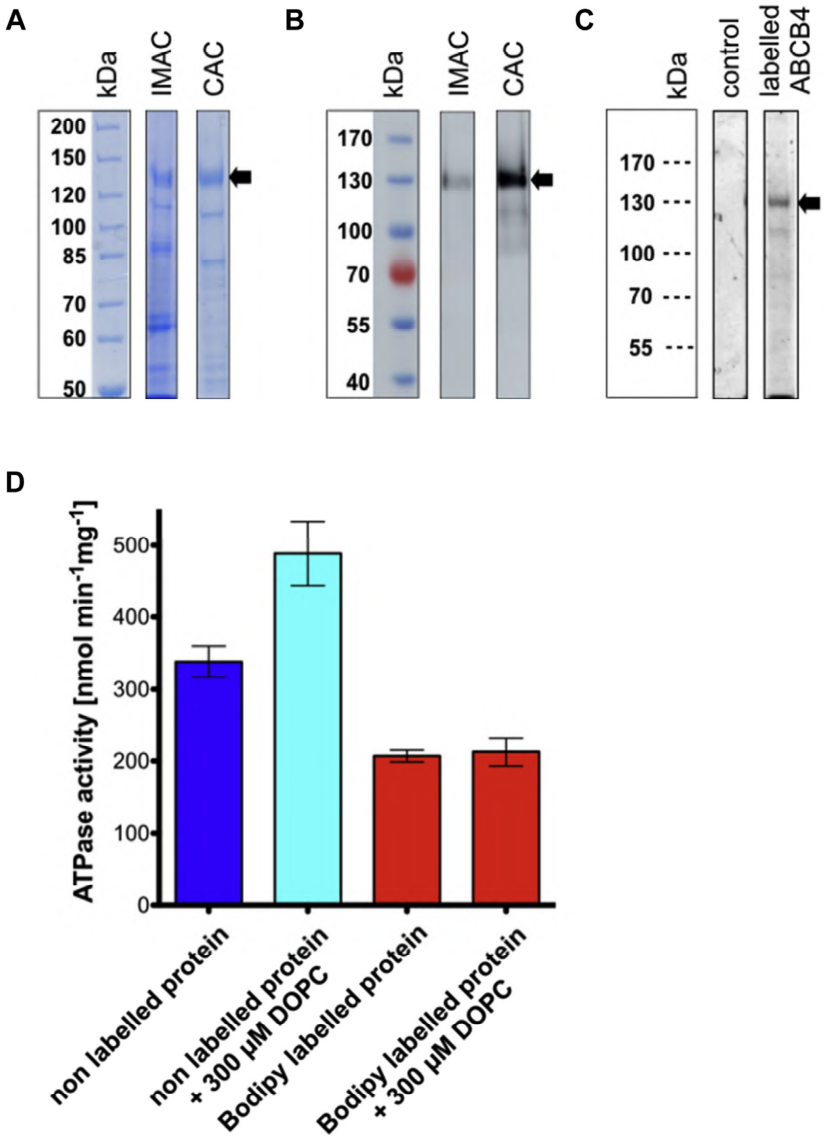

Fig. 2. ABCB4 purification, labeling, and ATPase activity. Human *wild-type* ABCB4 purified from *P. pastoris*. ABCB4 contains C-terminally a calmodulin binding peptide and two 6xHis-tag. It was purified by tandem affinity chromatography (first an IMAC, second a calmodulin affinity chromatography (CAC)). In total, 10 µl of the elution fractions of the IMAC and CAC was mixed with 40 µl SDS-buffer and injected on the gels for analysis by colloidal Coomassie brilliant blue staining (A) and immunochemiluminescence using the monoclonal anti P-gp C219 antibody (B). C: Purified ABCB4 was exceeded with 10-fold molar excess of BODIPY® FL maleimide (Bodipy). In total, 10 µl of an unlabeled control and the labeled ABCB4 were mixed with 40 µl of SDS-buffer and run on an 7% SDS-gel. Bodipy emission was detected under UV excitation. D: ATP hydrolysis activity using 5 µg of protein from the CAC elution fraction (blue) and with additional 300 µM DOPC (cyan). Red bars show the ATP hydrolysis of 5 µg of Bodipy®-labeled ABCB4 with and without the addition of DOPC. Data represent the mean and SD of three biological independent experiments.

values, in which the basal activity of ABCB4 (in the absence of PC lipids, bile acids, or cholesterol) was set to 0% and all other values are calculated as percent of stimulation of the basal activity. Thus, we determined comparable levels of stimulation and the EC_{50} for individual bile acids. Except for TLCA, all conjugated bile acids stimulated the ATPase activity of ABCB4 (Fig. 3A–D). Glycolithocholic acid precipitated at concentration higher than 100 µM and was not included in our quantitative analysis.

The highest stimulation was observed for the conjugated primary bile acids G/TCA (Fig. 3A). At concentration lower than 50 µM, these bile acids did not modulate ATP hydrolysis within experimental error. In

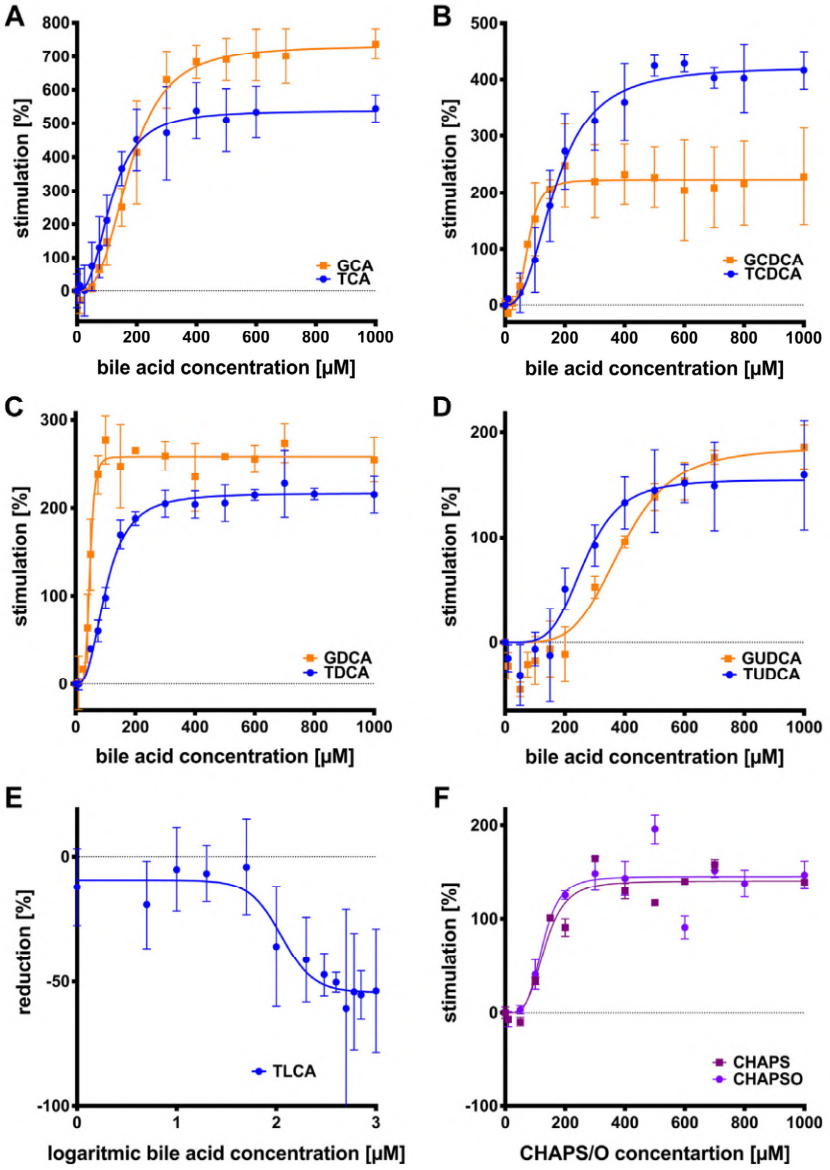

Fig. 3. Modulation of the specific ABCB4 ATP hydrolyzing activity by the different conjugated bile acids. Modulation presented as % stimulation/% reduction based on the basal activity at 0 µM bile acid, which was set to 0%. Modulation of the ATPase activity of ABCB4 was measured in concentration-dependent manner from 0 to 1,000 µM of the glycine (orange) and taurine (blue) versions of cholic acid. Please note the different scaling of the *y* axis in the different panels. A: Chenodeoxycholic acid (B), deoxycholic acid (C), and ursodeoxycholic acid (D). For lithocholic acid (E), only the taurine-conjugated version was soluble in 5% DMSO, while glycolithocholic acid precipitated at higher concentrations (>100 µM). Since the curve of TLCA presents an IC_{50} fit, the bile acid concentration (*x*-axis) is presented as their corresponding decadic logarithmic values. Additionally, the bile-acid-derived detergents CHAPS (dark violet) and CHAPSO (bright violet) were analyzed at the same concentration as bile acids (F). A–D: Data point represent the average of three independent experiments with the errors reported as SD and data were analyzed according to an allosteric sigmoidal fit (Equation 1). E: Bars and error bars represent the mean and SD of three independent experiments and were analyzed using Equation 2. F: Data are presented as mean and SD of two independent experiments and were fitted according Equation 1.

the range of 75–200 µM for TCA (EC_{50} = 114 ± 10 µM) and 75–300 µM for GCA (EC_{50} = 177 ± 10 µM) stimulation increased before reaching the maximal stimulatory effect at approximately 400 µM. Stimulation reached 733 ± 24% for GCA and 539 ± 24% for TCA, respectively (Table 1). The primary bile acid taurochenodeoxycholic acid (TCDCA) stimulated ABCB4 activity up to 423 ± 15% (Fig. 3B), which is the third highest stimulation that was observed. The corresponding glycine-conjugated bile acid, GCDCA,

TABLE 1. Kinetic parameters of ATPase activity of ABCB4 in the presence of conjugated bile acid

Bile Acid	Max. Stimulation (%)	EC_{50} (μM)	Slope
GCA	733.2 ± 23.5	177.0 ± 9.9	2.9 ± 0.4
TCA	538.5 ± 24.1	114.4 ± 9.7	2.6 ± 0.5
GCDCA	223.2 ± 10.1	77.8 ± 7.5	3.9 ± 1.5
TCDCA	422.7 ± 15.1	169.2 ± 11.0	2.6 ± 0.4
GDCA	258.5 ± 4.8	47.9 ± 1.5	5.9 ± 1.2
TDCA	216.5 ± 3.9	101.8 ± 4.2	2.7 ± 0.3
GUDCA	185.3 ± 12.9	393.2 ±23.3	4.5 ± 1.0
TUDCA	154.4 ± 10.8	266.5 ± 24.2	4.5 ± 1.4
CHAPS	142.6 ± 11.5	150.9 ± 20.8	3.7 ± 2.1
CHAPSO	145.4 ± 15.3	124.9 ± 23.3	4.3 ± 2.5
Bile acid mixture	566.0 ± 17.6	430.4 ± 11.2	4.5 ± 0.4
	Max. Inhibition (%)	IC_{50} (μM)	Slope
TLCA	−54.4 ± 5.8	113.1 ± 1.4	−2.7 ± 2.1

reached a maximal stimulation of 223 ± 10% (Fig. 2B). Parallel to G/TCA, the data of G/TCDCA showed no effect at concentration lower than 50 μM, followed by an increase until the maximal stimulation was reached at approximately 150 μM for GCDCA and approximately 500 μM for TCDCA. This implied that the conjugated primary bile acids act in a similar manner and only the maximal stimulation was affected due to differences in the structure and/or physiochemical properties of these bile acids. The secondary bile acids G/TDCA (Fig. 3C) differed not substantially from the primary bile acids. Glycine-conjugated DCA excelled the corresponding taurine-conjugated version by approximately 40%, with maximal stimulations for G/TDCA of 259 ± 5% and 217 ± 4%, respectively (Table 1).

Due to their high relevance in medicine, the conjugated versions of the bile acid UDCA were also considered. G/TUDCA are secondary bile acids in humans although the only difference from the primary G/TCDCA is the configuration of the hydroxyl moiety at position 7. A maximal stimulation of 185 ± 13% and 154 ± 11% was observed for G/TUDCA, respectively (Table 1). However, clear differences in the shape of the curves for G/TUDCA were apparent. The range, in which no stimulation occurred, was extended. The mean values at 50 μM showed a reduced activity of 30%–45%. Hence, stimulation of ABCB4 required higher concentrations of bile acid (Fig. 3D) and therefore EC_{50} values increased (Table 1). Half-maximal stimulation for G/TUDCA (EC_{50} values of 393 ± 23 μM and 267 ± 24 μM, respectively) was reached at significantly higher concentrations compared with the other bile acids. One possible rational for this behavior might be the unusual configuration of the hydroxyl moiety at position 7, which is beta in these cases. Nevertheless, all these bile acids showed the same modulation pattern. No modulation at lower concentrations followed by an increase, which finally resulted in a plateau value.

An opposite behavior was observed for TLCA. LCA makes up only ~1.5% of the total bile in healthy humans (8) and is more hydrophobic than the other bile acids, because it lacks two hydroxyl groups compared with, for example, CA. It is used as a model compound to induce cholestasis in rat liver (64–67). TLCA demonstrated inhibition of the ATPase activity of ABCB4 (Fig. 3E). Similar to the other bile acids, no effect on ABCB4 at concentrations below 50 μM was observed. At 100 μM ABCB4 ATPase activity was reduced by 36%. Maximal inhibition of approximately −55% was reached at 400 μM TLCA, which is in the range in which other bile acids had already reached v_{max}. The half inhibitory concentration (IC_{50}) was 113 ± 1.4 μM and therefore in comparable range to the EC_{50} values of bile acids, which stimulated ABCB4 (except for G/TUDCA).

To investigate the importance of conjugation, the two bile-acid-based detergents 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) and 3-[(3-cholamidopropyl)dimethylammonio]-2-hydroxy-1-propanesulfonate (CHAPSO) were tested. Both may be synthesized from CA and have a more bulky and complex conjugation than G/TCA. CHAPS and CHAPSO are presented as mean and error of duplicates (Fig. 3F). Within experimental errors, both compounds stimulated ATPase activity of ABCB4 in an identical pattern. Below a concentration of 100 μM, no stimulation was observed. Instead, slight inhibition occurred at 10 and 50 μM for CHAPS (–7.1 ± 3.6% and –9.9 ± 5.0%) and at 10 μM for CHAPSO (–7.5 ± 8.6%). At concentration higher than 50 μM, ATPase activity increased, until the maximal stimulation of 142.6 ± 11.5% and 145.4 ± 15.3% for CHAPS and CHAPSO, respectively, was reached. The plateau started at a concentration of 300 μM for both. Hence, the EC_{50} values for CHAPS and CHAPSO were in close range to each other (EC_{50}(CHAPS): 150.9 ± 20.8 μM and EC_{50}(CHAPSO): 124.9 ± 23.3 μM, Table 1).

ATPase activity of purified ABCB4 is modulated by unconjugated bile acids

The half synthetic bile acid Nor-UDCA, which is often employed as a new drug for cholestatic liver diseases, remains unconjugated, because its shorter side chain inhibits coenzyme A formation (68). To compare values of unconjugated Nor-UDCA, all major bile acids were tested in their unconjugated state. This also should provide insights in the importance of conjugation for the modulation of ATPase activity of ABCB4.

The highest stimulation for the unconjugated versions was obtained from CA (**Fig. 4**A) with a v_{max} value of 310.5 ± 16.4% (Table 2). Similar to the conjugated versions at concentrations lower 100 μM, CA did not modulate ATPase activity within experimental errors. The half-maximal stimulation (EC_{50}) was reached at a bile acid concentration of 390.5 ± 22.7 μM. The maximal stimulation was reached at approximately 700–800 μM. Similar results were observed for the other unconjugated primary bile acid CDCA (Fig. 4B). Within

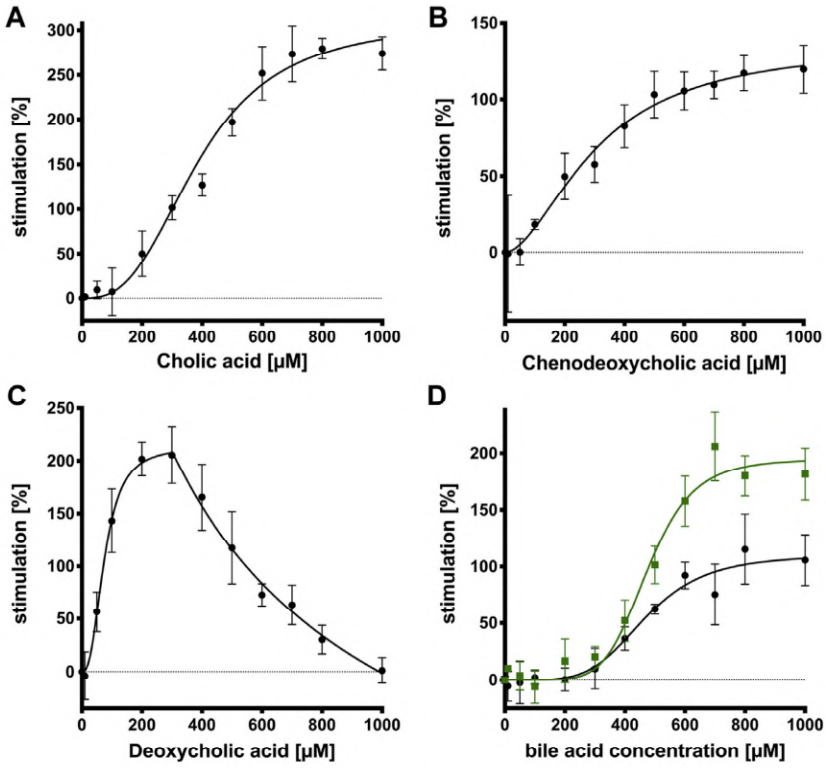

Fig. 4. Modulation of the specific ABCB4 ATPase activity by the different unconjugated bile acids. Modulation is presented as stimulation in percentage based on the basal activity at 0 µM bile acid, which was set to 0%. Modulation of the ATPase activity of ABCB4 was measured in concentration-dependent manner from 0 to 1,000 µM the bile acid cholic acid (CA). Please note the different scaling of the *y* axis in the different panels. A: Chenodeoxycholic acid (CDCA) (B), deoxycholic acid (DCA) (C), and ursodeoxycholic acid (UDCA) as well as the side chain shortened variant Nor-UDCA (D). Lithocholic was excluded since it was not soluble under the assay conditions at higher concentrations. For DCA kind of "substrate" inhibition after maximal stimulation was measured. For a better overview in panel D, Nor-UDCA was highlighted in green. Data point represents the average of three independent experiments with the errors reported as SD. Data were analyzed according to an allosteric sigmoidal fit (Equation 1) except for C. Here, stimulation was analyzed similar to others according to an allosteric sigmoidal fit (Equation 1), while the substrate-induced reduction is fitted according to Equation 3.

error no modulation occurred below 50 µM. Stimulation of the ATPase activity started at 150 µM and reached also its maximum at 700–800 µM CDCA with a maximal stimulation of 137.1 ± 16.8% and therefore is significantly lower than for CA (Table 2). The EC_{50} value of CDCA (308.4 ± 56.8 µM) is also decreased compared with CA (390.5 ± 22.7 µM). For the secondary bile acid DCA, a different modulation pattern was observed (Fig. 4C). Although no modulation within experimental errors below 50 µM occurs, the stimulation increased rapidly to a maximum of 213.6 ± 13.3% at a concentration of 300 µM. Therefore, the EC_{50} value of DCA (75.0 ± 7.3 µM) is significantly lower compared with the others. Unlike all previous bile acids, DCA has no plateau after reaching maximal stimulation. Starting at concentration of 400 µM, the ATPase activity is reduced to 0% within the errors if 1,000 µM of DCA is present. This might indicate "substrate" inhibition and is only observed for this bile acid. Next, the two medically relevant bile acids UDCA and Nor-UDCA were analyzed (Fig. 4D). Both bile acids have no modulatory effect within experimental errors up to 200 µM and share a nearly identical EC_{50} value of 470.3 ± 34.5 µM (UDCA) and 475.8 ± 18.5 µM (Nor-UDCA, Table 2). Additionally, both reach the maximal stimulation at the same concentration (700–800 µM). However, the v_{max} of Nor-UDCA exceeds the v_{max} of UDCA. More precisely, the maximal stimulation of Nor-UDCA with a value of 195.0 ± 10.8% is the highest of all unconjugated, two

TABLE 2. Kinetic parameters of ATPase activity of ABCB4 in the presence of unconjugated bile acid and cholesterol

Bile Acid	Max. Stimulation (%)	EC_{50} (µM)	Slope
CA	310.5 ± 16.4	390.5 ± 22.7	2.8 ± 0.3
CDCA	137.1 ± 16.8	308.4 ± 56.8	1.8 ± 0.4
DCA	213.6 ± 13.3	75.0 ± 7.3	2.6 ± 0.6
UDCA	110.3 ± 10.7	470.3 ± 34.5	4.6 ± 1.3
Nor-UDCA	195.0 ± 10.8	475.8 ± 18.5	6.3 ± 1.3
Cholesterol	103.5 ± 5.9	0.7 ± 0.05	7.8 ± 2.2

hydroxy bile acids (CDCA, DCA, UDCA, and Nor-UDCA) and second highest of all unconjugated bile acids, except CA. In contrast, UDCA only reaches a maximal stimulation of 110.3 ± 10.7% and therefore has the lowest stimulatory effect among the unconjugated bile acids. In summary, all unconjugated bile acids stimulate the ATPase activity of ABCB4. While CA, CDCA, UDCA, and Nor-UDCA show an overall similar curve, DCA is the first and only bile acid to show a reduction in stimulation after reaching the maximum (bell-shaped curve). One has to highlight again that stimulation by Nor-UDCA exceeds all other unconjugated bile acids, except for CA.

Modulation of ABCB4 by bile acids is correlated to their cmc

From a chemical point of view, bile acids differ only in one or two moieties (Fig. 1). All have a hydroxyl moiety at position 3 in common. The bile acid with the highest number of hydroxy moieties is CA and its derivatives. It is hydroxylated at position 7 as well as position 12. Importantly, CA showed the highest maximal stimulation regardless of conjugation or not. In contrast, LCA and its derivates lack both of these hydroxy moieties and TLCA was the only bile acid that inhibited basal activity of ABCB4 in our setup (Fig. 3E). All other bile acids possess a hydroxy moiety at position 7 or 12. Furthermore, their maximal stimulation was between the corresponding CA and LCA derivates. Additionally, the kinetics of G/TUDCA bile acids with a hydroxy group at position 7 in the β-conformation revealed a shift in the EC_{50} values (Table 1). Therefore, ABCB4 might recognize the cholesterol backbone and especially the hydroxy groups at position 7 and 12.

To analyze and compare all bile acids, we determined the cmc of each bile acid under the conditions of our assay. We used an assay, which relied on the increase of the fluorescence of Hoechst 33342 in the hydrophobic environment of a micelle (61). Although cmc values for many bile acids have been reported (69–74), cmc values are sensitive toward parameters such as temperature, pH, and/or ionic strength. For every bile acid used in the ATPase activity assay, a cmc was determined and summarized in Table 3. The primary bile acids G/TCA have the highest cmc values (4.1 and 3.5 mM), while for unconjugated CA a cmc of 2.3 mM was determined. Conjugated versions of CDCA and DCA exhibit a cmc in the range from 1.7 mM to 2.6 mM. The cmc of

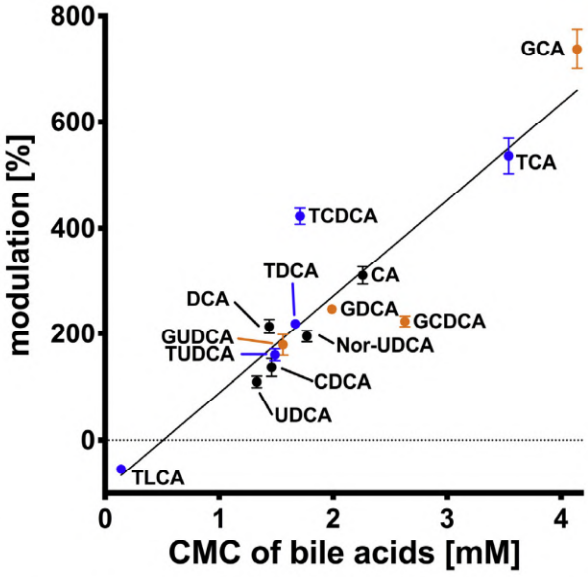

Fig. 5. Dependence of bile acid modulation of ABCB4 and cmc. Glycine-conjugated bile acids are shown in orange, taurine-conjugated bile acids are colored blue, and the unconjugated versions are in black. Linear regression revealed a slope of 181.8 ± 12.9 and a correlation coefficient r^2 of 0.83. Data point represents the v_{max} values and errors of individual bile acid kinetics (Figs. 3 and 4, Tables 1 and 2). CMC values were taken from Table 3.

unconjugated CDCA and DCA is lower than the corresponding conjugated versions but nearly identical (1.5 mM for CDCA and 1.4 mM for DCA, respectively). Interestingly, G/TUDCA and unconjugated UDCA displayed cmc in a narrow range. Among them GUDCA has the highest cmc (1.6 mM), second is TUDCA (1.5 mM), and hence, UDCA (1.3 mM) has the lowest of these three bile acids. Here, conjugation has less to no impact on hydrophobicity and cmc. Shortening the side chain of UDCA therefore resulted in an increase of the cmc. Nor-UDCA possessed a cmc of 1.8 mM. In literature, UDCA and its versions are considered to have one of the highest cmc (10, 75). However, we clearly observed an increase in fluorescence emission already in the range of 1.3–1.8 mM for our UDCA versions. The harmful bile acid TLCA possess a cmc of 0.14 mM and is therefore the only bile acid in our study with a cmc significantly below 1 mM. The determined cmc values of the bile acids demonstrate that monomeric bile acids

TABLE 3. Critical micelle concentration of bile acids under assay conditions

Bile Acid Conjugation	CA (mM)	CDCA (mM)	DCA (mM)	UDCA (mM)	Nor-UDCA (mM)	LCA (mM)
Glycine	4.1 ± 0.1	2.6 ± 0.1	2.0 ± 0.1	1.6 ± 0.1	–	Precipitate[a]
Taurine	3.5 ± 0.1	1.7 ± 0.1	1.7 ± 0.1	1.5 ± 0.2	–	0.14 ± 0.03
Unconjugated	2.3 ± 0.2	1.5 ± 0.1	1.4 ± 0.1	1.3 ± 0.1	1.8 ± 0.1	Precipitate[a]

Presented are the calculated values and the error of the mean derived from the fitting procedure. Data were evaluated using Equation 4. For Nor-UDCA, only the unconjugated version was considered, since it is not conjugated in hepatocytes.
[a]G/LCA precipitated at concentrations higher than 100 μM in our setup.

modulate the ATPase activity of ABCB4 as maximal stimulation was observed below a concentration of 1 mM bile acids, except for TLCA. Hence, the stimulatory effects are based on an interaction of ABCB4 with monomeric bile acids, not with the micelles. Additionally, the different maximal stimulations are reached due to the properties of bile acids and not because the free bile acid concentration is limited by the formation of micelles.

Plotting the maximal stimulation against the cmc values revealed a nearly linear relation with a correlation coefficient r^2 of 0.83 (**Fig. 5**). The highest stimulatory effect was observed for GCA, which also has the highest cmc value (4.1 mM). In contrast, TLCA was the only bile acid that reduced the ATPase activity of ABCB4 and exhibits the lowest cmc value (0.14 mM). Hence, formation of micelles already starts at 140 µM, and we cannot distinguish whether the observed reduction is due to TLC itself or because of micelle formation. In summary, we determined cmc values under the same conditions as in the ATPase activity assay for each bile acid in this study. Further and more important, a linear correlation between maximal stimulation and cmc values was observed.

ABCB4 accepts more than one bile acid

ABCB4 is clearly modulated by monomeric, unconjugated and conjugated bile acids. However, the question arises whether the observed effects are additive or whether ABCB4 has a preference for certain bile acids. To investigate this, we analyzed mixtures of TLCA with G/TCA. G/TCA concentrations were decreased in 200 µM steps starting at 1,000 µM, while TLCA concentration was increased in the inverse manner. Hence, the final bile acid concentration was always kept constant at 1,000 µM. Additionally, two conditions were tested. First, a setup with 967 µM of G/TCA and 33 µM of TLC was analyzed, this equals to a 30:1 ratio and matches approximately the in vivo human CA:LCA ratio (8, 76). Second a 50:50% mixture of both bile acids was included (**Fig. 6**A). In the case of only GCA and TCA (1,000 µM), the maximal stimulation was slightly reduced compared with Fig. 3A. This might be due to the presence of 5% DMSO, which was not present in the kinetic measurements (Fig. 3A), but is necessary for keeping TLCA in solution in this setup. In both assays (GCA + TLCA and TCA + TLCA, respectively), pure TLCA showed an inhibition of approximately 50%, which is in line with the previously observed reduction (Fig. 3E). Mixing the primary bile acids with TLCA at a ratio of 30:1 had no effect on the modulation of ABCB4. However, at 800 µM G/TCA and 200 µM TLCA, the stimulatory effect already decreased to approximately half. Furthermore, at a ratio of 3:2 (600 µM G/TCA and 400 µM TLCA) values were at the level of basal ATPase activity of ABCB4. The same holds true for the inverse ratio (400 µM G/TCA and 600 µM TLCA) as well as the 50:50 mixture. Increasing the amount of TLCA resulted in a decrease of the ATPase activity.

One has to consider that TLCA is present in micelles, while G/TCA exist as monomeric bile acids. Thus, three questions arose: (1) how do both bile acids interact; (2) if and how much of the primary bile acids were integrated into TLCA micelles; and therefore (3) what is the

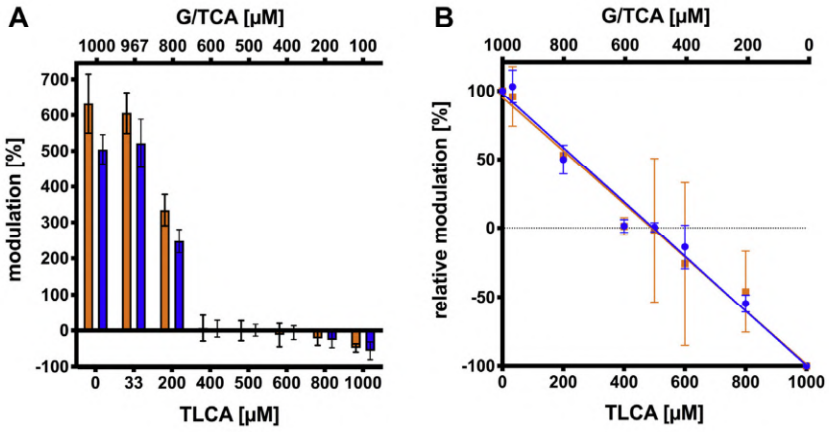

Fig. 6. Modulation of the ATP hydrolysis of ABCB4 by different ratios of mixtures of GCA and TLCA (orange) or TCA and TLCA (blue). Starting at 1,000 µM the concertation of the primary bile acid was decreased in steps of 200 µM and the concentration of TLCA was increased in parallel. Additional mixtures of 967 µM G/TCA and 33 µM of TLCA and 500 µM of both bile acids were tested. All reactions contained a total concentration of 1,000 µM of bile acids and 5% DMSO. In (A) the modulation of the different mixtures is presented as stimulation or inhibition in relation to ABCB4 basal activity, which was set to 0%. In (B) the relative modulation is displayed, in which the maximal stimulation was set to 100% and maximal inhibition was set to –100%, respectively. The graph demonstrates the linear correlation between the two bile acids. A and B: Bars and error bars represent the mean and SD of three independent experiments. B: Data were analyzed by a linear fit.

true concentration of G/TCA in the mixtures? With our assay, we cannot answer these questions, but after normalization of the data, a linear correlation was observed (Fig. 6B). This demonstrates that ABCB4 does not distinguish between bile acids. In case of higher G/TCA concentrations, the ATPase activity of ABCB4 was stimulated, while higher TLCA concentrations decreased the ATPase activity.

Modulation of the ATPase activity of ABCB4 by cholesterol

Cholesterol is abundant in every mammalian membrane and the substrate of a transporter of the bile triumvirate, ABCG5/G8. Therefore, one may assume a modulatory effect on ABCB4 similar to the one described for ABCB11 (47). Hence, this in vitro setup was used to address this hypothesis. However, a major problem of cholesterol is its solubility. To overcome this problem, chloroform is often used as a solvent, which is not compatible with our assay. Hence, cholesterol was mixed with DOPC in different ratios. In the ATPase assay, always a final concentration of 300 μM DOPC was present, mixed with cholesterol ranging from 0 to 1,000 μM resulting in molar ratios of 0–3.3 (cholesterol to DOPC). The DOPC concentration of 300 μM was chosen to ensure maximal stimulation by the PC lipid

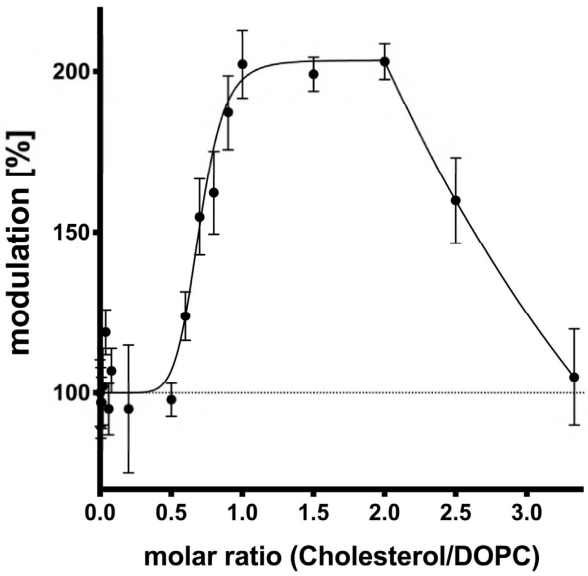

Fig. 7. Modulation of the ATPase activity of ABCB4 by increasing portions of cholesterol in the background of DOPC. Cholesterol was mixed with DOPC (300 μM) at different ratios. Cholesterol concentration ranging from 0 to 1,000 μM resulted in molar ratios of 0–3.3 (cholesterol to DOPC). The ATPase activity at a molar ratio of 0 (only 300 μM DOPC) was set to 100% (maximal stimulation by PC lipids). Data point represents the average of three independent experiments with the errors reported as SD. Data were analyzed according to an allosteric sigmoidal fit (Equation 1) as well as Equation 3 for substrate induced reduction.

(54). Therefore, the ATPase activity at a molar ratio of 0 represents stimulation by DOPC only, which was set to 100% (maximal stimulation by PC-lipids). Here, an increase in the ATPase activity is caused by cholesterol in addition to the maximal stimulation by the natural substrate. Actually, a similar behavior as for bile acid modulation was observed. Cholesterol at molar ratios (cholesterol over DOPC) higher than 0.5 enhanced ABCB4 activity in addition to the stimulation by DOPC (**Fig. 7**). Below this ratio, ATPase activity remained close to DOPC only activities (100%), except for 0.04, which showed a mean value of 119% stimulation. Due to the error in the measurements, we consider this value as no stimulation. Nevertheless, for ratios higher than 0.5 (equals 150 μM cholesterol over 300 μM DOPC), stimulation up to 203.5 ± 5.9% of basal ATPase activity was observed. Since DOPC stimulation was set to 100%, it demonstrates that cholesterol doubles the ATPase activity of ABCB4 in the presence of DOPC (Table 2). This effect is limited to ratios of 1–2 (cholesterol to DOPC). A further increase of the cholesterol content resulted in a gradual decrease of activity reaching 100% within experimental error, which equals the DOPC stimulation. Nevertheless, we could demonstrate that cholesterol in the environment of an actual substrate can increase the already stimulated ATPase activity of ABCB4.

ABCB4 is modulated in the presence of DOPC

It is now commonly accepted that ABCB4 only flops lipids of the PC family from the inner to the outer leaflet of the canalicular membrane of hepatocytes (2, 40). This study demonstrated that bile acids modulate the ATPase activity of ABCB4. One has to stress that this does not suggest that bile acids are a new family of substrates. To address this question, we analyzed the modulation of ATPase activity of ABCB4 in the presence of DOPC, bile acids, and cholesterol. First, DOPC and bile acids were used at concentrations at which both substances showed maximal modulation, but below the cmc of the bile acid (except for TLCA). Data is presented as relative modulation compared with DOPC alone, which was set to 100% (**Fig. 8**, cyan). Both glycine (orange) and taurine (blue) conjugated versions as well as the unconjugated versions (red) of all bile acids were analyzed. Further CHAPS/O (dark/bright violet) and Nor-UDCA (brown) were tested.

GCA, TCA and unconjugated CA increased the DOPC stimulated ATPase activity of ABCB4. GCA increased ATP hydrolysis to 171.2 ± 6.9%, while maximal stimulation was observed with 160.8 ± 26.1% for TCA and at 116.8 ± 8.9% for CA. In contrast, the two structurally related detergents CHAPS and CHAPSO decreased DOPC stimulation of ABCB4 to 52.6 ± 6.5% and 48.7 ± 3.0%, respectively. For the other primary bile acid, CDCA, a slight decrease to 89.7 ± 1.4% was measured, while the conjugated versions showed an additional stimulation to 127.2 ± 2.8% and 111.9 ± 8.2%

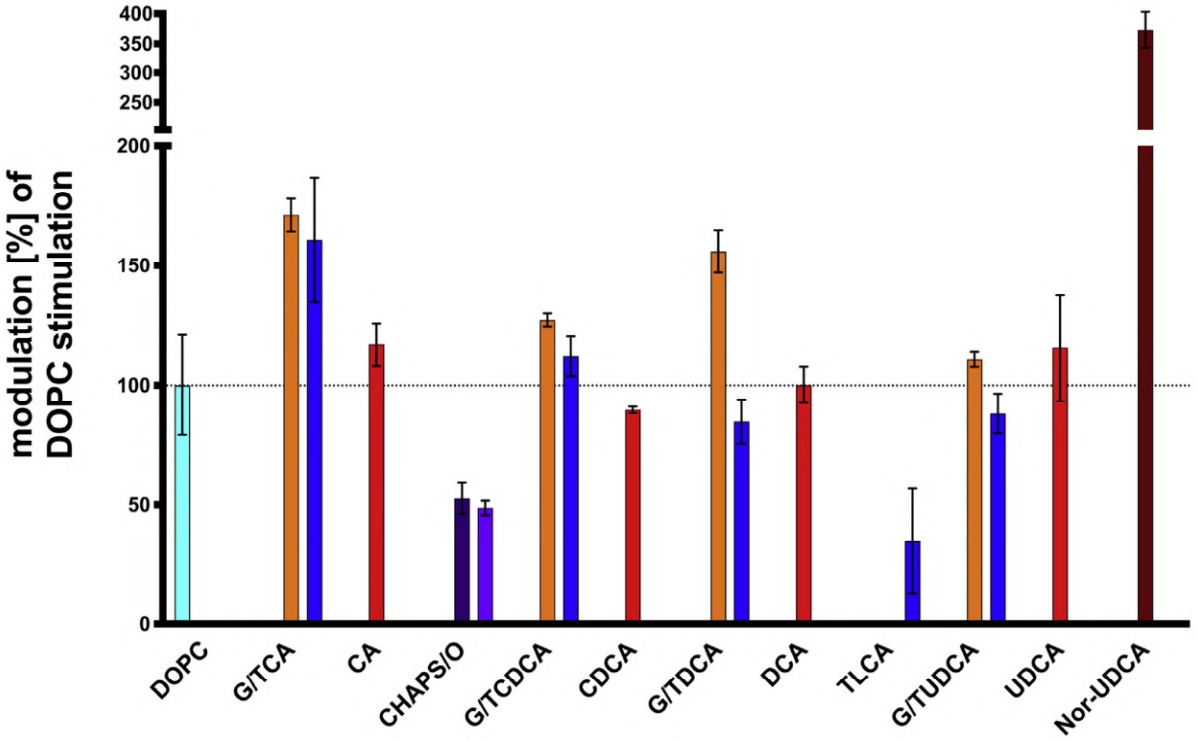

Fig. 8. Modulation of ATP hydrolysis of ABCB4 by the different bile acids in the presence of the naturally substrate DOPC. In total, 300 μM DOPC (cyan) stimulated the ATPase activity of ABCB4 and was set to 100%. 1,000 μM of each bile acid was mixed with 300 μM DOPC and ABCB4. Glycine-conjugated variants are colored in orange, taurine-conjugated are pictured in blue, and unconjugated bile acids are shown in red. Additionally, the effect of 1,000 μM CHAPS/CHAPSO (dark/ bright violet) and Nor-UDCA (brown) was analyzed. Concentration was chosen based on the v_{max} values (Figs. 3, 4 for bile acids and Kluth *et al.* (54) for DOPC). Discontinuous ordinate was chosen for a better overview of bars below 200%. Bars and error bars represent the mean and SD of three independent experiments.

for G/TCDCA, respectively. Also, for the glycine-conjugated version of the secondary bile acid GDCA, an increase in ATPase activity for DOPC-stimulated ABCB4 was observed at 155.9 ± 8.9%. In the case of GDCA, the taurine-conjugated version TDCA lowered the ATPase activity (84.8 ± 9.3%), while unconjugated DCA had no effect (100.1 ± 7.5%). The hydrophobic bile acid TLCA decreased DOPC stimulation by approximately 65% to a minimum of 34.8 ± 21.9%, which is still above the basal ATPase activity of ABCB4. For G/TUDCA and unconjugated UDCA, no further modulation could be measured within the errors. Relative ATPase activities were observed at 110.6 ± 3.1% and 88.2 ± 8.3% for G- and TUDCA, respectively. For unconjugated UDCA, a value of 115.4 ± 22.1% was measured. In contrast, Nor-UDCA bearing a shorter side chain revealed a significant stimulation of ATPase activity of ABCB4 in the presence of 300 μM DOPC to maximum of 373.1 ± 30.0%. This is the highest ATPase activity observed within this setup and reveals the potential of Nor-UDCA.

In this study, the effects of monomeric bile acids as well as monomeric bile acids in the context of DOPC and cholesterol in DOPC environment on the ATPase activity of ABCB4 were analyzed. Thus, we tried to combine these conditions. Therefore, a bile acid mixture (BAM) of GCDCA, TCDCA, GCA, TCA, GDCA, and TDCA (21:19:24:16:13:7) as described in literature was chosen (48, 77, 78). The effect of the BAM on the ATPase activity of ABCB4 was examined in a range from 0 to 1,000 μM similar to individual bile acids (**Fig. 9**A). For a second approach, the concentration of the BAM with highest stimulation (1,000 μM) on ABCB4 was used to investigate the effect of the BAM on ABCB4 in the presence of DOPC and cholesterol mixed with DOPC (Fig. 9B). DOPC concentration (300 μM) and cholesterol to DOPC ratio (1.5:1) were chosen based on the v_{max} values of their individual kinetics (Fig. 7 for cholesterol and Kluth *et al.* (54) for DOPC). The bile acid mixture increased ABCB4 ATPase activity in all approaches. For comparison basal ABCB4 ATPase activity was set to 0% similar to the kinetics of individual bile acids. Adding BAM resulted in a v_{max} of 566.0 ± 17.6% (Fig. 9A, Table 1), which is in good agreement with the individual kinetics of the bile acids (Fig. 3). However, in comparison to the individual conjugated bile acids

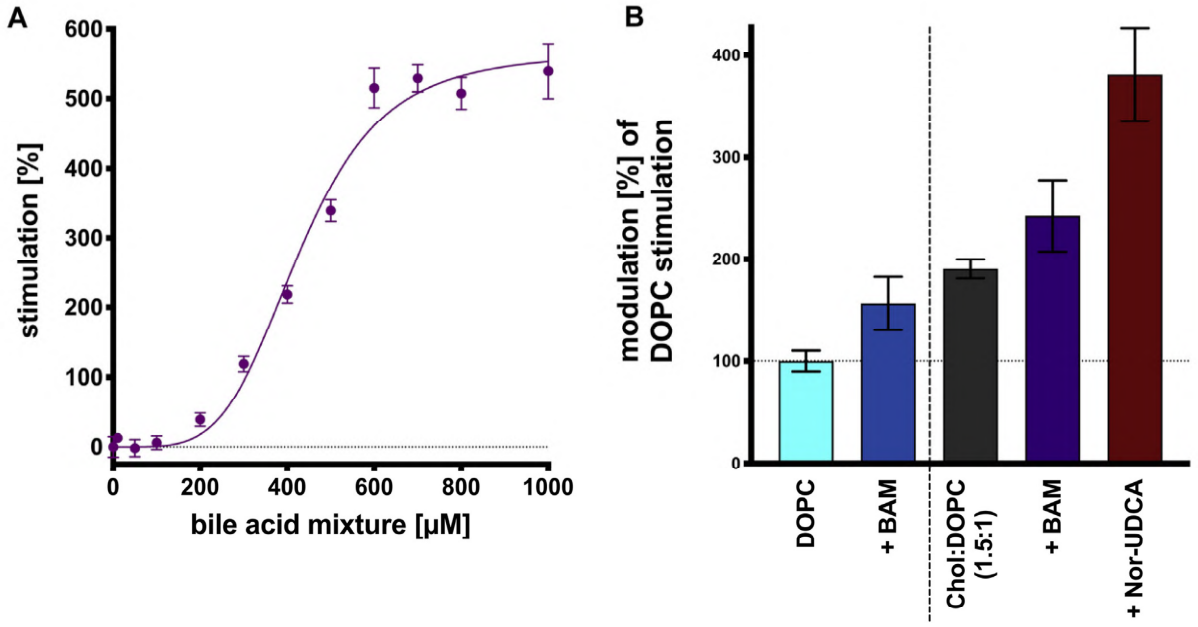

Fig. 9. Stimulation of ABCB4 ATPase activity by a bile acid mixture (A) and stimulation of ABCB4 ATPase activity in the presence of DOPC and the bile acid mixture, cholesterol, and Nor-UDCA (B). A: The bile acid mixture (BAM) consists of GCDCA, TCDCA, GCA, TCA, GDCA, and TDCA with a ratio of 21:19:24:16:13:7 as described in literature (48, 77, 78). The BAM was tested in range from 0 to 1,000 µM similar to individual conjugated bile acids (Fig. 3). Data were analyzed according to an allosteric sigmoidal fit (Equation 1). B: Stimulation of the ATPase activity of ABCB4 by DOPC at a concentration of 300 µM (cyan bar) was set to 100% substrate stimulation (54). In all further approaches DOPC concentration was always 300 µM. Modulation of DOPC stimulated ATPase activity by the BAM is presented by the dark blue bar. Next cholesterol was added in a 1.5 M excess to DOPC (dark gray bar), so that maximal stimulation by cholesterol was achieved (Fig. 7), before the BAM (dark violet bar) or Nor-UDCA (brown bar) was added. A and B: Consider different *y*-axes. Bars and error bars represent the mean and SD of three independent experiments.

present in the BAM, v_{max} is not reached until 600–700 µM. Furthermore, the EC_{50} value of BAM is significantly increased to a concentration of 430.4 ± 11.2 µM (Table 1). For the second approach, DOPC (300 µM) stimulation was set to 100% (Fig. 9B, cyan bar) similar to Fig. 8. Adding BAM to DOPC (dark blue bar) increased the ATPase activity to a maximum of 156.4 ± 26.1%. To investigate the effect of the bile acid mixture in the presence of cholesterol, first only cholesterol and DOPC in a ratio of 1.5:1 were measured (Fig. 9B, dark gray bar). Stimulation by cholesterol (190.4 ± 9.4%) is in good agreement with the v_{max} value (203.5 ± 5.9%) of the DOPC cholesterol kinetic (Fig. 7). Hence, BAM was added to the DOPC cholesterol mixture (Fig. 9B, dark violet bar), which increased the ATPase activity further to 242.6 ± 35.1%. Since Nor-UDCA demonstrated the highest effect on ABCB4 ATPase activity in a DOPC environment, the bile acid mixture was exchanged to Nor-UDCA in the latest approach (Fig. 9B, brown bar). With a maximum of 380.7 ± 45.6%, Nor-UDCA exceeded the bile acid mixture by ~250%. Nor-UDCA, on the other hand, was unaffected by cholesterol since stimulation in only DOPC (373.1 ± 30.0) and cholesterol:DOPC environment (380.7 ± 45.6%) was equal within the experimental error. However, the results in Figs. 8 and 9 demonstrated that maximal DOPC stimulation does not reflect the maximal stimulation of ATPase activity of ABCB4 that was observed in this study. Adding cholesterol and bile acids (independent or as mixture) increased the ATPase activity of ABCB4 beyond the DOPC value. Therefore, we conclude that bile acids are not substrates, but act as enhancers or modulators of the ATPase activity of ABCB4.

DISCUSSION

ABCB4 is modulated by bile acids

The aim of this work was to answer the question if bile acids interact with ABCB4. This question derives from data demonstrating that PC lipid concentrations rises, when conjugated bile acid was added to the medium of cells or vesicles that contained ABCB4 (19, 57–59). The increased amount of PC lipids could be due to interaction with PC lipids, ABCB4, or both. For the first time, it was shown in this study that monomeric bile acids directly modulate ABCB4 (Figs. 3, 4).

We chose an in vitro setup (53), in which ΔABCB4 is detergent-solubilized and the composition and concentration of the compounds under investigation can be regulated under defined conditions. By tandem affinity purification, a yield of 5–6 mg of ABCB4 (Fig. 2A,

B) was reached (54). Due to Bodipy labeling, it was possible to measure specific ATPase activity of ABCB4 in a background that likely contained other ATPases (Fig. 2D) (54). Although, an ATPase activity assay is not directly measuring substrate translocation, it is now generally accepted that substrates increase the protein's ATPase activity, since the NBDs change conformation and are temporally in close proximity (79).

This provided a starting point to measure ATP hydrolysis of ABCB4 in the presence of different bile acids (Figs. 3, 4). In humans, a number of bile acids are present. They vary in the number of hydroxy groups (one to three), conjugations (Fig. 1), and physical properties such as hydrophobicity. This diversity results in different cmc values for each bile acid. But the cmc of bile acids, like any other detergent, is also depended on the chemical and physical properties of the solution. Therefore, we determined the cmc of each bile acid used in this study under the conditions of the ATPase assay (Table 3). The bile acids with three hydroxy moieties G/T/CA exhibited the highest cmc (2.3–4.1 mM) and for G/TCA cmc values were in good agreement with published values (73, 74), although temperature and ionic strength were different. With decreasing numbers of hydroxy moieties, the average cmc decreased. For example, bile acids with two moieties exhibited cmc values in a range of 1.3–2.6 mM, while the bile acid with only one hydroxy moiety (TLC) displayed a cmc of 0.14 mM. Compared with literature values, our TCDCA and TDCA values are in good agreement, but cmc values of GCDCA and GDCA were slightly increased (74). In contrast, our value for TLCA is lower than that reported in a study by Hofmann and Roda (10). We could also observe that conjugation of bile acids increased the cmc for CA, CDCA, and DCA. To be more precise, the glycine-conjugated version of these bile acids exhibited always the highest cmc. All conjugated versions possessed higher cmc values than their unconjugated analogues. From a chemical point of view, this makes sense, since conjugations increase the hydrophilicity of the bile acids and as a result their cmc values. In nature conjugation of bile acids is performed to increase their water solubility (9). Regarding the importance of the position of the hydroxy moiety within the back bone and its stereochemistry, no conclusion can be drawn. While for GCDCA and GDCA, there is a difference in the cmc values between hydroxylation at position 7 (GCDCA, 2.6 mM) and position 12 (GDCA, 2.0 mM), for TCDCA (1.7 mM), TDCA (1.7 mM), CDCA (1.5 mM), and DCA (1.4 mM), no differences regarding the influence of the position of the hydroxy moiety was observed. Switching the hydroxy moiety at position 7 from the α-position (G/T/DCA) to the ß-position (G/T/UDCA) resulted in a decrease of the measured cmc for the conjugated versions (Table 3). However, shortening the side chain of UDCA by one methylene moiety (Nor-UDCA) significantly increased the cmc to 1.8 mM, which is the second highest cmc for all unconjugated bile acids.

To the best of our knowledge, the bile acid concentrations in human hepatocytes are not known. Bile concentrations in the gallbladder of healthy persons are in the range of approximately 100–150 mM (8). But gallbladder bile is highly concentrated. Therefore, we tested the effect of all bile acids on the basal ATPase activity of ABCB4 in a range of 0–1,000 μM. Indeed, all bile acids showed a modulation of the basal ATPase activity. All tri- and dihydroxy bile acids as well as the bile-acid-derived detergents CHAPS and CHAPSO demonstrated stimulation of basal ATPase activity in a concentration-dependent manner (Figs. 3, 4). Concentrations required for maximal stimulations ranged from 200 to 800 μM. More importantly, however, the maximal stimulation was always observed below their corresponding cmc values. Therefore, stimulation derives from monomeric bile acids. Furthermore, the data revealed that except for G/TCDCA, the glycine-conjugated bile acid exceeded the taurine conjugation. Interestingly, glycine conjugation is the predominant form in human bile acid. The question arises whether mice ABCB4 has an inverted preference, since taurine is the mayor conjugation in rodents. In contrast, studies investigating the effect of bile acids on lipid release in the presence of ABCB4 demonstrated that taurine-conjugated bile acids exceeded glycine-conjugated versions (76, 80, 81). However, modulation of ABCB4 and lipid extraction by bile acids are two different events that have to be considered separately. Unconjugated versions demonstrated the least maximal stimulation within the same group of bile acids. One interesting exception is Nor-UDCA, which exhibited the highest v_{max} of all UDCA variants and the second highest of all unconjugated bile acids indicating a different effect on ABCB4. All four UDCA versions had in common that their EC_{50} values are significant higher compared with the others. This is most likely due to the fact that their hydroxy moiety at position 7 is in ß-position compared with all other bile acids. In summary, monomeric tri- and dihydroxy bile acids stimulated ATPase activity of human ABCB4 in a concentration-dependent manner. Glycine-conjugated bile acids reached the highest maximal stimulation within the same group of bile acids, except for CDCA, where taurine conjugation (TCDCA) exceeded glycine conjugation (GCDCA). Additionally, higher concentration of UDCA versions was required to observe stimulation. Shortening the side chain of UDCA by one carbon atom (Nor-UDCA) revealed an increase of approximately 85% of ATPase activity.

The only exception was TLCA, a bile acid with only a hydroxy moiety at position 3. TLCA clearly decreased the activity of ABCB4. However, TLCA is also the only bile acid with a cmc significant smaller than 1 mM and therefore has to be considered separately. Up to 50 μM no effect was detected within experimental error. Approximately at the cmc, the half inhibitory concentration (IC_{50}) was reached (Table 3) implying a

reduction of the basal ATPase activity by monomeric TLCA (from ~50 to 140 μM) as well as micelles (~140–500 μM). However, after the basal activity is reduced to 50%, no further reduction was observed. This indicates that TLCA may not be able to fully inhibit ABCB4. This is in a good agreement with the fact that TLCA triggers cholestasis in rats (64–67).

Interestingly plotting the maximal stimulation against the cmc values revealed a linear correlation (r^2 = 0.83). This correlation shows that ABCB4 recognizes bile acids by their physical properties. From a point of evolution, the most common bile acid in humans is GCA (8, 76), which also possessed the highest cmc and degree of stimulation. In contrast, UDCA and LCA represent the smallest part of the human bile acid pool (76) and displayed the lowest level of stimulation or even a reduction of basal ATPase activity. However, we could clearly demonstrate that monomeric bile acids modulate ATPase activity in dependence of their cmc. Due to the limitations of the assay, it still remains unclear how bile acids modulate ATP hydrolysis of ABCB4. In case of ABCG5/G8, it was suggested that bile acids promote an active conformation of ABCG5/G8 by acting as a chemical chaperone (48). In contrast to ABCB4, purified ABCG5/G8 did not show any ATPase activity in the presence of the natural substrate cholesterol. Only after adding bile acids, ATP hydrolysis was detected. The authors therefore concluded that bile acids act as a chemical chaperon. However, bile acids modulated the activity of both transporters at different concentrations. ABCB4 was stimulated in the μM range, while ABCG5/G8 becomes active at concentrations higher than 1 mM suggesting that micelles are required for the stimulation of ABCG5/G8. Therefore, a different mode of action of bile acids on both ABC transporters should be considered.

Modulation of ATPase activity of ABCB4 in the presence of DOPC

It is now commonly accepted that ABCB4 only flops lipids of the PC family from the inner to the outer leaflet of the canalicular membrane of hepatocytes (2, 40). This study demonstrated that bile acids modulated the ATPase activity of ABCB4, which does not necessarily suggest that bile acids are a new family of substrates. To address this question, we analyzed the modulation of ATPase activity of ABCB4 in the presence of DOPC, cholesterol, and bile acids. In a previous study, it was shown that DOPC stimulates ATPase activity of human ABCB4 similar to a liver PC–lipid mixture (54). The effect of cholesterol and bile acid should be measured at maximal stimulation by DOPC, which corresponds to 50–60 μM. Thus, a concentration five to six times higher (300 μM) was chosen in our setup. The ATPase activity for maximal DOPC stimulation was set to 100% and modulation of cholesterol (Fig. 7) or bile acids (Fig. 8) as well as a combination of both (Fig. 9) was analyzed. A doubling of the ATPase activity was observed for cholesterol in a range of 300–600 μM (cholesterol to DOPC molar ratio ranging from 1:1 to 2:1, Fig. 7). Higher ratios resulted in a reduction to the initial value. Below a ratio of 1:2, no modulation was observed within experimental error. Thus, cholesterol is capable to enhance the stimulation beyond its maximal value induced by DOPC. Increasing amounts of cholesterol in purified membrane vesicles containing human ABCB11 (BSEP) resulted in a duplication of the ATP-dependent transport of different bile acids (47).

The modulation of ATPase activity of ABCB4 was also analyzed in the presence of DOPC and bile acids (Fig. 8). Glycine-conjugated versions of CA, CDCA, and DCA enhanced stimulation of the ATPase activity of ABCB4 in the presence of DOPC. In the case of taurine-conjugated bile acids, only TCA displayed a clear additional stimulation, while all others did not show an enhanced stimulation within experimental error. Interestingly changing the conjugation to a nonnatural conjugation as the one in CHAPS and CHAPSO resulted in a decrease of DOPC-stimulated activity by approximately 50%. Most importantly, Nor-UDCA revealed a higher increase of the DOPC-stimulated ATPase activity of ABCB4 than all other bile acids. ATPase activity was nearly four times higher if both were present Nor-UDCA and DOPC compared with only DOPC. Additionally, this stimulation remained the same if cholesterol was included in the analysis (Fig. 9B, brown bar) suggesting a new mode of action of Nor-UDCA. So far, the positive effects of Nor-UDCA were assigned to shortening of the biliary hepatic circulation, promoting a bicarbonate-rich choleresis, direct anti-inflammatory, antiproliferative, or antifibrotic effects (30). Here we demonstrate that unconjugated Nor-UDCA is capable of increasing the ATPase activity in the presence of the natural substrate DOPC. Although Nor-UDCA cannot be conjugated in the hepatocytes, glycine-conjugated Nor-UDCA may be considered in further studies, since we observed that glycine conjugation exceeded all other bile acids suggesting a new mode of interaction of ABCB4 and Nor-UDCA.

In a last approach, a BAM as close as possible to the in vivo situation was tested. Therefore, a BAM of GCDCA, TCDA, GCA, TCA, GDCA, and TDCA with a molar ratio of 21:19:24:16:13:7 as described (48, 77, 78) was investigated on ABCB4 (Fig. 9A) and in the presence of DOPC as well as in the presence of DOPC and cholesterol (Fig. 9B). The BAM stimulates ATPase activity of ABCB4 by ~566% ± 17.6%. This is in the between the v_{max} values for individual G/TCDCA and G/TCA bile acids (Table 1), which make up the majority of this mixture. Therefore, the mixture stimulates ATP hydrolysis of ABCB4 similar to the individual bile acids. In contrast the EC_{50} value of the BAM is significant higher than for individual G/TCDCA and G/TCA kinetics. This may be to the fact that the specific concentration of individual bile acids in the mixture is 4–5 times lower. This also explains why v_{max} is reached at higher concentration (500–600 μM) of BAM compared with

individual conjugated bile acids (Fig. 3). In the case of the BAM/DOPC sample (Fig. 9B, dark blue bar), an additional increase of 56% ± 26 compared with only DOPC (cyan bar) was detected, which is in the range of the individual bile acids (Fig. 8). Adding the BAM to the cholesterol:DOPC mixture (Fig. 9B, dark violet bar) revealed again an additional stimulation compared with only the cholesterol:DOPC mixture (Fig. 9B, dark gray bar). But the last approach including Nor-UDCA instead of BAM (Fig. 9B, brown bar) displayed the highest stimulation similar to Fig. 8. Here once more we observed that Nor-UDCA has the highest impact on ABCB4 ATPase activity and should be considered in future ABCB4 research.

In summary, we have demonstrated that ABCB4 is a PC–lipid translocase, whose ATPase activity is enhanced in the presence of bile acids and cholesterol. Obviously, this enhanced activity might result in an increased rate of lipid flop from the inner to the outer leaflet of the canalicular membrane. This clearly indicates an intricate cross talk of the substrates of the transporters of the bile triumvirate and their regulation to fulfill their physiological function.

Data availability

All data are contained within the article.

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Author contributions

T. K., S. H. J. S., and L. S. conceptualization; T. K., S. H. J. S., and L. S. validation; T. K. investigation; L. S. resources; T. K. and S. H. J. S. writing-original draft; S. H. J. S. and L. S. writing-review and editing; S. H. J. S. and L. S. supervision; L. S. project administration; L. S. funding acquisition.

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Conflict of interest

The authors declare that they have no conflicts of interest with the contents of this article.

Abbreviations

ABC, ATP binding cassette; BAM, bile acid mixture; BSEP, bile salt export pump; CA, cholic acid; CDCA, chenodeoxycholic acid; CHAPS, 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate; CHAPSO, 3-[(3-cholamidopropyl)dimethylammonio]-2-hydroxy-1-propanesulfonate; DCA, deoxycholic acid; DOPC, 1,2-dioleoyl-sn-glycero-3- phosphocholine; G/T/CA, glyco-/tauro-/cholic acid; G/T/CDCA, glyco-/tauro-/chenodeoxycholic acid; G/T/DCA, glyco-/tauro-/deoxycholic acid; G/T/UDCA, glyco/-tauro-/ursodeoxycholic acid; LCA, lithocholic acid; MDR3, multidrug-resistant protein 3; NBD, nucleotide binding domain; NTCP, sodium taurocholate transporting peptide; PC, phosphatidylcholine; TCA, taurocholic acid; TCDCA, taurochenodeoxycholic acid; TLCA, taurolithocholic acid; TMD, transmembrane domain; UDCA, ursodeoxycholic acid.

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4 Discussion

In this thesis, the understanding of a cross-talk between bile acids and the PC lipid translocator ABCB4, as part of the bile triumvirate, was addressed. Bile triumvirate and its importance regarding bile formation was first introduced in the introduction and chapter 1. The importance of mutations within these transporters and related diseases were outlined. While in the introduction, a general outline of structure and function of ABC transporters was given, in the first chapter, the structure and function of ABC transporters was described in more detail with respect to ABCB4. Furthermore, its role in bile formation, the protection mechanism of PC lipids, ABCB4 related diseases and current treatment options were described in more detail. Lastly, the challenges in ABCB4 research were depicted and detergent-solubilized and purified ABCB4 as a possible solution was presented. In chapter 2, four new discoveries about ABCB4 were presented: 1) monomeric bile acids modulate ABCB4 in concentration-dependent manner; 2) maximal modulation by bile acids corelates with their cmc; 3) bile acids still exhibit their modulatory effect in presence of substrate DOPC; and 4) Nor-UDCA highly stimulates ABCB4 in presence of DOPC (and cholesterol), which represents a new and medically-relevant mechanism of action.

Within this discussion, the major focus is: 1) ABCB4 expression and purification, 2) lipid secretion and 3) bile acid modulation.

4.1 General outline

Human bile formation is a unique function of the liver, which is essential for the survival of the organism. Bile is a complex product of the secretion of different compounds that originates from hepatocytes. Its composition and the concentration of the individual compounds are modified by absorptive and secretory transport systems in the bile duct epithelium. Subsequently, bile either enters the gallbladder where it is concentrated or is delivered directly to the intestinal lumen. There bile performs several functions: (I) Bile is the major excretory route for a number of partial harmful substances or endogenous metabolic substrates like exogenous lipophilic toxins,

bilirubin, bile acids or compounds which are not readily filtered or excreted by the kidney. (II) The highly concentrated bile acids in the bile are necessary to emulsify dietary fats and facilitate their intestinal absorption. (III) Cholesterol elimination via bile is the major and most important route in cholesterol metabolism. (IV) By excretion of immunoglobulin A and inflammatory cytokines, bile shields the organism from enteric infections. Additionally, it is stimulating the innate immune system in the intestine. (V) Many hormones and pheromones are excreted in bile, and contribute towards growth and development of the intestine in some species.[8] Since bile is an aqueous solution, it consists of ~90% water in which a number of endogenous substances are dissolved. These compounds are mainly bile acids, phosphatidylcholine (PC) lipids and cholesterol. These three compounds form mixed micelles in the bile canaliculi to protect the cells from the detergent activity of bile acids and crystallization of cholesterol. Furthermore, bilirubin and organic anions are also present in bile at low quantities. Distribution of this mixture varies from species to species and is also slightly varying based on gender. For example, in humans, bile consists of 5% bile acids in woman while only 4% in men. However, the composition of bile in both is approximately 2% phospholipids and 1% cholesterol.[9] Even bile acids distribution is different in women and men.[9] Since bile is a mixture of the above stated compounds with a quite high predefined equilibrium, this “synthesis” mixture needs highly specific pathways and a unique organ / cell type to fulfill this task. In mammals and especially in humans, this place is the liver, precisely the hepatocytes. These cells have a unique polarity and face different environments within their membrane. The basolateral membrane faces the endothelia cells and the space of Disse. The apical membranes of two or more adjacent hepatocytes contribute to a capillary-like structure or tubule, called bile canaliculus. Therefore, the apical membrane of hepatocytes is also called canalicular membrane. The canalicular membrane is functionally sealed by tight junctions and constitutes ~13% of total hepatocyte plasma membrane.[10,11] Typically, the membrane can be subdivided into individual components having different functions and tasks. By having a look at the distribution of transporters in the hepatic membrane (see introduction, Figure 1), it becomes obvious that mainly symporters or importers are located at the basolateral, while at the canalicular membrane mainly exporters are found, indicating the different functions of the two membranes. Hence, six out of nine transporters at the canicular membrane are ABC transporters. All of them are exporters

and most of them contribute towards bile formation. For each of the major compounds of bile, a unique ABC transporter is present. Bile acids are secreted via ABCB11 (also called bile salt export pump, BSEP) [12], while ABCB4 (historically also called multidrug resistance protein 3, MDR3) translocates specifically PC lipids [13] and the heterodimer ABCG/G8 transports cholesterol [14]. Hence, these transporters are summarized as bile triumvirate. The minor components of the bile are secreted via ABCC2 (also called multi resistance protein 2, MRP2).[15] The two remaining ABC transporters are ABCB1 (also called multidrug resistance protein 1, MDR1) and ABCG2 (also known as breast cancer resistance protein, BCRP). Both are expressed in several tissues and organs, have a broad spectrum of substrates and play a key role in the detoxification process of humans. For detailed information about these ABC transporters, please refer to the review about hepatobiliary ABC transporters[16], presented in the introduction.

ABCB4 was first identified in 1987 by sequence analysis.[17] Due to its high sequence identity and homology with ABCB1, it was first considered as a multi drug resistance protein like ABCB1 and consequently termed MDR3 in human and mdr2 in rodents. Due to their similarity, it was assumed that ABCB4 has a similar function and broad substrate spectrum like ABCB1. Different *in vitro* studies were conducted to investigate the effects of the various substrates and inhibitors of ABCB1 on ATPase or transport activity of ABCB4.[17-22] However, the works of Smit *et al.* [13], Smith *et al.* [23,24] and van Helvoort *et al.* [25] clearly demonstrated that ABCB4 specifically translocates PC lipids from the inner to the outer leaflet of the canalicular membrane and has a very different and unique function of all MDR proteins. Thereby, ABCB4 maintains the lipid equilibrium of the canalicular membrane and provides steady supply of PC lipids for bile acid extraction. PC lipids are an essential part of the bile. They reduce the detergent activity and cytotoxicity of the bile acids by forming mixed micelles and consequently protect the biliary ducts. Furthermore, PC lipids are important for cholesterol homeostasis and prevent cholesterol crystallization by integrating cholesterol into the mixed micelles (see introduction, Figure 11). Dysfunctions of ABCB4, ABCB11 or ABCG5/G8 lead to a change in the bile equilibrium and therefore, often to health problems or even serious diseases. Dysfunction of ABCB4 due to mutations often results in a reduced PC lipid pool, or in some cases to complete absence of PC lipids in the bile. Hence, bile acid protection and cholesterol crystallization are affected or absent. Mutations in ABCB4 can alter its expression,

folding, stability, localization and activity.[26,27] Hence, PC lipid concentration varies in the bile, resulting in mild to severe diseases (see chapter 1, Figure 2). Progressive familial intrahepatic cholestasis type 3 (PFIC-3) is the most severe form of ABCB4-related liver diseases. Here accumulation of free bile acids leads to bile duct injuries.[28] PFIC -3 occurs in patients in the early years of life and transiently progresses to liver cirrhosis in childhood [29], normally resulting in liver transplant.

The present thesis focusses on the *in vitro* analysis of human ABCB4. Although it is universally accepted that ABCB4 specifically translocates PC lipids in a unidirectional manner within the canalicular membrane, published data [30-32] displayed an effect of bile acids on PC lipid extraction and a change in PC lipid concentration of the extracellular medium in presence and absence of bile acids. Furthermore, it is known that bile acids are required to activate purified ABCG5/G8 [3], while cholesterol content in the membrane effects ABCB11.[2,33] In 2003, Small suggested a specific interplay between these three ABC transporters crucial for bile formation and their substrates.[1] However, there hasn't been further investigation on the same whether bile acids just interact with PC lipids or also with ABCB4 and may affect its activity. The same is true for cholesterol and ABCB4. Hence, the aims of this thesis were to investigate the effect of all bile acids present in humans, either unconjugated or conjugated, as well as the therapeutically-relevant *urso* bile acids *in vitro*. Furthermore, bile acids with contradictory effects were mixed and investigated towards preferences of ABC4. Additionally, cholesterol was tested and in the last two approaches the effect of individual, mixed bile acids and cholesterol in the presence of the natural substrate DOPC was examined.

4.2 Expression and purification of ABCB4

The aim of this thesis was to analyze ABCB4 *in vitro*, precisely the analysis of solubilized and purified ABCB4. This approach has the advantage that the target protein is isolated and direct effects of molecules or other proteins on the target can be measured. Subsequently, all measured changes can be described as an interaction of the target and the added compound. In contrast, the absence of any effect directly emphasizes the absence of any interaction under the chosen conditions. A disadvantage of this approach is that the target protein is not in its natural environment,

which always has to be considered. Furthermore, the results depend on the chosen conditions. While the later ones can often be changed easily and adapt as close as possible to the natural environment, the bottleneck of purified membrane proteins is their expression and purification itself.

4.2.1 Expression of ABCB4

For heterologous expression one of the first approaches is to use *Escherichia coli* (*E. coli*) as their strains are easy to handle (S1), have short expression times and high expression levels. However, for expression of human ABC transporters in *E.coli* such as ABCB11 [34] and a number of other ABC transporters[35], various parts of the cDNA sequence were lost. Therefore, an alternative expression host had to be selected. Subsequently, the expression of ABCB4 in the yeast *Pichia pastoris* was established by Stindt *et al* [36] and Ellinger and Kluth *et al* [6] and it has been used since. However, the question arises if *P. pastoris* is the best expression system for subsequent purification and biochemical analyses of ABCB4. Therefore, different established expression systems of the other ABC transporters of the bile triumvirate and ABCB1, a highly related ABC transporter to ABCB4, are compared.

Human ABCB11 was expressed in HEK cells [37] to solve the structure, while for characterization often insect cells [2,34,38], human cell lines [39-41] and *P. pastoris* [41] were chosen. For ABCG5/G8 expression and subsequent purification *P. pastoris* was also chosen.[3,42] In case of ABCB1, HEK cells [43], insect cells [44] and the yeasts *P. pastoris* [45,46] and *S. cerevisiae* [47,48] are established expression systems. In general, the yeast *S. cerevisiae* is also known for successful expression of eukaryotic ABC transporters.[49] Hence, the yeast *S. cerevisiae* was tested but resulted in low yields of ABCB11 and was not sufficient for subsequent approaches.[36] However, *P. pastoris* displayed good results for expression of both ABCB11 and ABCB4.[6] In general *P. pastoris* is a well-established system for ABC transporter expression, it can reach high cell densities in fermentation and expression is strongly induced by the AOX1 promoter.[46,50,51] In this thesis, the established fermentation protocol of *P. pastoris* was used for expression of ABCB4 as described in chapter 2.

In conclusion, *P. pastoris* is a well-established and accepted expression system for a variety of ABC transporters and the fermentation protocol of *P. pastoris* is an excellent method to reach the high cell density needed for ABCB4 purification. Hence, no change of the current system was needed.

4.2.2 Purification of ABCB4 – significance of other tags?

The second major step to consider while working with purified ABCB4 is of course the purification itself. In case of membrane proteins like ABCB4, purification can be divided into three separate steps: i) cell disruption and membrane preparation, ii) solubilization, and iii) the actual purification step which is often performed via one or more chromatography methods. In case of ABCB4, the latter one is performed by a tandem affinity purification (TAP) consisting of an immobilized metal affinaty chromatography (IMAC, Ni^{2+}-NTA) and a calmodulin binding affinity chromatography (CBA). The goal of these steps is to obtain the target protein as pure as possible, optimally to a purity level of 99% and higher. Unfortunately, purification of membrane proteins, especially the ones exhibiting more than one transmembrane domain, often seems to be more difficult than soluble proteins. In general, for membrane proteins there is less space within the cell (membrane versus cytosol) and an additional solubilization step is necessary. In case of ABCB4, a purity of approximately 90% or higher was achieved (see chapter 2, Figure 2 A). However, the minor impurities present in the purified protein displayed ATPase activity (see chapter 2, Figure 2 D), which is often the chosen readout for characterization of ABCB4. Fortunately, ABCB4 has a cysteine in its Walker A motif. Cysteine residues are rare and not present in all ABC transporters. In the expression host *P. pastoris,* there are no ABC transporters harboring a cysteine in their Walker A motif except for ABCB4. Therefore, a method to specifically inhibit ABCB4 amongst other impurities present in the purified protein was established. It was first described for ABCB1, which shares 100% amino acid identity with ABCB4 in the NBDs.[52] Using a maleimide linked to a large chemical compound resulted in covalent binding of the maleimide to the cysteine in the Walker A motif. Subsequently, this lead to occupancy of the ATP binding site and thereby, inhibition of

ATP hydrolysis by ABCB1.[53] This method was adopted for ABCB4 and allowed determination of ABCB4-specific ATPase activity.[7] Although, this method helped to overcome the hindrance caused by the impurities present in the purified protein, but removal of the impurities would result in a more direct readout of ABCB4. Hence, analyzing and improving the different steps of ABCB4 purification would be beneficial.

Cell lysis of yeast, especially *P. pastoris*, often relies on bead-mills.[54-58] Although, these mills allow sufficient lysis [55-57,59,60] , they often leak, break and are unsuitable for large scale approaches. Another alternative is cell disruption, for example using the TS1 multi shot cell disruption system by Constant Systems LTD, which has a high pressure (up to 2.7 kbar). In comparison to the bead mill, it is easier to handle and is more efficient for disruption of large volumes of *P. pastoris* cell culture. This system demonstrates an efficiency comparable or even better to bead-mills. Due to the advantage of high pressure and lysis of large volumes, this system is successful in *P. pastoris* lysis and no change in the current protocol (chapter 2) is advisable.

Preparation of crude membrane vesicles of ABCB4 is usually performed by several differential centrifugation steps (see chapter 2).[6] However, the separation of membranes by gradient centrifugation was also tested and it didn't improve the purification significantly. Rather, it only elongated the purification step. Thus, there is no potential for optimization of the crude membrane preparation step.

The next crucial step is solubilization of ABCB4. Here, the published data strongly supported the use of FC-16 as detergent for ABCB4 which is expressed in *P. pastoris*.[6] In comparison, for the structure of ABCB4, the protein was obtained from Flp-In T-Rex 293 cells and was solubilized in 1% DDM and 0.2% CHS[61]. However, in case of *P. pastoris* expression, DDM was not effective at all.[6] Hence, using FC-16 is the best possible option.

The last step is the tandem affinity purification (TAP) performed in two steps consisting of Ni-NTA chromatography followed by CBP affinity chromatography. Normally, a one-step purification with a high affinity chromatography like CBP should be suitable to purify a protein. In case of ABCB4, one-step purification with only CBP affinity chromatography resulted in target protein with higher contaminations consisting of other proteins and lipids. Hence, TAP was chosen to remove major impurities and the lipid contamination. Actually, Ni-NTA affinity is suitable to remove lipids and

reduces impurities to just five major bands on SDS gel (Bands at ~100 kDa, 85 kDa, 2x at 65 kDa and 55 kDa, see chapter 2, Figure 2A). Subsequently, CBP affinity chromatography results in two strong bands at ~ 100 and 85 kDa and some minor bands below 80 kDa (see chapter 2, Figure 2A). Overall, the intensity of the impurities is reduced compared to the IMAC. The band at ~110 kDa is a degradation product of ABCB4 as confirmed via immunoblotting (see chapter 2, Figure 2B). Unfortunately, the impurities exhibit a background ATPase activity (see chapter 2, Figure 2D). Here, switching the tags might have an impact on the purification efficiency. A general overview of common tags frequently used for TAP is provided in Table 1 based on the work by Terpe [62] and Li [63]. TAP was originally used a combination of two synthetic Fc-region-binding domains (also called double Z domain; derived from the B domain of ProtA) and the CBP tag.[64] But a huge disadvantage of the double Z domain tag is that denaturing conditions (low pH) are necessary for elution.[65] Therefore, a TEV cleavage site has to be integrated for elution under native conditions. Hence, a ProtA-based purification and a subsequent CBP affinity chromatography was generally recommended.

Table 1: Comparison of common tags for protein purification by affinity chromatography.

Tag	Affinity [nM]	Binding capacity [mg/ml Resin]	Eluent	Ref.
His-Tag & Ni-NTA	10,000	80	Imidazole, Histidine	62,66
CBP	3	2	EGTA	66-68
Flag	100	1	DYKDDDDK peptide	66,69
Strep(II)	1,000	9	Desthiobiotin, biotin	66,70,71
rho1D4	20	3-4	Protease digest	72
Z domain	n.s.	n.s.	Protease digest	65

Alternative tag combinations from literature [63] usually used for yeast-derived systems are: i) His-HA-tag [73], ii) His-FLAG-tag [74,75], iii) His-Myc-tag [76], iv) His-ProtC-tag [77] and v) His-biotinylating-tag [78]. While the His-Strep(II)-tag combination is recommended for proteins purification derived from mammalian cells.[63,79] Furthermore, a number of His-based TAP tags for a soluble protein (derived from *E.coli*) was compared regarding their purification capacities, costs and yields.[66] CBP-, HPC- and Strep(II)-His-tag combination exhibited the highest yield (above 60%). Additionally, CBP-His-Tag exhibits the highest binding capacities and the lowest cost, among the compared tags. In contrast, the HPC-tag has the lowest capacity and is the most expensive.[66] The yield of the different tags varies from protein to protein, especially between soluble and membrane-bound proteins. Hence, purification methods for human ABCB1, ABCB4, ABCB11 and ABCG5/G8 were compared for more detailed information.

In case of ABCB1, only a His-tag and subsequent single IMAC is sufficient for purification.[45,46,80] Alternative affinity tags such as GFP antibody tag [81] and the rho1D4-tag [43] also resulted in highly pure ABCB1. Also ABCB11 can be purified by single affinity chromatography via a FLAG-tag, followed by a size exclusion chromatography.[37] Furthermore, for ABCB4 derived from Flp-In T-Rex 293 cells, a single affinity chromatography with the newly rho1D4-tag was also established for purification.[61] Purity of ABCB4 was sufficiently high to solve the structure by cryo-EM. In contrast, for ABCG5/G8, a TAP combination of His- and CBP-tags was necessary for purification from *P. pastoris* [3,42], similar to the present ABCB4 protocol.

It is evident from the literature that TAP is not necessary. However, the combination of His-and CBP-tag is the most favored combination for TAP. Furthermore, the new rho1D4-tag appears to be frequently used and could be the first choice for ABC transporter purification in the future. With an affinity of approximately 20 nM [72], the rho1D4-tag has one of the highest affinities and is in the range of the CBP-tag (~3 nM)[67,68]. Furthermore, the rho1D4-tag was specifically designed for proteins with low abundance like the membrane proteins. A disadvantage of this tag is that either a low pH is necessary to denature the tag or a protease is required for elution. Hence, the protease has to be removed in a subsequent step.

In case of purification protocol for ABCB4 in this thesis (see chapter 2, Figure 2), IMAC reduced the number of impurities significantly (from a smear to just ~ 5 bands). However, CBP affinity chromatography did not reduce number of impurities significant, suggesting to possibly switch the CBP tag. Here, the FLAG- and Strep(II)-tag seem to be the best choice. The FLAG-tag has an affinity of ~ 100 nM [69], while the affinity of the Strep(II)-tag is approximately ten times higher (1 µM) [70,71]. The affinities of the tags and the usage of the FLAG-tag for ABCB11 purification [37], suggests to first try the FLAG-tag. However, based on the binding capacity, the Strep(II)-tag would be the better choice. Yet, it seems to be possible to purify ABCB1 [43] or ABCB4 [61] only via the rho1D4-tag.

Due to the constraints of this thesis, testing the different tags was not possible. Based on the analysis of different tags above, the order to test new tags should be rho1D4-, FLAG- and then Strep(II)- tag. But due to the establishment of a third step or a completely new purification protocol in case of the rho1D4-tag, both other tags might be tested faster. Here, no major changes in buffer and purification protocol are needed, if the CBP-tag in the ABCB4 TAP is replaced by either FLAG- or Strep(II)-tag.

4.3 Role of ABCB4 in bile formation

4.3.1 Lipid translocation in general

A biological membrane is a lipid bilayer, which marks the boundary between the different compartments of a cell, the intracellular space and the extracellular environment.[82] The lipid bilayer is formed by different lipids, facing the intracellular or extracellular space with their hydrophilic heads. Hence, the fatty acids face each other building a hydrophobic space, the actual boundary, which prevents diffusion of polar ions and molecules; maintaining the electrochemical gradient. Eukaryotic cells display an asymmetric distribution of lipids in the membrane, especially the plasma membrane. While the inner leaflet mainly consists of phosphatidylserine (PS), phosphatidylethanolamine (PE) and phosphatidylinositol (PI); phosphatidylcholine (PC) and sphingomyelin (SM) are located in the outer leaflet.[83-86] The so-called lipid asymmetry is essential for the cells and correspondingly disintegration of the asymmetry is related to macrophage recognition and apoptosis.[86,87] Hence, maintaining the lipid asymmetry and distribution is essential for maintaining functionality and survival of the cells.

In a biological membrane, lipid movement can happen in two dimensions. First is the lateral motion of lipids within the same leaflet and second is the transverse exchange between the different leaflets, also called flip-flop. While the first one is rather frequent and a fast (~10 million lipids/s) event driven by diffusion [88,89], the latter one is limited and requires the help of membrane proteins [90-92]. These proteins are named translocases and can be subdivided into two groups: energy-independent and energy-dependent lipid translocases (see Figure 1). Although, this chapter will focus on the second group, especially ABC transporters and ABCB4, the first group will be also described briefly.

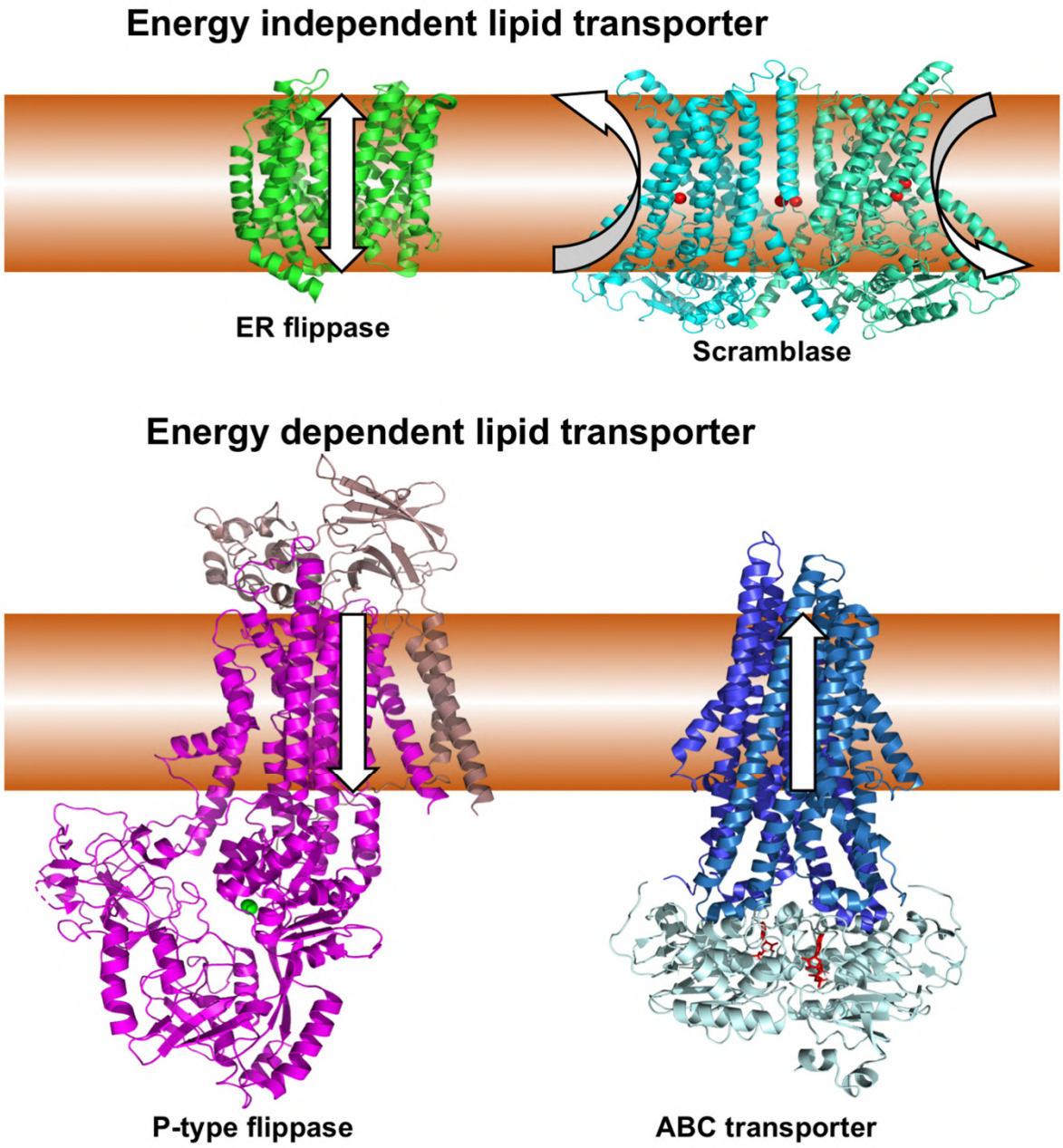

Figure 1: Examples for energy-independent and energy-dependent lipid transporters.
Top: Example of an ER flippase (green) and scramblase (turquoise). The structures display the endoplasmic located Lipid II flippase MurJ from Thermosipho africanus (PDB 6NC9)[93] in the outward-facing conformation and the human scramblase TMEM16K (PDB 6R7X)[94] in the calcium ion-bound (red dots), closed form. **Bottom:** Example of a P-type flippase and an ABC transporter for energy-dependent lipid translocation. In violet is the structure of human P4-type flippase ATP8A1 with bound CDC50 (brown) (PDB 6K7N)[95]. Human ABCB4 serves as an example for the structure of an ABC transporter (PDB 6C0V)[61]. TMDs are represented in dark blue and NBDs in light blue. These Figures were created with Pymol version 2.3.4 and PowerPoint version 16.16.

The energy-independent transport of lipids through the bilayer is ensured by two protein families, the flippases and scramblases. The major difference between these translocases is their location within the cells. Flippases were identified in the ER (origin of phospholipids), Golgi and bacterial cytoplasmic membranes.[96-100] They often have no specificity for any kind of lipids or direction (bi-directional), but mainly translocate newly synthesized lipids.[96,101,102] Scramblases also transport rapidly all kinds of lipids in both directions. But in contrast to flippases, they are conserved in eukaryotes and located in the plasma membrane. Furthermore, they are regulated by calcium ions, responsible for destroying the asymmetry of the plasma membrane and therefore, involved in apoptosis.[103-106]

While activated scramblases destroy the asymmetry of plasma membranes, the energy-depended translocases are in charge of formation and maintenance of bilayer asymmetry. These can be sub-divided into two classes of protein families: the P-type ATPases subfamily and the ABC transporter family. Both translocate phospholipids uni-directionally via ATP hydrolysis. In general, P-type ATPases can be divided in five subfamilies (P1-P5) and share the same role of pumping ions and forming a phosphorylated intermediate during their catalytical cycle.[107,108] While P1 to P3-type ATPases are found in all three domains of life, P4-type ATPases are only present in eukaryotes. They are so-called flippases and transport specific lipids (mainly PC, PE and PS) from the outer to the inner leaflet of membranes against a concentration gradient energized by ATP hydrolysis.[109] In mammals, 14 P4-type ATPases are present. Among them, the best studied member is ATP8B1, also called familial intrahepatic cholestasis 1 (FIC-1). ATP8B1 is also expressed among others in the hepatocytes, where it is specifically located in the canalicular membrane (see Introduction, Figure 1), similar to ABCB4. ATP8B1 specifically flips lipids of the PS family from the outer to the inner leaflet.[110-112] Here, ATP8B1 plays a pivotal role in the initiation and maintenance of the transversal lipid asymmetry of the plasma membrane and mutations lead to progressive familial intrahepatic cholestasis type 1 (PFIC1).[113,114] Due to the loss in function, membrane asymmetry is disintegrated and the protection against harsh bile acid detergent action is lost.[115]

The second energy-dependent translocases are ABC transporters. The ABC transporters are huge and diverse in prokaryotes and eukaryotes. For example, in the human genome there are 48 genes encoding ABC transporters which can be divided

in seven subgroups (A-G).[116,117] The general features and architecture of ABC transporters are well described in the introduction section.[16] Therefore, the lipid transport mechanism of ABC transporter in general and especially ABCB4 will present the focus here. Not all ABC transporters are translocases. A list of human lipid ABC transporter is provided in [118], but it includes transporters, which are nowadays known to not transport lipids. Namely, these are ABCA2 and ABCG5/G8, which recognize cholesterol. Additionally, ABCB11 and ABCC2 transport bile acids and the latter one also transports bilirubin, while ABCD1 translocates fatty acids, but none of them is a lipid floppase. Hence an updated list is provided in Table 2.

Table 2: Overview of lipid translocating ABC transporters.

name	distribution in human cells	Substrate(s)	Related disease	Ref.
ABCA1	Ubiquitous	Phospholipids, cholesterol	Tangier disease	119,120
ABCA3	Lung	Surfactant lipids	Surfactant metabolism dysfunction 3	121,122
ABCA4	Retina	Phosphatidyl-ethanolamine	Stargardt disease	123-127
ABCA12	Lung, skin	Lipids including ceramides	Harlequin ichthyosis	128-130
ABCB1	Ubiquitously, in various tissue	Glycosphingolipids Short chain lipids (low transport efficiency)	Inflammatory bowel disease	131-133
ABCB4	Hepatocytes	Phosphatidyl-choline lipids	PFIC3	23,24,134-136

The transport mechanism of ABC transporters and especially how they flop lipids is not yet completely understood. In general, the “alternating access” model is still the most favored one for transport by an ABC transporter.[137,138] The model is described in detail in section 1.2. of the introduction.[16] However, the development in molecular dynamic simulations and the progress in cryo-EM has led to a number of important structures of ABCB1 in different conformations.[43,81,139,140] Hence, the transport mechanism of ABCB1 was refined.[141,142] A recent publication postulates a ”twist and squeezing” mechanism for ABCB1.[143] Hence, each ABC transporter might resemble an individual transport mechanism and the general model has to be adopted regarding the specific task of each ABC transporter.

For lipid transport, two models for ABC transporters have been proposed: i) the vacuum cleaner model and ii) the floppase model (see Figure 2). The vacuum cleaner model postulates that the lipids are extracted from the inner leaflet, enter the binding cavity provided by both TMDs and released into the extra cellular space.[144-146] The problem with this model is the amphiphilic character of lipids and the complete exposure to water after the release from the transporter. Therefore, a complete hydration of the lipid has to be assumed. But the energy needed for a complete hydration of a lipid cannot be provided by an ABC transporter, assuming that only one transport cycle is sufficient for lipid export. Hydrolysis of one to two ATP molecules does not provide the energy needed for transport and hydration of the lipid.[146,147] Hence, the vacuum cleaner model was extended to the activated model of ABC transporter. Here, the transport pathway is similar to the vacuum cleaner model. The lipid enters the binding cavity from the inner leaflet and is transported to the extracellular space. But instead of hydration of the energetically-activated lipid, it is taken up by an acceptor.[1,146,148] One well accepted example for this model is the heterodimer ABCG5/G8 (Figure 2), which secretes cholesterol from the inner leaflet into the bile canaliculi, where it is taken up by the mixed micelles. [1]

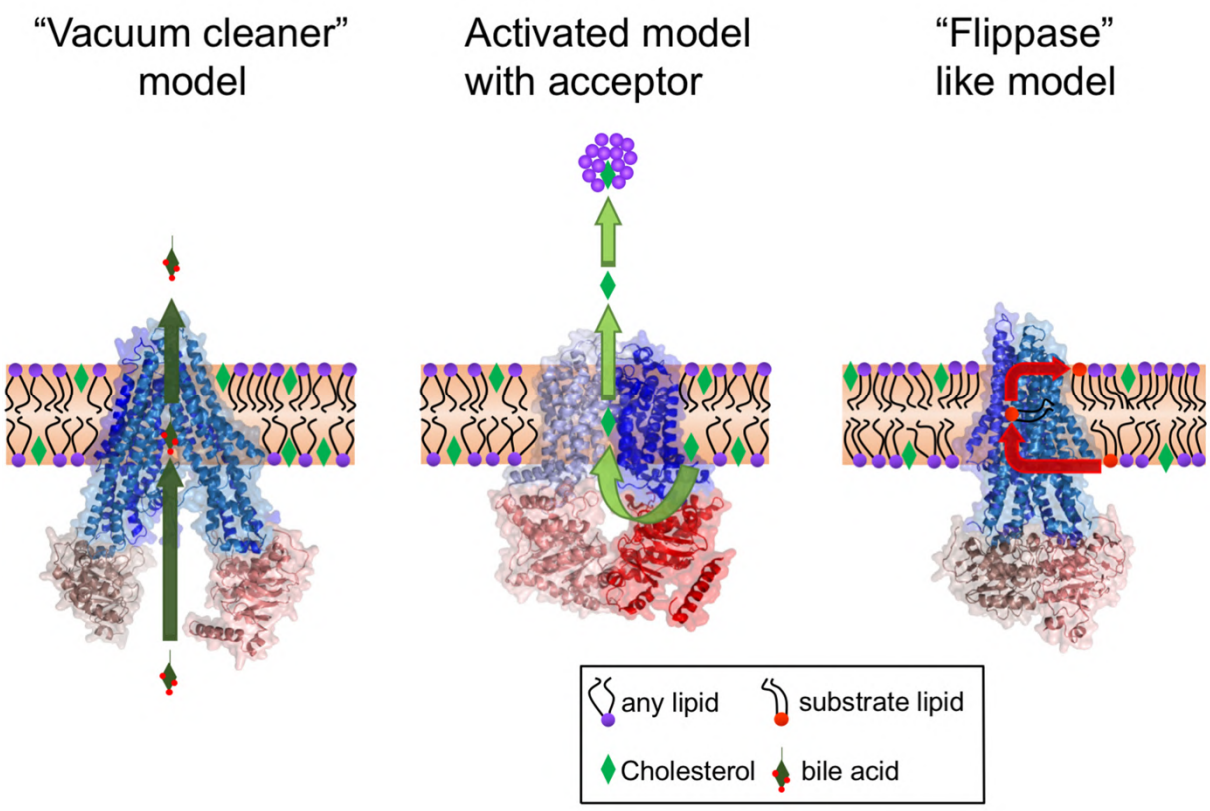

Figure 2: Representation of the different models of transport by ABC transporters. Left: The vacuum cleaner model for possible bile acid transport by ABCB11 (PDB 6LR0)[37]. TMDs are represented in blue and NBDs in brown color. The substrate enters the binding cavity from the cytosol and is released into the extracellular medium. **Middle:** The activated model with acceptor proposed for ABCG5/G8. Structure is taken from PDB 5DO7[42]. ABCG5 is colored in light blue (TMD) and red (NBD), while ABCG8 is highlighted in dark blue (TMD) and red (NBD). Here, the substrate activates the transport and enters the binding pocket laterally from the lower leaflet. Hence, it is secreted into the medium, where it is taken up by an external acceptor. **Right:** Transport of lipids as proposed for ABCB4 (PDB 6S7P)[61]. TMDs are shown in blue, while NBDs are represented in brown color. The specific lipid (red head) is taken up via a lateral gate into the binding pocket and switched by 180° to be released into the upper leaflet. The Figures were created with Pymol version 2.3.4 and PowerPoint 16.16.

The second model is the floppase mechanism of lipid transport by ABC transporter. It is less favored and often not postulated for ABC transporters, because it requires the lipid to perform a 180° turn within the transport step. Similar to the other models, the lipid enters the binding cavity and is then assumed to flop due to the conformational change of the ABC transporter. Subsequently, the lipid is not released but rather integrated into the extracellular leaflet.[149] The most intriguing point in this model is how does the flop occur within the binding cavity. Recent work published by Prescher *et al.* [150] demonstrates an alternative pathway for the floppase model of ABCB4.

4.3.2 Lipid translocation by ABCB4

ABCB4 is an ABC transporter specifically located in the canalicular membrane of hepatocytes of mammals where it has only one function. It flops lipids of the PC family from the inner to the outer leaflet of the bile canaliculi, where PC-lipids form mixed micelles with bile acids and cholesterol, secreted by ABCB11 and ABCG5/G8, respectively. [12,23,134,151-153] While most other ABC transporters accept a broader spectrum of substrates, the three ABC transporters of the bile triumvirate (ABCB4, ABCB11, ABCG5/G8) share a high substrate specificity. Detailed information about the three ABC transporters and bile formation are provided in the introduction.[16] Further information and a more detailed summary about ABCB4 is provided in the current thesis as a second review (see chapter 1).[154] In this section, only the recent updates regarding the transport mechanism of ABCB4 are discussed.

To measure lipid transport in an environment of lipids, like membranes or mixed micelles, is rather challenging. One common way is to measure the lipid content of the medium in absence and presence of an acceptor medium, either of the cells expressing ABCB4 or membrane vesicles containing ABCB4.[27,30,31,155-158] Actually, all the studies demonstrated that in presence of an acceptor medium (often albumin or bile acids), the PC content in the medium was significantly increased, while in its absence, no PC could be measured[31]. Hence, the activated vacuum cleaner transport method was postulated for ABCB4. However, nowadays there is evidence in mouse and rat about the formation of phospholipid patches at the canalicular membrane.[159,160] Furthermore, the formation of fluid microdomains consisting mainly of PC lipids [159-162] was proven. These microdomains would then be solubilized by the bile acids and thereby form the mixed micelles without disintegration of the membrane. This theory became more evident as it was observed that large portions of the bile triumvirate ABC transporter gather in specific microdomains of the canalicular membrane of rat hepatocytes.[163] Additional bioinformatic analysis provided mathematical evidence for this alternative model.[4] Briefly, it described that monomeric bile acid enter the external leaflet of the canalicular membrane, when the cmc is reached in the bile canaliculus. The bile acids form liquid disordered patches that pull off from the external leaflet as nanometer-scale mixed micelles. This model is consistent with crucial experimental findings and may

provide further details about formation of bile and mixed micelles. Subsequently, this means that measuring the PC content in the media informs only about the amount of PC that is extracted by the acceptor medium (i.e., bile acids in this case) and does not provide any detail about the export mechanism or its rate. To overcome this issue, other methods are required which directly measure activity or transport of ABCB4. ATPase activity measurement is an indirect evidence for transport, which in general is used for ABC transporters and it is feasible to conclude that ATPase stimulation by a certain substrate is linked to its transport.[164] However, the measurement of ATPase activity in native membranes is challenging due to the background activity of other ATP-hydrolyzing proteins, without knowledge of a specific inhibitor for ABCB4. Here, solubilization and purification of ABC transporters offers a solution. In case of ABCB4, it is possible to purify the transporter which enables the direct measurement of kinetic parameters.[6,7]

This was recently used to study the mechanism and selectivity of ABCB4.[150] Structural and amino acid comparison of the two highly identical transporters, ABCB1 and ABCB4, revealed the main differences are clustered within the transmembrane helices (TMHs) facing the lipid bilayer. One cluster was found in the TMH1, constituting about 18% of all differences. Especially, the exchange of three non-polar amino acids in ABCB1 to polar equivalents in ABCB4 was surprising. From a thermodynamic point of view, it is not favorable to place these types of amino acids into the bilayer. They may function as anchor points for the choline headgroup during the transport cycle. Furthermore, sequence alignment revealed that this exchange of amino acids in ABCB4 is conserved in up to 50 different species. Subsequently, TMH1 was postulated to be favorable to fulfill a credit card like floppase mechanism. Hence, a new way of lipid flop was postulated (see Figure 3), which does not need the binding cavity of classical ABC transport.

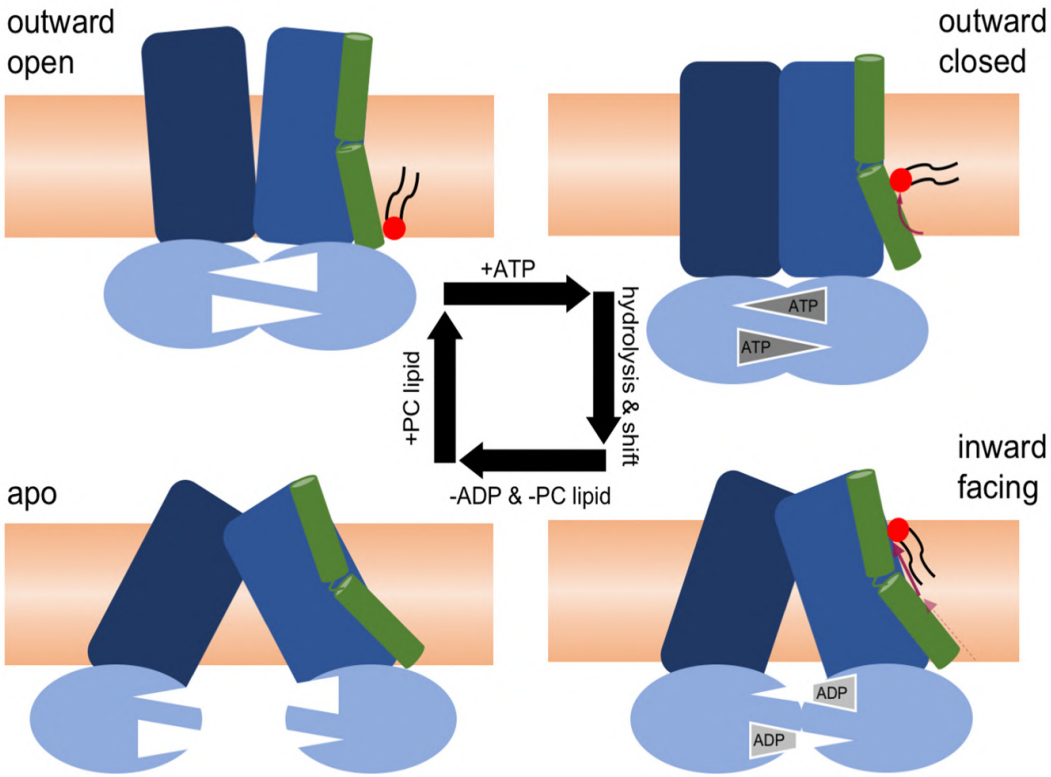

Figure 3: Different stages of possible translocation of PC lipids in a long TMH1 of ABCB4. The ABC transporter is represented in blue color. TMH1 is highlighted in green and the PC lipid with a red head. Starting at the upper left corner, PC lipid is recognized and “loaded” while ABCB4 is in the outward open confirmation. Next (upper right) due to ATP binding and NBD dimerization, ABCB4 is in the outward closed confirmation. Hence, PC lipid is shifted upwards along TMH1. In the next step (lower right corner), the PC lipid is pushed into the upper leaflet by the movement of the TMDs into inward-facing conformation. This movement is indicated by ATP hydrolysis and NBD dissociation. In the last step, ADP is released (apo state), a new PC lipid can be recognized at the lower end of TMH1 and the transport cycle can restart. The Figure was created with PowerPoint version 16.16.

Starting from the apo form, PC lipids are recognized and ABCB4 is `loaded´ with the PC lipid at Q52 of TMH1, pushing the transporter into an outward open conformation. Since, no ATP is bound, the NBDs are in contact but not yet dimerized. Next, ATP is loaded and NBDs dimerize. This movement is transferred to the TMDs, especially TMH1, and allows the choline headgroup to move to S58. By hydrolyzing ATP, the transporter changes from the outward closed to an inward facing position, which in this case leads to a conformationally change in TMH1 to literally push the choline headgroup towards S69 and the lipid into the outer leaflet. Lastly, ADP is released and the cycle can restart.

Based on all the research findings, a mode of bile formation can be postulated (see Figure 4). ABCB4; ABCB11 and ABCG5/G8 are located in close proximity in a specific lipid raft. By flopping PC lipids into the outer leaflet via a credit card mechanism, ABCB4 creates a high abundance of PC lipids there. Subsequently, they gather in a specific microdomain of the external leaflet. Next, bile acids secreted by ABCB11 into the bile canaliculi are accumulated until the cmc is reached. Then they start to solubilize the microdomain / external leaflet by entering the PC-rich domain as monomeric bile acids. They form disordered patches of bile acid and PC lipids, which pinch off as mixed micelles on the nanometer-scale. However, it is rather uncertain if cholesterol is secreted into the external medium and then accepted by the mixed micelles or it is only lifted into the outer leaflet and bile acid extracts PC lipids and cholesterol. Since, the vacuum cleaner mode of action is suggested for ABCG5/G8, it might be that cholesterol is directly released into the bile canaliculi and the mixed micelles are the acceptor medium. Hence, crystallization of cholesterol and gallstone formation is prevented. This could explain why purified ABCG5/G8 is only active in presence of bile acids above their cmc.[3] Additionally a study observed a correlation between cholesterol content of the membrane and ABCB11 activity.[33]

This indicates an interplay of both transporters and their substrates. Less amount of bile acids in the bile canaliculus would result in less mixed micelles and a less active ABCG5/G8. Subsequently, cholesterol accumulates in the canalicular membrane, which then would increase again the release of bile acids by ABCB11, to provide higher amount of bile acids and mixed micelles in the bile canaliculus. Hence, enough acceptor medium (mixed micelles) is present to ensure cholesterol is transported without the risk of crystallization. As a consequence, ABCB4 has to constantly provide fresh PC lipids and should also be capable of recognizing bile acids and cholesterol.

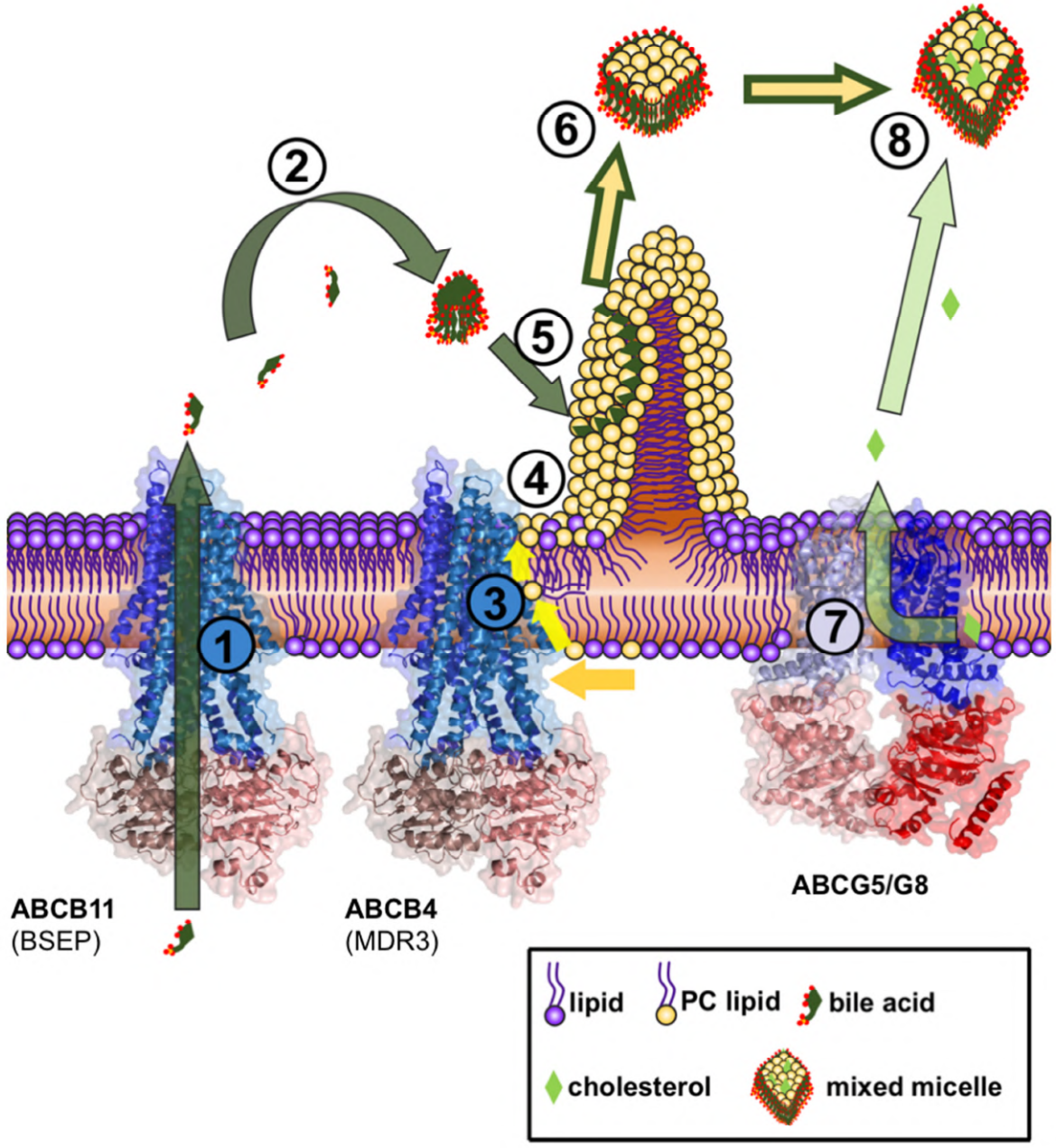

Figure 4: Scheme of bile formation at the canalicular membrane of hepatocytes. For a better overview, biological composition, proportions and distribution of various molecules within the bilayer are omitted. 1) Bile acids are transported across the canalicular membrane by ABCB11 (PDB 6LR0) likely by the vacuum pump mechanism. 2) Subsequently, bile acid concentration accumulates in the bile canaliculus and bile acid micelles are formed if the cmc is exceeded. 3) Parallelly, ABCB4 (PDB 6S7P) flops specifically PC lipids from the inner to the outer leaflet by the recently proposed credit card like mechanism. 4) Subsequently, the PC lipid accumulates in close range to ABCB4 and form microdomains mainly consisting of PC lipids provided by ABCB4. 5) Bile acid with their harsh detergent mechanism enter the PC-rich lipid rifts and form a nanometer scale patch of PC lipids enclosed by bile acids. 6) Next, these patches pull off in form of mixed micelles. 7) Cholesterol is secreted into the bile canaliculus by ABCG5/G8 (PDB 5DO7) via the activated model transport mechanism and 8) lastly, cholesterol is incorporated into the mixed micelles, which acts as necessary acceptor medium. The Figure was created with Pymol version 2.3.4 and PowerPoint 16.16.

4.4 ABCB4 recognizes substrates of other bile triumvirate transporters

As stated above, with progress in bile triumvirate research, it became more evident that the proposed orchestrated ensemble of these ABC transporters is true, at least for ABCB11 and ABCG5/G8. Furthermore, one had to consider that measuring the lipid concentration in the external media with an acceptor medium like BSA or bile acid provides only information about the extraction potential of these acceptors and not the actual ABCB4 transport activity. Presently, the transport of PC by ABCB4 and extraction into the media are viewed as two different processes. Hence, the question is raised whether ABCB4 is activated in a similar manner by the substrates of its neighbors? And how to measure this in an environment of lipids which are preferably extracted by the testing molecules (e.g. bile acids). Here, using solubilized and purified ABCB4 to measure the modulation of ATP hydrolysis offers an elegant solution. It is the main aim of the current work to investigate the effect of all bile acids present in humans, as well as the medically-relevant versions of UDCA, including new nor-UDCA (see chapter 3).[165]

4.4.1 Bile acid origin, composition and chemical properties

Bile acids are divided into primary and secondary bile acids based on their chemistry and origin. The primary ones are cholic (CA) and chenodeoxycholic acid (CDCA) (see chapter 3, Figure 1) and derived from cholesterol. Synthesis takes place exclusively in the hepatocytes of liver. Bile acid synthesis exhibit two pathways: i) the neutral one, which either ends in CA or CDCA, and ii) the acid pathway, which only leads to the formation of CDCA. The enzymes and the individual steps involved are extensively explained in a review by Norlin and Wikvall.[166] Hence, it is not surprising that with ~ 47%, CDCA is the major bile acid in a healthy female. CA makes up ~ 33% of the bile in the gallbladder. Interestingly, the two pathways are active differently in males. Here, CA is the major bile acid with ~53%, while CDCA makes up only ~33% of the bile present in the gallbladder of males.[9] After synthesis, primary bile acids are

conjugated with either taurine or glycine. Astonishing, the conjugation with both amino acids is performed by the same enzyme.[167,168] However, there is a difference regarding the major conjugation present in different mammals and animals. A study investigating the conjugation in different animals and humans demonstrated two possibilities[167]: i) either the bile acid-CoA:amino acid N-acyltransferase (BAT) displays the highest affinity towards the major conjugation or ii) BAT displays a higher affinity towards the non-major conjugation. However, in the latter case the amino acid of the minor conjugation is present in low concentrations to ensure conjugation with the major amino acid. An example for this latter behavior is exhibited by rabbits. Rabbits exclusively form glycine conjugations, but BAT displays a high affinity towards taurine *in vitro*. Due to the absence of taurine in rabbit liver, they form glycine-conjugated bile acids.[167] Human BAT displays three times lower K_m towards taurine (K_m = 1.8 mM) than glycine (K_m = 5.6 mM).[168] Consequently, one has to consider second behavior of BAT and a higher abundance of glycine in human hepatocytes.

Secondary, bile acids, namely deoxycholic acid (DCA) and lithocholic acid (LCA) (see chapter 2, Figure 1), are derived from the primary ones. As part of the hepatic circulation, bile acids are secreted into the intestine where bacteria convert the primary ones into the corresponding secondary ones.[152,169] By a multi-step pathway, the primary bile acids are dehydroxylated at position 7. Subsequently, DCA derives from CA, while LCA is the corresponding secondary bile acid of CDCA. Additionally, bacteria perform isomerization and other changes to bile acids. Since these changes only constitute a minuscule part of the bile acid composition, these are often referred to as “others”. Furthermore, bacteria deconjugate bile acids up to almost 100%. All details about intestinal biotransformation of bile acids are well summarized in a review by Ridlon, Kang and Hylemon *et al*.[169] Interestingly, secondary bile acid distribution in the gallbladder is quite similar in females and males. DCA makes up ~14% / ~9%, while LCA constitutes ~ 1.6% /~ 1.5%, in female and male, respectively.[9] However, it is hard to explain the discrimination between the two as approximately 95% of bile acids are taken up again. Analysis of human faeces provides an explanation. Faeces comprises of only ~ 2% of each primary bile acid, while secondary bile acids (including the “others”) make up more than 90% of faecal bile pool. DCA and LCA are present in high quantities, representing 34% and 29%, respectively. Hence, a high conversion rate of primary bile acids and a discrimination in reuptake is concluded.[170]

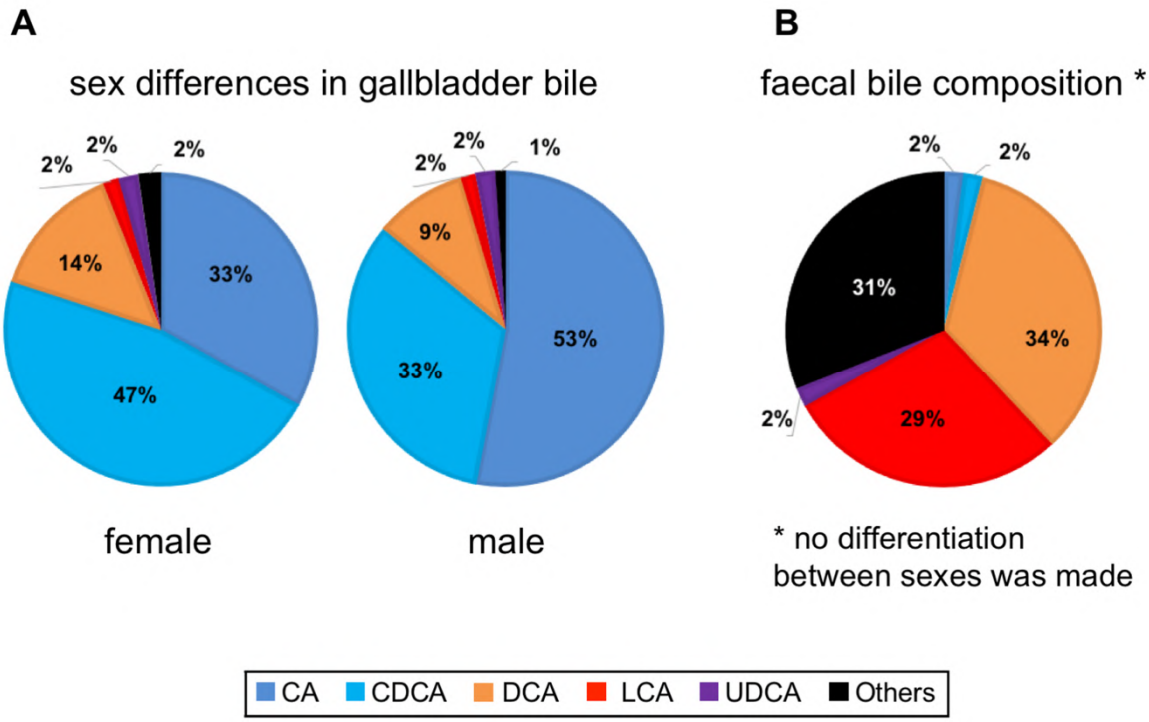

Figure 5: Bile acid composition in the gallbladder and faeces in healthy humans. Numbers are adapted from [9,169,170]. Pie diagrams were created with Excel 2016.

The special bile acid ursodeoxycholic acid (UDCA, see chapter 3, Figure1) and its variants sometimes are called tertiary bile acids. In humans, UDCA can be found in low amounts (~2%) in bile, nearly the same amount is found in faeces (~2%).[166,170] This suggest a negligible uptake of UDCA in the colon and insignificant portion of the bile pool in hepatocytes. Hence, under natural circumstances, the bile triumvirate is not affected by versions of UDCA. On the other hand, UDCA and the shortened Nor-UDCA are used as treatments in different liver diseases.[171-180] In case of UDCA treatment, it replaces the majority of bile acids and becomes the most prominent one. For UDCA, it is known that it is conjugated with glycine and taurine in hepatocytes, but Nor-UDCA is not recognized by BAT due to the shortened side chain. However, one study demonstrated that conjugation is essential for the anti-cholestatic effect.[181] Subsequently, both bile acids and its variants are interesting to test on ABCB4. Some Nor-UDCA was kindly provided by Prof. Dr. Dieter Häussinger, University Hospital Düsseldorf, to test its effect on ABCB4. Due to the availability of limited quantities, it was possible only to test the unconjugated version.

One essential characteristic of all bile acids is the formation of micelles due to their amphipathic nature. Since, bile acids differ in number of hydroxy groups, their position and stereo chemistry, bile acids display a broad spectrum of physical differences. The concentration at which micelle formation starts is called critical micelle concentration (cmc). One unique quality of bile acids is that they have two cmcs.[182] The first one displays a kind of intermediate state, where the concentration of free bile acids still rises but is not equal to the concentration of added bile acids, as the bile acid micelles also increases in size and number. Finally, at the second cmc, bile acids behave like a classic detergent and an increase of total bile acid concentration does not increase the concentration of free bile acids anymore. However, micelles of the first cmc also have a detergent force. Since the aim was to investigate the effect of monomeric bile acids and avoid the detergent effect of bile acid micelles in the assay, it was performed below the first cmc. Because the environment also influences the cmc of detergents, the cmc of all applied bile acids in the current work were determined under the same conditions as in the ATPase activity assay. Only the detergent FC-16 present in the ATPase activity assay was omitted, as it created a high background signal. For measuring the cmc, a fluorescence-based assay was chosen containing the hydrophobic dye Hoechst 33342.[183] The fluorescence of the dye increases proportionally to the micelle concentration. Subsequently, the different bile acids were first dissolved in the ATPase assay buffer (without FC-16), diluted and then the dye was added. In case of more hydrophobic bile acids, DMSO was added but a constant proportion of DMSO (1%) was ensured in all dilutions. Fluorescence was measured at 37°C with a Tecan M200 plate reader and after the background correction data was analyzed with equation 4 (chapter 2, Material and Methods, Determination of the critical micelle concentration). In general, the assay can be described as easy and fast, since it is possible to measure either a number of different detergents or a broad concentration spectrum in a single 96 well plate. In case of water-soluble bile acids, a single measurement exhibited the expected curves (see Figure 6A). In contrast, preparation of the dilutions in DMSO of bile acids were more time consuming and complex. Additionally, the result sometimes displayed an increasing background, resulting in a moderate slope below the actual cmc (see Figure 6B). However, when the cmc is reached the slope displayed a significant increase and it is possible to

calculate the actual cmc. The cmc was determined for each bile acid used (see chapter 2; Table 3).

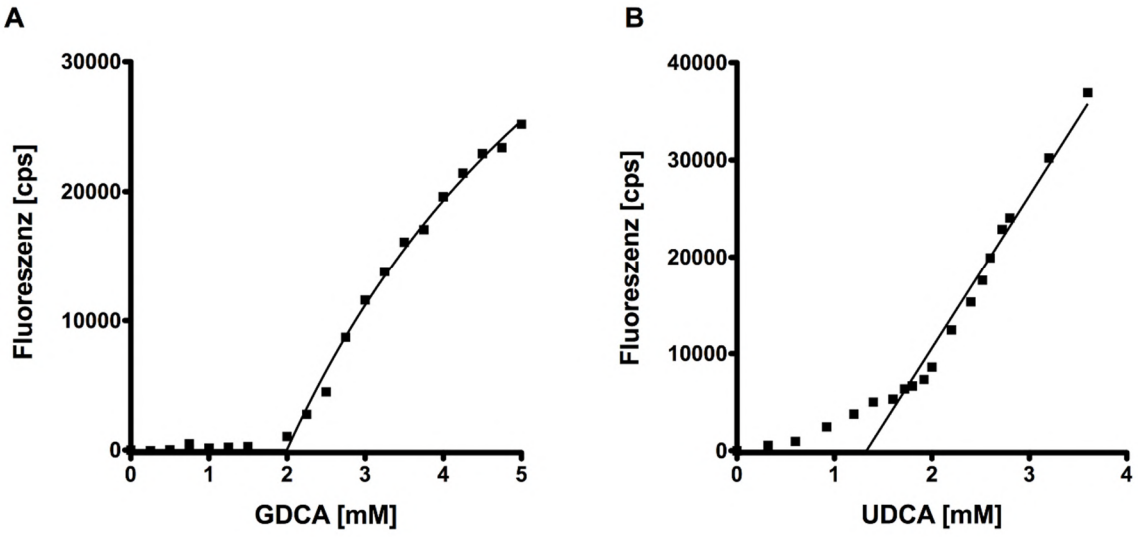

Figure 6: Examples of bile acid cmc determination by increasing fluorescence of the dye Hoechst 33342. After background correction, fluorescence counts are plotted against the bile acid concentration and fitted with equation 3 of chapter 3 using GraphPad Prism. A: An example fit of a fully water-soluble bile acid (GDCA). B: Example of a bile acid dissolved in 1% DMSO in solution. An increase of background fluorescence in the range from 0 to 2 mM can be observed. The fit displays the actual cmc based on the slope starting at ~ 2 mM. These fits and Figures were created using GraphPad Prims 5.

All bile acids have a cmc higher than 1 mM, excepting TLCA, which displays a cmc in the range of 140 µM. Unfortunately, GLCA and LCA could not be dissolved under the current conditions, even though DMSO concentration was increased up to 5%. Higher DMSO concentrations were not suitable for purified ABCB4. However, based on cmc of the other bile acids, cmc of G/LCA can be estimated to be in a range of 100 to 200 µM. It is not totally unexpected that LCA versions have the lowest cmc. TLCA is used to initiate liver cholestasis in rat.[181,184] In general, cmc increased with number of hydroxy moieties. Primary GCA exhibited the highest cmc with 4.1 mM. Furthermore, conjugation of bile acids has a higher impact than the position of the hydroxy moiety. Within the same core bile acid, glycine conjugation shows the highest cmc. While the change of hydroxy moiety from position 7 (CDCA) to 12 (DCA) does not result in any change for the taurine and unconjugated versions. However, a minor decrease of the cmc for glycine conjugated bile acids was observed if the hydroxy moiety position was

changed from 7 (GCDCA) to 12 (GDCA). Additionally, the switch from the α- to the β-position at the carbon moiety 7 (CDCA→ UDCA) reveals a further decrease in cmc values. In case of glycine conjugation, a decrease of ~ 400 µM is observed. In contrast the switch from the α- to the β- position at the carbon moiety 7, is negligible within the error range for taurine conjugated and unconjugated bile acids. The surface distribution of the hydroxy moieties may provide an explanation (Figure 7).

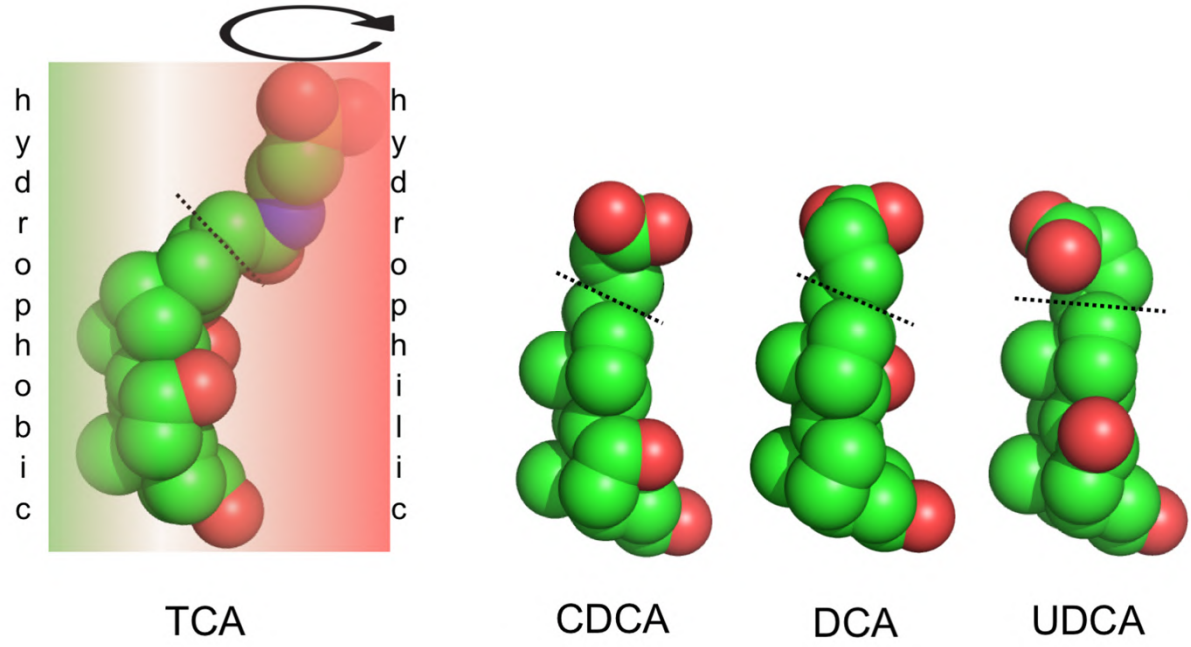

Figure 7: Comparison of the surface of bile acids. Bile acid surface representation was established with ChemDraw Professional 16.0. Green represents carbon atoms, while in red is oxygen, blue represents nitrogen and sulfur is displayed in yellow color. Hydrogens were hidden for a better overview. Black dashed lines represent the beginning of the side chain, which either harbors a carboxyl group or can be conjugated to taurine or glycine. Although, the side-chains can move freely in theory, in aqueous solution it is proposed to align with the hydroxy moieties of the core and form a hydrophilic side. In contrast, the carbon atoms form a hydrophobic side as indicated for TCA. Bile acids are aligned to highlight the major differences due to hydroxy moiety positioning. The Figure is adapted from Hofmann [185].

While the side chain (acid group or conjugation) of bile acids can theoretically turn 360°, the hydroxy moieties at positions 3, 7 and 12 are fixed due to their stereochemistry. It is proposed, that the hydroxy moieties of the side-chain orient towards the side of the fixed hydroxy moieties, as indicated in Figure 7 for TCA. Subsequently, a hydrophobic and hydrophilic side would form. Of course, in case of

CA versions, this side is filled with polar hydroxy moieties. In CDCA, the two remaining hydroxy moieties are in close range, while in case of DCA, the polar groups are further apart. This could explain the decrease in cmc of DCA. Furthermore, the hydroxy moiety at carbon atom 7 of UDCA is in ß-position compared to all other bile acids (α-position). In Figure 7, the differences through this change became obvious. The hydroxy moiety is now placed at the border of the originally hydrophilic and hydrophobic sides. Hence, the hydrophilic surface is shifted which might explain the decrease in the cmc. However, the surface distribution of the hydroxy moieties in the pictured bile acid cannot explain why UDCA is considered as the most hydrophilic bile acid.[171] Indeed the order of bile acids regarding their cmc does change, if the second cmc is taken into account. Here, UDCA exhibit the highest cmc (~17 mM), followed by CA (~13 mM), while CDCA and DCA display similar cmc values of 7-8 mM. LCA was not measured.[186] However, in this study the first cmc is not taken into account at all. The determined cmc values in the current thesis (chapter 2, Table 3) are in good agreement with the values of first cmc measured under similar conditions.[187,188]

4.4.2 Monomeric bile acids modulate the ATPase activity of ABCB4

ABCB4 research could clearly demonstrate that ABCB4 specifically transports lipids of the PC family [23,134,161] and contributes to the formation of asymmetry at the canalicular membrane. Subsequently, ABCB4 is the major reason behind the specific lipid composition in mixed micelles and bile. Furthermore, it seems obvious that bile acids play an important role in ABCB4 and bile formation. There are evidences which indicate the function of bile acids as an acceptor for lipids and important for lipid extraction. However, recently it could be demonstrated for the first time that monomeric bile acids modulate solubilized and purified ABCB4 ATPase activity. Furthermore, the current study demonstrates a correlation between the level of stimulation and bile acid cmc (see chapter 2).[165] Here the *in vitro* system of a solubilized and purified ABCB4 displayed its advantages in contrast to a system where ABCB4 is still in a lipid environment. As stated in chapter 2, the first assays only consisted of ABCB4 solubilized in FC-16 with minor impurities, buffer, salt and bile acids in a concentration

range of 0 to 1000 µM and ATP/Mg^{2+}. Hence, it was possible to measure ABCB4 specific activity. ABCB4 ATPase activity was modulated by all tested bile acids, including the unconjugated versions and the two bile acids derived detergents CHAPS and CHAPSO (see chapter 2, Figure 3 and 4, as well as Table 1 and 2). In case of the hydrophobic LCA bile acids, only TLCA was not precipitating within the set-up and therefore, could be tested. Unfortunately, for Nor-UDCA, no conjugated versions were available and subsequently only unconjugated Nor-UDCA was tested. However, examining at least Nor-UDCA was of utmost importance, as stated later in section 4.4.3.

The results in chapter 2 demonstrated that conjugation is not necessary for stimulation but enhances the modulatory effect of bile acids significant. In case of the two primary bile acids CA and CDCA, this effect is more distinct than for the secondary bile acid DCA and the medically relevant bile acid UDCA. Here, conjugation increased maximal stimulation from 3 to 75%, while conjugation of primary bile acids resulted in an addition from ~86 to 423%. However, conjugation is favored and contributes to higher stimulation levels in case of all tested bile acids. Regarding the differences within the conjugations, glycine conjugation displayed a stronger effect, except in case of CDCA. In case of CDCA, the highest maximal stimulation was observed for taurine conjugation. Compared to GCDCA, TCDCA displaying an approximately 200% higher stimulation. A preference regarding conjugated, especially glycine conjugation, for human ABCB4 can be easily explained with the bile acid distribution amongst humans (see Figure 5). However, one has to also consider distribution of the amino acids for conjugation. In section 4.4.1, the enzyme responsible for conjugation BAT was introduced. It sometimes displays an *in vitro* affinity towards taurine, although the host organism mainly exhibits glycine conjugation. Hence, the actual distribution of conjugated bile acids is regulated by the concentrations of both amino acids within hepatocytes. A similar case could be true for ABCB4 and TCDCA. Because *in vivo* GCDCA is present in high amounts, TCDCA has to stimulate ABCB4 stronger than its counterpart to exhibit the same effect on total PC lipid transport. However, then it should also be true for the other bile acids or at least the other primary bile acids G/TCA, but it is not. Unfortunately, the current set up cannot provide an answer. An actual transport assay is needed, but is not yet established and exhibits several problems (see section 4.5 Outlook).

To understand the importance of conjugation, the two CA-derived detergents CHAPS and CHAPSO were investigated. They exhibit the same basic atomic skeletal structure as CA but are conjugated with 1-propanesulfate or 2-hydroxy-1-propanesulfate, respectively. Both detergents exhibited nearly identical maximal stimulations and EC_{50} values. However, in comparison to the unconjugated CA, maximal stimulations were approximately halved. Hence, this unnatural conjugation is not preferred. Taken together, conjugation is not necessary for stimulation but definitely recognized and natural conjugations are favored by ABCB4.

Another chemical property which definitely has an influence on ABCB4 modulation is the stereo center at position 7. CDCA and UDCA versions only differ in the steric position of the hydroxy moiety at carbon atom 7. However, modulation of ABCB4 and EC_{50} values vary between these two bile acids. G/TUDCA and unconjugated UDCA displayed a significant lower maximal stimulation but an increased EC_{50} value, compared to the corresponding CDCA versions. Interestingly, Nor-UDCA, which is only shortened by one methylene group, displayed double maximal stimulation compared to UDCA and an even higher maximal stimulation than the unconjugated CDCA. However, the EC_{50} value for Nor-UDCA was identical to UDCA. Hence, the stereo chemistry of bile acids alters both the maximal stimulation and the EC50 value, but side chain shortening only alters the maximal stimulation. Subsequently, ABCB4 might recognize the hydroxy moiety at position 7 and at least human ABCB4 favors the α-position. In literature, shortening of the side-chain is known to have a major effect on the hydrophobicity of bile acids.[189]

Dehydroxylation at position 7 results in the harmful LCA bile acid. TLCA represents all LCA versions in the current setup, since GLCA and LCA are not soluble. TLCA was the only bile acid demonstrating a reduced ABCB4 activity. However, TLCA was also the only bile acid exhibiting a cmc below 1 mM. Therefore, ATPase activity was measured in presence of monomeric as well as micelle-forming TLCA. The cmc TLCA exhibited under the current conditions is ~140 µM. Hence, between 50 and 140 µM monomeric TLCA reduces the ATPase activity of ABCB4. At higher concentrations (400 to 500 µM), a 50% reduction is observed and reaches a saturation (600 to 1000 µM). This might be due to the special intermediate between first and second cmc of bile acid. At the first cmc bile acids start to form micelles, mostly consisting of a small number of bile acids (maximum 10 ions), but also the concentration of free bile acids

can increase.[190] Only if the second cmc is reached, bile acids behave like normal detergent and form large secondary micelles without further increase of free bile acids.[190] In between the range of the first and second cmc, it remains unclear what is actually happening. Hence, it is rather complicated to postulate what is happening in case of TLCA. From the current experimental setup, it can only be stated that monomeric TLCA in a range of 50 to 140 µM, reduces the ATPase activity by ~35 to 40%. A further increase in TLCA concentrations up to 500 µM led to in a saturation at -50% of basal ATPase activity. However, TLCA results emphasize the importance of hydroxy moieties within bile acids regarding the modulation of ABCB4 ATPase activity. As a last approach, a bile acid mixture identical to bile acid composition in a healthy human was tested. The mixture comprised of GCDCA, TCDCA, GCA, TCA GDCA and TDCA in a ratio of 21:19:24:16:13:7 as described in literature.[3,191,192] Similar to individual bile acids, the effect of the mixture was investigated in a concentration range of 0 to 1000 µM total bile acids. It exhibited a similar sigmoidal stimulation pattern as the individual bile acids, especially the primary ones. Also, maximal stimulation with 566% is in good agreement of primary bile acids. This was expected since the conjugated version of the two primary bile acids comprises 80% of the mixture. Yet, the EC_{50} value was significantly shifted towards higher concentrations. But this can be explained with the lower concentration of individual bile acids present within the mixture. Subsequently, higher concentrations of the mixture are necessary to reach the concentration, where the bile acid starts to stimulate ABCB4 in our setup. Nevertheless, the bile acid mixture represents the natural composition and no significant difference to the kinetics of individual bile acids was observed. Hence, it can be used for further investigations.

Next, the different maximal modulation values were plotted against the given cmc under the same conditions (see chapter 2, Figure 5). The plot revealed a linear correlation between cmc and maximal stimulation of all tested natural bile acids and a correlation coefficient r^2 of 0.83. This correlation shows that ABCB4 recognizes bile acids by their physical properties. It also represents the approximate composition of bile acids within the bile. Less harsh CA and CDCA versions are the major bile acids in both sexes of the human race. Hence, the prioritization of bile acids by ABCB4 might result from the evolution in bile composition of humans.

It is feasible for ABC transporters to link an increase in ATP hydrolysis to substrate transport. In case of ABCB1, most substrates indeed stimulate its ATPase activity.[164] This raises the question whether bile acids are a substrate of ABCB4? The simple answer is no. *In vivo* data and analysis of PFIC3 patients clearly demonstrated that ABCB4 contributes only to the PC lipid content of the bile. However, from the results of chapter 2, a modulator- or chaperone-like action of bile acids was concluded. A similar action of bile acids was also concluded for ABCG5/G8.[3] Furthermore, an orchestra-like interplay is assumed for the transporter and substrates of the bile triumvirate.[1] Hence, it would make sense if the substrates of specific other ABC transporters naturally modulate each other in any kind of way. This could contribute to the maintenance of the sensitive equilibrium in bile.

However, it remains still unclear whether ABCB4 is active in the absence of bile acids *in vivo*. A study demonstrated that the lipid extraction into the extracellular medium is similar if ABCB1 is expressed (control) or ABCB4 is expressed but cells are not treated with bile the bile acid (TCA was used in this study).[31] Only if ABCB4 was expressed and medium contained 1 mM TCA PC lipid was detected in the extracellular medium. Subsequently, one possibility could be that in the absence of bile acids, ABCB4 only contributes to the asymmetry of the canalicular membrane but PC lipid lifting is enhanced in presence of bile acids. However, under natural conditions, ABCB4 is always in an environment containing bile acids at both sides of the lipid bilayer.

To summaries this important section of discussion, ABCB4 is modulated by bile acids and this might contribute to the abundance of PC lipids in the outer leaflet which subsequently are extracted by the bile acid micelles to reduce the harsh detergent properties.

4.4.3 ABCB4 recognizes bile acids in presence of DOPC

Although bile acids constitute the majority of bile present in gallbladder with nearly 70%, cholesterol, which comprises only 10% of gallbladder bile, should not be underestimated. Improperly incorporated cholesterol easily crystallizes in the bile or

gallbladder (gallstone disease). Detailed information about the gallstone disease are summarized in the review [193]. However, PC lipid containing mixed micelles are the acceptor for secreted cholesterol in humans. Hence, the question arises whether cholesterol can also modulate ATPase activity of ABCB4 in the current setup? Although complicated but the answer is yes. Cholesterol is insoluble in water and a variety of organic solvents, which do not form two phases with an aqueous buffer. But cholesterol can be dissolved into a lipid environment. Since DOPC (naturally absent in the canalicular membrane) displayed similar kinetic ATPase parameters as liver PC extract[7] and the highest amongst a number of tested PC lipid variants[194], it is feasible to choose it as a substrate analog for ABCB4 research. DOPC concentration in in the current setup was set to 300 µM to ensure maximal stimulation by an actual substrate.[7] This maximal stimulation by DOPC was then set to 100%. This should ensure that any modulation of ATPase activity of ABCB4 is a result of cholesterol.

Since cholesterol is insoluble in water and organic solvents are unacceptable for the ATPase assay, it was mixed as powder with DOPC powder in different ratios. Subsequently, the mixture was dissolved in chloroform and used for formation of small unilaminar vesicles (SUVs), as stated in the protocol by Geertsma *et al.* (Step 1-4).[195] Hence, these SUVs contained different cholesterol ratios and were added to the ATPase activity assay. ABCB4 was stimulated by the cholesterol present in DOPC SUVs. Since DOPC is a substrate of ABCB4 but was always present at 300 µM, ABCB4 ATPase activity is increased above the maximal stimulation, which can be achieved by DOPC. This means DOPC, representing PC lipids in this setup, have a maximal effect on ABCB4 ATPase activity. However, other compounds like cholesterol can increase this activity above the DOPC maximal stimulation, starting at a ratio of 0.5 (cholesterol/DOPC). At ratios of 1.0 to 2.0 of cholesterol to DOPC, ABCB4 ATPase activity was doubled by cholesterol compared to DOPC maximal stimulation (see chapter, 2 Figure 7). However, an increase in the cholesterol/DOPC ratio to 3.5 did not result in a further increase of this additional stimulation of ATPase activity. This is in line with the published data for mouse ABCB11 and ABCBC2.[2,33] For example, the transport of the actual substrate of ABCB11 (bile acids) was significantly increased in presence of higher cholesterol levels [2], similar to the findings presented in this thesis (chapter 2).

Subsequently, it was tested if monomeric bile acids exhibit an effect on ATPase activity of ABCB4 in presence of DOPC, similar to the approach with cholesterol. Indeed, G/TCA, GCDCA, GDCA and Nor-UDCA significantly stimulated ATPase activity of ABCB4 on top of the maximal DOPC stimulation (which was set to 100%, see chapter 2, Figure 8). Among these, Nor-UDCA exhibited the highest stimulation in presence of DOPC. For the bile acids CA, TCDCA, GUDCA and UDCA, it is questionable if they actually stimulate ABCB4 further within the experimental error range. However, CDCA, TDCA and TUDCA exhibited no effect on the ATPase activity of ABCB4 in presence of DOPC. In contrast, CHAPS/O and TLCA definitely reduced the ATPase activity to half in presence of DOPC. Comparing these results with the outcome of the kinetics of induvial bile acids, a more distinctive picture of human ABCB4 is presented.

Glycine-conjugated bile acids and TCA increased the ATPase activity of ABCB4 in presence of DOPC. This confirms that the glycine-conjugated versions are favored by ABCB4. Furthermore, the enhancement by G/TCDCA is inversed in presence of DOPC compared to the induvial kinetics of these bile acids. As discussed in section 3.3.3.2, TCDCA exhibited a higher maximal stimulation compared to its counterpart GCDCA, but in presence of DOPC, GCDCA exhibited the higher effect. However, taurine versions of primary bile acids still stimulate ABCB4 in presence of DOPC. In contrast, the taurine version of the secondary bile acids DCA and UDCA displayed no effect. Nevertheless, one has to consider the actual contribution of these bile acids. In humans, it is considered that only ~40% of bile acids are conjugated with taurine. Hence, the secondary bile acid TDCA for example only makes up approximately 3.5% to 5.5% of total bile present in the gallbladder. In case of the secondary bile acid TUDCA, it is only ~0.8%. In contrast, TCA makes up 13% and 21% of total bile present in the gallbladder of females and males, respectively. Subsequently, there might be less need to specifically recognize taurine-conjugated secondary bile acids. Taken together, bile acid stimulating ATPase activity of ABCB4 in presence of DOPC is more in line with the distribution of bile acids in human bile. ABCB4 favored bile acid naturally present in high abundance in human, which ensures high amount of PC lipids at the extracellular leaflet of the canalicular membrane.

The effect of TLCA on the ATPase activity of ABCB4 was similar in presence and absence of DOPC. In both cases it was reduced by 50%. This might be to characteristic

of TLCA forming micelles at significantly lower concentrations compared to other bile acids. This result could explain how TLCA induces cholestasis in TLCA-treated rat liver.[181,184] The bile pool of these rats is changed and consists of mainly TLCA, which then reduced the PC lipid concentration as stated in one of the studies.[184] Subsequently, cells of the bile canaliculi are not protected from the harsh detergent mechanism of bile acid, especially TLCA.

The two bile acids derived detergents CHAPS and CHAPSO exhibited a reversed effect in presence of DOPC compared to their kinetics. While in absence of DOPC, both exhibited a stimulation, they displayed a reduction of nearly 50% in presence of DOPC. Subsequently, in presence of PC lipids, unnatural conjugation is less favored and exhibit a negative effect on ATPase activity of ABCB4.

UDCA and its conjugated versions, are all well-established treatments for liver diseases.[172,174,175] However, they exhibited a rather small to no effect on the ATPase activity of ABCB4 in presence of DOPC. This is in good agreement with another study that demonstrated UDCA did not increase the PC release of ABCB4 WT and a mutant.[27] Additionally, among the four mechanisms of UDCA treatment, none has a direct effect on ABCB4. The known mechanisms of UDCA are: (I) protection of injured cholangiocytes against toxic effects of bile acids, (II) stimulation of impaired biliary secretion, (III) detoxification of hydrophobic bile acids, and (IV) inhibition of apoptosis of hepatocytes. However, it is not clearly understood which of these mechanisms plays a primary role in providing beneficial effects of UDCA in cholestatic liver diseases.[196] The results of this thesis only emphasize that UDCA versions exhibit no direct effect on ABCB4.

In contrast, the shortened Nor-UDCA version exhibited the highest stimulation of ATPase activity of ABCB4 in presence of DOPC among all tested bile acids. It increased the maximal stimulation of ABCB4 in presence of DOPC by ~400%. This is more than two times higher compared to the stimulation by GCA in presence of DOPC, which exhibited the highest maximal stimulation of all human bile acids. Additionally, a definite difference between the two UDCA versions becomes obvious. Subsequently, another mechanism of action for Nor-UDCA has to be considered. Nor-UDCA becomes more promising in ABCB4 research and its related diseases. Furthermore, conjugation of Nor-UDCA and Nor-*urso* versions of other bile acids (especially primary

bile acids) should be considered in future. However, it was not possible to test these within the current thesis due to the limitation of these bile acids and their conjugations.

In a last approach, the effect of bile acids (represented here by the bile acid mixture) and especially Nor-UDCA, should be tested in presence of DOPC and cholesterol. This would represent the natural environment of ABCB4 as close as possible in the current set up. Furthermore, it allowed testing the limitations of ATPase activity of ABCB4. Therefore, maximal cholesterol stimulation (1.5:1 cholesterol: DOPC, based on the findings from the current research work, chapter 2 Figure 7) was ensured in all approaches. Indeed, the bile acid mixture could further stimulate the ATPase activity of ABCB4 in presence of DOPC as well as in presence of both DOPC and cholesterol (see chapter 2 Figure 9B). The activity was enhanced by further 50%. Hence, bile acid can further increase the maximal ATPase activity possible by DOPC and cholesterol. Subsequently, it can be interpreted that both bile acid and cholesterol exhibit each an individual additive effect. It was possible for the first time to demonstrate a direct effect of all three major parts of the bile (bile acids, PC lipids and cholesterol) at the same time. This is the proof that the orchestra theory is at least true for ABCB4. However, Nor-UDCA exhibited a significant higher effect on the ATPase activity of ABCB4 in presence of DOPC and cholesterol. Adding Nor-UDCA to DOPC and cholesterol doubled the ATPase activity of ABCB4. The effect of Nor-UDCA is unique and outstanding within all tested bile acids, whether in presence of DOPC or DOPC and cholesterol. As stated above, this represents a novel mechanism of Nor-UDCA and can lead to various approaches for bile acids and ABCB4 research in the future. Even a clinical trial on patients with liver PC absence due to the failure of ABCB4 is considered in a future cooperation with the group of Professor Keitel-Anselmino from the university hospital Düsseldorf.

Taken together, all tested bile acids modulate ABCB4, both as individual monomers and within the bile acid mixture. Furthermore, a correlation between the cmc and maximal modulation of individual monomeric bile acids could be presented. Additionally, a stimulatory effect of cholesterol content in DOPC SUVs was demonstrated. More importantly, the modulatory effect by bile acids persisted even in the presence of DOPC and exhibited a stimulation pattern similar to natural bile acid composition in humans. Bile acids mixture was also able to stimulate ABCB4 further in

presence of DOPC and cholesterol. However, the new and outstanding effect of Nor-UDCA on ABCB4 displays the potential for ABCB4 and bile acid research in the future.

4.5 Outlook

Although, it is not always necessary that new insights or findings culminate in a solution as it rather forms a foundation for further research. Modulation of ABCB4 by monomeric bile acids as presented in chapter 2, is a totally new contribution to the field of ABCB4 research. However, two major questions arised:1) What is the location of interaction and from which side do bile acids reach towards ABCB4? 2) What is the detailed mechanism of ABCB4 stimulation by bile acids? In this outlook it is briefly described whether a transport assay could answer the above questions. Additionally, the major challenges and limitations in different transport-based assays for lipids in a lipid environment, are addressed.

4.5.1 Reconstitution of ABCB4

Although working with solubilized and purified membrane proteins like ABC transporters has a variety of advantages as stated above, it also has a few disadvantages: i) since it does not represent the natural environment, it could result in an artificial behavior; ii) the current set up is not feasible to distinguish if interaction takes place from the cytoplasm or extracellular medium and iii) detergents might affect the transporter. An example for the later one would be LmrA, which displays approximately four times higher activity in FC-16 than in DDM.[197] This might be even worse if the detergent and the substrate share a similar structural element which is specifically recognized, like in case of ABCB4 (DOPC and FC-16). Of course, this problem can be overcome by testing the effect of detergent on the transporters as it was performed with ABCB4. FC-16 inhibited ABCB4 at a concentration higher than three times the estimated cmc.[194] Hence, the FC-16 concentration in all assays was chosen below this level. Nevertheless, it is not that easy to overcome the other disadvantages. Although working with solubilized and purified ABC transporters is a well-established and accepted method for characterization but the optimum best option

is to return to the natural environment. One way is the reconstitution of ABC transporter into lipid vesicle or synthetic membranes. Advantage of reconstitution is that one can specifically chose the environment, like lipid composition, cholesterol content and labeling of lipids. Disadvantage of this system is that the orientation of ABC transporter has to be managed.[198] A good starting point for ABCB4 reconstitution might be provided by the protocols used for ABCB1 reconstitution.[132,199-202]

However, is reconstitution the best way to study the effect of bile acids on ABCB4? Reconstitution might allow to incorporate ABCB4 in a controlled lipid environment but how does one observe PC lipid flop in a lipid environment? One elegant solution might be the use of NBD-labeled PC lipids.[22,25] However, one must ensure to maintain the membrane integrity, by creating a mixed membrane with a limited amount of NBD-labeled PC lipids and also limiting the time of transport. Further disadvantages of SUVs are the size and the effect of curvature on translocation velocities. Both problems might be solved with giant unilamellar vesicles (GUVs). Size, curvature and extraction can be ignored in GUVs, but unfortunately GUVs are more unstable and difficult to handle than SUVs.[203]

Regarding an experiment with bile acids, one has to ensure the proteoliposome integrity. Based on the current data (see chapter 2), bile acids should be used below their cmc. Since bile acids tend to integrate into the leaflets or accumulate at the leaflet surface, they could create a local high concentration although overall concentration is below the cmc. Hence, they might destabilize the liposomes or extract lipids. Therefore, testing the effect of bile acids on the liposomes in advance is necessary and can also serve as a control experiment.

4.5.2 Transport in a cell-based assay

To completely understand the transport by ABCB4 and to investigate the exact mechanism of bile acids and cholesterol, a suitable transport assay is needed. Since ABCB4 is a PC lipid floppase, a classical transport assay that measures export across a membrane is not appropriate for ABCB4. One possibility via cell-based assay is the extraction of lipids by BSA.[25,27] However, as stated at the beginning of the discussion (4.5), lipid translocation, surface presentation and extraction seem to be different steps

and there is no guarantee that extraction is equivalent to transport. It may allow monitoring the general differences in the abundance of PC lipids in the outer leaflet but not the translocation rate by ABCB4. It gets even more complex if bile acids are added, since bile acids extract lipids Hence, similar to the reconstitution system discussed in the section above (4.5.1), an assay to directly monitor PC flop is required. One futuristic and not yet tested alternative might be the use of brominated PC lipids and quenching by tryptophan incorporation. Since tryptophan quenching occurs in a range of four to five times less than the size of a membrane (~8Å [204] vs 30Å to 40Å [205]), a well-positioned tryptophan mutant would allow to monitor the position of the lipid. Especially, if PC flop is really performed along TMH1, one could introduce tryptophan mutants to create an asymmetry at different ends of TMH1. This would enable the analysis of the possible crucial position in TMH1. Of course, the mutants have to be tested with regards to the protein function. In case these ABCB4 mutants can also be integrated in a membrane (e.g., a cell membrane, liposomes or membrane vesicles), this should allow a proper transport monitoring. Subsequently, bile acids may be added to the system at levels which do not destroy the membrane. This would enable to investigate the effect of bile acids on the translocation process.

What about the polarized cell system with stable transfected Na^+-taurocholate cotransporting polypeptide (NTCP), ABCB4, ABCB11 and ABCG5/G8? In 2016, the group of Bruno Stieger managed to stably transfect the LLC-PK1 cell line with the three ABC transporters of the bile triumvirate and NTCP, which is necessary for bile acid uptake.[30] It was possible to cultivate these cells in trans-well plates creating a pseudo basal and apical membrane. The transporters were correctly located within the membranes and also active. This system was further used to study the effect of antifungal drugs on ABCB4 and its role in DILI. However, it is remarkable how it was possible to create a cell-based system representing a hepatocyte with a functional bile formation system. Not only does it enable studying the passage of bile acids through the cell but it might also enable us to study ABC transporters as integrated entities. Additionally, the response of the three ABC transporters towards their mutual neighbors' substrates could be investigated. The system has a high potential to investigate the different scenarios occurring during bile formation. Furthermore, it allows to test the effect of the absence, inhibition or changed activity of the ABC transporters. Furthermore, the important mutants in ABCB4, ABCB11 and the change

in bile can be studied. It has the potential to provide deeper insights into the mechanism of protection by (Nor-) UDCA or other drugs. Due to the NTCP and bile acid passage through the cell, the side of the ABC transporter through which bile acids effect ABCB4 and ABCG5/G8 could be identified. However, this system might not provide insight about the mechanism of action of bile acids on these two transporters. In both cases, a detailed transport assay is required. If the assay (for example bromide quenching for ABCB4) can be transferred into this cell line, it would be a convenient tool which might allow more detailed study of the bile triumvirate than done earlier. Nevertheless, it is imperative to first understand the mechanism behind transport and it would also provide some insights into ABCB4 research. Monitoring the transport could allow better differentiation between translocation, accumulation and extraction of PC lipids and subsequently solve the mystery behind modulation of ABCB4 by bile acids and cholesterol.

5 Literature

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Appendix

I. Curriculum vitae

Personalis notitia

Name of Birth	Tim Kroll
Date of Birth	29.10.1990
Place of Birth	Mönchengladbach, Germany

Educati universitates

April 2016 – recent	**Doctorate** Heinrich-Heine-Universität, Düsseldorf Institute of Biochemistry Head: Prof. Dr. rer. nat. Lutz Schmitt Interaction network and regulation of the human ABC transporter MDR3/ABCB4
October 2013 – January 2016	**Master of Science – Biochemistry** Heinrich-Heine-Universität, Düsseldorf Institute of Biochemistry Head: Prof. Dr. rer. nat. Lutz Schmitt “Purification and characterization of the NsrP loop”
October 2010 - September 2013	**Bachelor of Science – Biochemistry** Heinrich-Heine-Universität, Düsseldorf Institute of Biochemistry Head: Prof. Dr. rer. nat. Vlada Urlacher “Permeabilization of cell membrane of Pseudomonas putida for hole cell catalysis “

Educatio scholaris

Till June 2010	**Abitur - higher education entrance qualification - Germany** Highest school graduation possible in Germany, qualifying for university admission Pascal Gymansium, Grevenbroich

II. List of publications

Year of publication

2021	Monomeric bile acids modulate the ATPase activity of detergent-solubilized ABCB4/MDR3 **Tim Kroll**, Sander H.J. Smits, Lutz Schmitt Journal of Lipid Research
2020	Structure and function of hepatobiliary ABC transporter **Tim Kroll** [#], Martin Prescher [#], Sander H.J Smits, Lutz Schmitt Chemical Reviews
2019	ABCB4 in heath and disease – at the crossroads of biochemistry Martin Prescher [#], **Tim Kroll** [#], Lutz Schmitt Biological Chemistry

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IV. Declaration

Ich versichere an Eides Statt, dass die Dissertation von mir selbstständig und ohne unzulässige fremde Hilfe unter Beachtung der „Grundsätze zur Sicherung guter wissenschaftlicher Praxis an der Heinrich-Heine-Universität Düsseldorf“ erstellt worden ist. Darüber hinaus ist die Dissertation, in der vorgelegten oder in ähnlicher Form, bei keiner anderen Fakultät eingereicht worden, noch hat es einen erfolglosen oder erfolgreichen Promotionsversuch gegeben.

(Ort, Datum) (Tim Kroll)