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Review

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ABC transporters involved in respiratory and cholestatic diseases: from rare to very rare monogenic diseases

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Abstract
ATP-binding cassette (ABC) transporters constitute a 49-member superfamily in humans. These proteins, most of them being transmembrane, allow the active transport of an important variety of substrates across biological membranes, using ATP hydrolysis as an energy source. For an important proportion of these ABC transporters, genetic variations of the *loci* encoding them have been correlated with rare genetic diseases, including cystic fibrosis and interstitial lung disease (variations in *CFTR/ABCC7* and *ABCA3*) as well as cholestatic liver diseases (variations in *ABCB4* and *ABCB11*).
In this review, we first describe these ABC transporters and how their molecular dysfunction may lead to human diseases. Then, we propose a classification of the genetic variants according to their molecular defect (expression, traffic, function and/or stability), which may be considered as a general guideline for all ABC transporters' variants. Finally, we discuss recent progress in the field of targeted pharmacotherapy, which aim to correct specific molecular defects using small molecules.
In conclusion, we are opening the path to treatment repurposing for diseases involving similar deficiencies in other ABC transporters.

Keywords: ABCA3; ABCB4; ABCB11; CFTR; Classification; Targeted pharmacotherapy.

List of Abbreviations

4-PBA	4-phenylbutyrate/phenylbutyric acid
ABC	ATP-binding cassette
AECII	Type II alveolar epithelial cells
ASO	Antisense oligonucleotide
ASL	Airway surface liquid
BA	Bile acid
BF	Bezafibrate
BRIC	Benign recurrent intrahepatic cholestasis
BSEP	Bile salt export pump
CF	Cystic fibrosis
CFTR	Cystic Fibrosis Transmembrane conductance Regulator
COPD	Chronic Obstructive Pulmonary Disease
Cryo-EM	Cryogenic electron microscopy
DILI	Drug-induced liver injury
DMSO	Dimethylsulfoxide
ECD	Extracelllular domain
EH	Extracellular helix
ENaC	Epithelial sodium channel
ER	Endoplasmic reticulum
FDA	Food and drug administration
GM-CSF	Granulocyte-macrophage colony-stimulating factor
ICP	Intrahepatic cholestasis of pregnancy
IH	Intracellular helix
ILD	Interstitial lung disease
LB	Lamellar body
LPAC	Low phospholipid-associated cholelithiasis
MDR	Multi-drug resistance
MSD	Multispanning domain
NBD	Nucleotide-binding domain
NMD	Nonsense-mediated mRNA decay
PC	Phosphatidylcholine
PCL	Periciliary liquid
PDZ	Postsynaptic density protein-95, Disc large tumor suppressor, Zonula occludens-1
PFIC	Progressive familial intrahepatic cholestasis
PTC	Premature termination codon
RD	Regulatory domain
RDS	Respiratory distress syndrome
SAHA	Suberoylanilide hydroxamic acid
SNP	Single nucleotide polymorphism
SP	Surfactant protein
TAP	Transporter associated with antigen processing
TMD	Transmembrane domain
TMAO	Trimethylamine-N-oxide
UDCA	Ursodeoxycholic acid

1. Introduction

A majority of the molecules essential for cell survival are not able to cross lipid membrane bilayers on their own. Thus, transmembrane proteins allowing their transport through these membranes are necessary for all living organisms. Around 10% of all genes encode proteins predicted to have a role in membrane permeability and regulating the traffic of molecules and ions entering/leaving the cell or organelles (1). Whereas most ion channels or transmembrane facilitators do not use ATP hydrolysis as an energy source (they are named secondary transporters) for the transport of molecules/ions, primary active transporters require energy from ATP consumption to carry out such transport. Among them, a large family of genes encoding ATP-binding cassette (ABC) transporters are found in all domains of life (archaea, bacteria and eukaryotes) and are able to transport various substrates, molecules and ions using the energy released by ATP hydrolysis (2).

Most ABC transporters are highly substrate-specific (lipids, small molecules, drugs), while some of them are multi-specific (e.g. ABCB1/MDR1/Pgp; P-glycoprotein). The transport of specific substrates through these transporters is mostly unidirectional (import or export). For example, they are responsible for the import of essential nutrients (mainly in prokaryotic cells) or the export of essential lipids or cytotoxic compounds (3). Some of them are involved in lipid transport among which the ABCA, ABCD (except for ABCD4 involved in intracellular processing of cobalamin/vitamin B12) and ABCG subfamilies, as well as some ABCB subfamily transporters (ABCB1 and ABCB4) (for reviews, see (4,5)).

Both importers and exporters are found in prokaryotes (6,7), whereas in eukaryotes, ABC transporters are mainly exporters; even if some importers have been identified, they seem to have disappeared during evolution (8,9). ABC transporters share common features such as sequence and structure but they allow the specific transport of different substrates across biological membranes thanks to conformational changes allowed by ATP hydrolysis as an energy source, as recently shown by crystallography analyses and cryogenic electron microscopy (cryo-EM) (10,11).

A total of 49 ABC transporters have been identified in humans (including the *ABCA11* pseudogene) (3). They are divided into seven sub-families (from *ABCA* to *ABCG*), six of them being conserved in *S. cerevisiae* (12), mostly based on the sequence and organization of their domains (13). More recently, a classification based on the organization of transmembrane domains (TMDs) defined seven types of ABC transporters (14). With the exception of the ABCA and ABCG subfamilies which are type V transporters, having TMDs separately embedded in the membrane, without swapping (meaning the TMDs remain distinct and do not intersect within the membrane), and short intracellular helices, the other human ABC transporters belong to the type IV category, with swapped transmembrane helices, causing the TMDs to cross over each other within the membrane, and long intracellular loops (14).

An ABC transporter is usually composed of four domains, including two TMDs (each containing six transmembrane helices), embedded in the membrane lipid bilayer and forming a passage through the membrane, and two cytoplasmic nucleotide-binding domains (NBDs) in their cytosolic part allowing ATP binding and hydrolysis (15,16). Bacterial transporters are mainly expressed as half-transporters, which contain a TMD fused with a NBD, and dimerization (homo- or hetero-dimerization) is then necessary to obtain a functional protein, thus forming a full and functional transporter (17).

On the contrary, for human ABC transporters, there is a majority of full transporters for which the four domains are encoded as a single polypeptide: four ABCB transporters (ABCB1, B4, B5 and B11), all ABCA and ABCC (3,13). The different membrane topologies of human ABC transporters are represented in Figure 1. Then, less than half of human ABC transporters are synthesized as half-transporters and need to form homodimers or heterodimers to be

functional; this is the case for seven ABCB transporters (ABCB2, B3, B6, B7, B8, B9 and B10), all ABCD and ABCG transporters. Heterodimerization of half transporters mainly concerns ABCB2/ABCB3 (TAP1/TAP2; transporters associated with antigen processing) and ABCG5/ABCG8 (3,18,13) ; oligomerization of half transporters (forming dimers or higher order of oligomers) concerns ABCB9 and ABCG2 (18). In addition, previous studies showed that among peroxisomal ABC transporters which are all half transporters, ABCD1, ABCD2 and ABCD3 are also able to form homodimers, heterodimers or tetramers (19). Moreover, it is important to note that even full transporters are able to form oligomers such as ABCA1, ABCA3, ABCC1 and ABCC7 (20–22,18,23,24). About half of ABCB and ABCC transporters have an additional domain called TMD0 which consists of three to five additional membrane helices at the N-terminus of the protein. Depending on the transporter, it serves as an addressing signal (ABCB9/TAPL, TAP-like) or allows interaction with other proteins (ABCB6, ABCB9, ABCC8, ABCC9) (25,26). In the case of ABCC, TMD0 is bound to the rest of the protein through a cytoplasmic loop (L0) (14,27).

ABCA members differ from other ABCs by the length of their TMDs forming an elongated hydrophobic tunnel, and they have two large extracellular domains (ECDs), between the first two membrane helices of each TMD (28); these extracellular loops contain glycosylation sites essential for protein trafficking and stability (29,30) and are thought to serve as anchors for apolipoproteins in the case of ABCA1 (31). Moreover, the presence of two regulatory domains (RDs), one after each NBD, is specific to ABCA transporters and well conserved into this subfamily (32). This ABCA subfamily gathers the largest ABC transporters as they range in size from 1543 to 5058 amino acids (versus a maximum of 1581 amino acids for the other subfamilies) (3,33–35). Interestingly, ABCA4 is the only human ABC importer (36).

Then, the ABCB subfamily includes transporters involved in various processes such as multidrug resistance (MDR), immune response (TAP complex), bile acid (BA) homeostasis (ABCB4 and ABCB11) or iron metabolism in mitochondria (ABCB7, ABCB8 and ABCB10) (3,35). In addition to ABCB1/MDR1/Pgp, ABCG2 and ABCC subfamily members are involved in MDR, except for ABCC8/9 (SUR1/2, sulfonylurea receptors 1 and 2) and ABCC7 (CFTR, cystic fibrosis transmembrane conductance regulator), which is an ATP-gated anion channel. Finally, transporters of the ABCE and ABCF subfamilies are only composed of two fused NBDs (37,38) and do not appear to be involved in the transport of substrates; some studies showed their implication in the regulation of protein translation initiation, in chemoresistance of cancers, antibiotic resistance or in antiviral defense (39–44).

Molecular defects in several human ABC transporters are correlated with rare diseases (Table 1; for a review see (13)), the best described being cystic fibrosis (CF), for which defects in CFTR are identified. The present review focus on four members of the ABC superfamily involved in respiratory and cholestatic liver monogenic diseases: CFTR, ABCA3, ABCB4 and ABCB11 (Table 2), excluding the role of other ABC transporters, such as ABCA1 or ABCC2 which have been identified as risk factors to develop chronic obstructive pulmonary disease and intrahepatic cholestasis of pregnancy, respectively (45,46). It is of note that genetic variations of ABCC2 are also directly involved in the pathogenesis of Dubin-Johnson syndrome (47), which is outside the focus of the present review. After a brief description of these transporters and their pathophysiology, we will propose a classification of their genetic variants identified in patients and we will discuss pharmacological tracks that are under investigation for these patients in the frame of personalized medicine approaches.

2. ABC transporters implicated in respiratory diseases

2.1. ABCC7/CFTR

The ATP-binding cassette subfamily C member 7 (*ABCC7*), also called *CFTR,* is a type IV ABC transporter gene discovered in 1989 and named as such because genetic variants (locus 7q31.2) cause CF (48–50) (Table 2). The CFTR protein has conserved motifs based on the assembly of five domains: two cytoplasmic NBDs (NBD 1 and 2), two TMDs (TMD 1 and 2), each containing six transmembrane helices, and the two homologous (TMD1-NBD1 and TMD2-NBD2) are linked by a large cytosolic regulatory R region (see 3D structure in Figure 2). This R region contains numerous consensus phosphorylation sites (serine and threonine residues), which constitute targets for various cytosolic protein kinases, among them protein kinases A and C (51). CFTR function is therefore regulated by two complementary processes: first, it is phosphorylated on its R region (52); and second, the gating of phosphorylated CFTR is driven by ATP binding to cytosolic NBDs and hydrolysis (53). The 1,480 amino acid CFTR protein is the only member of this family functioning as an ion channel (54).

The physiological roles of CFTR in epithelial cells are enabled by two salient properties. First, its ability as a cAMP-dependent and ATP-gated ion channel to conduct bicarbonate and chloride anions through the apical plasma membrane constitutes its main physiological signature; CFTR regulates the amount and composition of epithelial secretions throughout the body (55,56) and variations in the gene can lead to multisystem pathologies of the lung (sticky mucus in bronchi), digestive tract (abnormal intestinal absorption, loss of exocrine pancreatic function) (55,57,58), and other systems (reproductive, salivary and renal) (59). Second, CFTR being in complex with multiple enzymes as well as scaffolding and signaling proteins (e.g. membrane receptors, ion channels and transporters), it creates a multifunctional platform linking the plasma membrane to the architecture of the cells, for example with cytoskeleton actors. The C-terminal end of CFTR interacts with PDZ (Postsynaptic density protein-95, Disc large tumor suppressor, Zonula occludens-1 (ZO-1)) domain-containing proteins (e.g. NHERF1, NHERF2, PDZK1, PDZK2, Shank2, and CAL), which affect its stability and lifetime (reviewed in (60)). On the other side of CFTR, its N-terminus also interacts with proteins such as syntaxins and SNAREs, thus regulating its traffic towards the plasma membrane (reviewed in (61)).

Hereafter, we will focus on the role of CFTR in airway physiology and diseases. Airway epithelial cells possess a tandem of apical ion channels fine tuning transport of chloride and sodium ions: CFTR and the epithelial sodium channel (ENaC). The functional interaction of CFTR and ENaC in airway epithelium generates a sustained inhibition of ENaC (62), also driving water flow by osmosis, and controls the airway surface liquid (ASL). ASL is made of two phases: the periciliary liquid (PCL) and the mucus. The PCL, surrounding the cilia of airway ciliated cells (most of them are in the surface epithelium), is necessary for cilia beating. Above the cilia lies the hydrated mucus that traps debris in inhaled air, a process required for mucociliary clearance (mucus composition: 97.5% water, 1% ions, and 1.5% organic molecules) (63,64). The water and ion content of ASL is thus fine-tuned by the activity of these two ion channels to maintain a PCL height of approximately 7 µm and a water-saturated mucus to facilitate its elimination by cilia beating towards the pharynx (64). CFTR also regulates other transport proteins, like chloride and potassium channels, which are important for exocytosis and the formation of molecular complexes at the plasma membrane (65). Thus, the role of CFTR in epithelial cells is not restricted to its anion channel activity but non-channel-dependent roles can also be attributed to CFTR. As a whole, CFTR is thus a master controller of transepithelial ion and water transport.

CF is a monogenic autosomal recessive disease caused by variants of the *CFTR* gene (Table 2). It is still considered as the most common genetic life shortening disease that causes severe damages to lungs, gastrointestinal tract and other organs (55). The *CFTR* gene encompasses

approximately 180,000 base pairs on the long arm of chromosome 7. CF is diagnosed in European-derived populations with a carrier frequency of approximately 1:25 and a mean incidence of 1:2,000-3,000 live births worldwide. Overall, 31,000 people are affected in the USA (57), almost 50,000 people in 38 European countries (67) and a total of more than 160,000 individuals worldwide.

CF leads to progressive dysfunctions of the airways and digestive tract, with abnormal transepithelial electrolytes and water transport, dehydration of lung mucus and generalized perturbation of fluid homeostasis. The accumulation of dehydrated mucus reduces the efficacy of mucociliary clearance allowing pathogens to colonize the airway respiratory tract, leading to recurrent infections and inflammation and thus resulting in reduced respiratory capacity (64). CF is progressive and multiform with different stages ranging from mild to severe respiratory failure and/or to pancreatic sufficiency and insufficiency aggravating the disease. Whereas more than 2,100 variants in the *CFTR* gene have been identified (66–69), only 312 are reported as CF-causing. They affect CFTR protein through a variety of molecular mechanisms at the origin of different functional defects.

The most frequent *CFTR* pathogenic variant is the deletion of a phenylalanine at position 508 (F508del), which is present in approximately 85% and 81% of the individuals in the USA (57) and in Europe (67), respectively. F508del-CFTR shows an inefficient maturation, a reduced plasma membrane expression (70,71), a gating defect (72), a reduced stability at the plasma membrane (73) and a thermal instability at physiological temperature (56,74,75). For clarity, one-letter code is used for genetic variations throughout this review.

2.2. ABCA3

ATP-binding cassette transporter A3 (ABCA3) is a type V ABC transporter, consisting of two NBDs, two TMDs and includes two large ECDs (32). Recently, the presence of four extracellular helices (EHs), four intracellular helices (IHs) and two RDs have been characterized in its protein structure – see Figure 2 (76,77). IHs are essential for TMD-NBD interactions by forming many salt bridges and H-bonds, whereas RDs can act as structural latches, stabilizing the interaction between the two halves of the transporter, but their roles remain to be clarified (76). ABCA3 is a 1,704 amino acid protein involved in pulmonary surfactant formation and secretion, mainly expressed in lungs, in type II alveolar epithelial cells (AECII) (78). Although its highest expression is in lungs, some transcripts have been detected in other tissues, such as the trachea, liver, stomach, kidneys, adrenal glands, pancreas and brain (79), in which its role is not defined yet, *ABCA3* variants exclusively leading to pulmonary symptoms. This protein has two N-glycosylation sites in the first ECD, which are essential for protein trafficking and stability (Asn124 and Asn140) (29). Thanks to the xLxxKN (or xLxKN) motif ($L^{9}LLWKN^{14}$ in ABCA3), a Golgi exit signal presents in most ABCAs, the protein is addressed to endosomes to further reach the membrane of multivesicular bodies (MVBs), which are lysosome-related organelles and precursors of mature lamellar bodies (LBs) (80). The extracellular loops of ABCA3 protein are directed towards the lysosomal matrix which contains many proteins, among which cathepsin L cleaves ABCA3 N-terminal part, just after Lys174 located in the first ECD. Then, the ABCA3 protein becomes cleaved and has a molecular weight of approximately 150 kDa. The role of this proteolytic cleavage is not yet known, but it seems to be specific for ABCA3 among all human ABC transporters (81,82). It is important to note that for the ABCA3 protein, the so-called 'extracellular loop' is actually located intracellularly in this transporter. This misnomer can lead to confusion, but it refers to a domain that, despite its name, is located within the cell.

The pulmonary surfactant is a tensio-active film made of a complex mixture of phospholipids (about 80%) and surfactant proteins (SP), mainly SP-A, SP-B, SP-C and SP-D, covering the air-liquid interface of the alveoli (83). It prevents alveolar collapses at the end of expiration and has a protective role against pathogens. Pulmonary surfactant is produced by AECII and stored in LBs, before being secreted at the air-liquid interface by exocytosis. ABCA3, located at the limiting membrane of LBs (84,85), is essential for LB biogenesis and phospholipid translocation (mainly phosphatidylcholine and phosphatidylglycerol) from the cytoplasm into LBs. Once LB reach the apical pole of the cell, surfactant is secreted into the alveolar space by Ca^{2+}-dependent exocytosis (86). ABCA3 is indirectly involved in SP-B and SP-C maturation since the latter depends on LB formation. Accumulation of surfactant protein precursors was observed in ABCA3-deficient patients and mice (87). Through its role as a cholesterol transporter for LB formation in AECII, ABCA3 additionally prevents the potentially toxic accumulation of free cholesterol in the cell (88,89). Therefore, ABCA3 is crucial for pulmonary surfactant biogenesis and homeostasis (90,91). When ABCA3 protein expression is impaired, evidence shows abnormal LBs and no surfactant production, causing lethal respiratory diseases in humans and mice (92–94).

ABCA3 is implicated in several respiratory disorders, ranging from pediatric to adult forms, with an autosomal recessive hereditary transmission (Table 2). In 2004, variations in the *ABCA3* gene affecting both alleles were identified as the most common cause of congenital pulmonary surfactant deficiency associated with lethal respiratory distress syndrome (RDS) in full-term newborns (92). RDS is the most frequent and severe form of ABCA3-associated diseases. While two-thirds of the patients die early in life, partial recovery following RDS during the neonatal period can often occur, followed by interstitial lung disease (ILD) in children (95,96). Nowadays, onset of ILD during childhood or adulthood is observed only in very few patients and little is known about the clinical course of these cases (97–99). However, whatever the age of onset is, the disease can slowly evolve to lung fibrosis (100). In fact, in this recent review focused on children surviving beyond their first birthday without lung transplantation, Li and coll. highlighted that it mainly concerns patients carrying variants with a residual function of ABCA3 protein. This study also emphasized the progressive nature of ABCA3-related lung disease over time and suggests the use of disease-modifying treatments (100). The phenotypic variability, including variable onset, is mainly due to the type of variants involved, but environmental factors also seem to influence the onset of pulmonary fibrosis (98). ABCA3-associated diseases are rare, and patients present homozygous or compound heterozygous variants, most of them being private, thus making phenotype-genotype correlation studies difficult to perform.

Current therapeutic strategies for infants and children with progressive respiratory failure consist of oxygen supplementation, exogenous surfactant, glucocorticoids, hydroxychloroquine, and azithromycin administration (101–105). However, the response to these non-specific treatments is highly variable and, despite a rapid improvement in lung compliance and a decrease in oxygen requirements, there are still many deaths before 1 year of age, making them mostly ineffective. The only definitive treatment is lung transplantation, but morbidities and mortality remain significant after transplantation (106). A recent report explores emerging therapies, including personalized pharmacological treatments and gene therapy, aimed at addressing the specific genetic variants associated with these disorders (105). The article also stresses the need for ongoing research to develop these targeted therapies, highlighting the potential of gene therapy as a promising approach for some of these severe conditions.

3. ABC transporters in cholestatic liver diseases

Bile is a watery fluid that performs several essential functions in the body, including elimination of xenobiotics and fat emulsification (107). The ABC transporters ABCB4 and ABCB11 allow the active flop/transport of phosphatidylcholine (PC) and bile salts at the canalicular membrane of hepatocytes, respectively (35). Cholesterol is also a bile component secreted by the ABCG5/G8 heterodimeric transporter, which will not be discussed in this review. Therefore, functional defects of these transporters may lead to bile secretion defects, which are the cause of several cholestatic diseases (Table 2).

3.1. ABCB4/MDR3

ABCB4, also known as multi-drug resistance protein 3 (MDR3), is a transmembrane glycoprotein of 1,279 amino acids located at bile canaliculi of hepatocytes (Table 2). Its expression is restricted to hepatocytes, although mRNA traces have been detected in other organs, including adrenal gland, heart, striated muscle, spleen, and tonsil (108). ABCB4 basic structure is consistent with the prototypical structure of an ABC transporter: the protein is organized in two TMDs, each composed of six α-helices, and two ATP-binding and hydrolyzing NBDs, a structure that has been confirmed by cryo-EM analyses – see Figure 2 (109). Two N-glycosylation sites, essential for folding and maturation processes of ABC transporters in general (110), are also present in the first extracellular loop connecting the first two α-helices of TMD1 (111). A 'linker' region provides the link between TMD1 and TMD2 (109). In addition, the cytosolic N-terminal region of ABCB4 is rich in phosphorylation sites, which are essential for transport function (112), while its C-terminal part has been shown to interact with the PDZ-domain containing protein EBP50 (ezrin-radixin-moesin (ERM)-binding phosphoprotein 50), regulating plasma membrane trafficking of ABCB4 (113), also regulated by the interaction of the transporter with several molecular partners, including the small GTPase RAB10 and the serine/threonine kinase myotonic dystrophy kinase-related Cdc42-binding kinase isoform α (MRCKα) (114,115).

ABCB4 is responsible for the translocation of PC, a fundamental bile component, from the inner to the outer leaflet of the canalicular membrane of hepatocytes (116,117). Through the formation of mixed micelles, the role of PC is to solubilize cholesterol and protect the biliary epithelium from the detergent action of free hydrophobic bile salts (118). However, the mechanisms allowing PC flop and secretion into bile canaliculi are still unclear, even if recent cryo-EM studies suggested two alternative mechanisms using a “credit-card swipe” on the external side of the transporter (119), or an internal binding pocket involving a critical tryptophan residue – Trp234 (120).

ABCB4 dysfunctions result in cholesterol crystallization with increased biliary lithogenicity and damage to the biliary epithelium (121,122). Several hepatobiliary diseases are associated with variants in the *ABCB4* gene, including low phospholipid-associated cholelithiasis (LPAC) syndrome, intrahepatic cholestasis of pregnancy (ICP) and progressive familial intrahepatic cholestasis type 3 (PFIC3) (122–124). PFIC3 is a rare, inherited, autosomal recessive disease, with predominantly homozygous or compound heterozygous *ABCB4* variants. It usually manifests in the first year of life, but may occur later during childhood, and is the most severe form of the diseases associated with ABCB4 deficiency, frequently progressing to cirrhosis and death due to liver failure (123,125,126). Interestingly, in some rare PFIC patients, no *ABCB4* variant was identified (126). This might be explained by variants in ABCB4 molecular partners, factors, or regulatory genes. LPAC syndrome affects young adults (under 40 years of age) often carrying heterozygous variants and developing less severe clinical phenotypes (127). ICP is a transitory cholestatic disease that occurs during the third trimester of pregnancy and symptoms usually disappear after delivery (128,129).

In addition to various hormonal and environmental factors, variations in both *ABCB4* and *ABCB11* genes have been identified in ICP (128,129). It is detected in 3% of pregnancies and can lead to serious health effects for both the mother and the fetus (35). In addition to the

diseases mentioned above, variants in the *ABCB4* gene have been identified in other conditions, such as ductopenic liver disease (130) and chronic fibrosing cholestasis (131). Moreover, in a recent study conducted by Avena and colleagues, in which they investigated unexplained cases of cholestasis and gallstones, *ABCB4* heterozygous variants were detected, suggesting that *ABCB4*-related diseases are underdiagnosed (132). Thus, patients of any age with cholestatic symptoms of unknown etiology should be considered for screening potential genetic variations in the *ABCB4* gene (130,132).

3.2. ABCB11/BSEP

ABCB11, also known as bile salt export pump (BSEP), is also a transmembrane glycoprotein located at the bile canaliculi of hepatocytes, with a tissue distribution restricted to the liver (133,134). Structurally, the N-terminal part of this 1,321 amino acid transporter is extended and inserted into the substrate-binding cavity (135). The rest of the cryo-EM resolved structure is quite similar to ABCB4, except that four N-glycosylation sites are present on the first extracellular loop of ABCB11 – see Figure 2 (109,136).

ABCB11 is a BA transporter from the hepatocyte to bile canaliculi with a preference for taurine-conjugated BA (133). A significant part of the biliary flow is dependent on the BA-generated osmotic force, described as bile salt-dependent flow (107,133). Furthermore, it appears that ABCB4 function and/or PC secretion are closely related to ABCB11 function since PC secretion is increased in the presence of taurocholate (137). The molecular mechanisms involved in ABCB11-mediated BA transport are still poorly described even if cryo-EM studies provided evidence that the transporter could bind up to two taurocholate molecules using two independent internal transport cavities (138).

Variations in the *ABCB11* gene are associated with several cholestatic liver diseases, ranging from the mildest forms, ICP and benign recurrent intrahepatic cholestasis type 2 (BRIC2), to the most severe one, namely progressive familial intrahepatic cholestasis type 2 (PFIC2) (35,139) (Table 2). PFIC2 is a rare, inherited autosomal recessive disease, which, unlike PFIC3, manifests in early infancy or even in the first days of life with clinical signs of cholestasis, including jaundice and severe pruritus (126,140). Its pathophysiology is characterized by an accumulation of bile salts in the hepatocytes, resulting in hepatocellular damage and an increased risk of developing hepatocellular carcinoma (141,142). BRIC2 patients have a clinically less severe phenotype characterized by recurrent episodes of cholestasis, which, unlike PFIC2, does not cause liver damage (143,144). Another particular disorder associated with ABCB11 and ABCB4 defects is the drug-induced liver injury (DILI), which is induced by the use of drugs that can potentially inhibit the activity of hepatic transporters and cause cholestatic disease symptoms that are not related to genetic variations of these transporters (145). Because the liver is the main organ of detoxification and drug metabolism, DILI may be the cause of many drug development failures, considering the importance of hepatobiliary ABC transporters.

For many years, the treatment of PFICs has been limited to the use of ursodeoxycholic acid (UDCA), or Ursodiol™, in combination with rifampicin and cholestyramine for the symptomatic treatment or surgical treatment such as biliary diversion (146). Nevertheless, more than 50% of patients have no or insufficient response to this treatment, making liver transplantation the last therapeutic option before adulthood for these young patients (123,147). Alternatively and beyond the scope of the present review, it is interesting to note that the apical sodium-dependent bile acid transporter (ASBT) inhibitors Maralixibat (Bylvay™) and Odevixibat (Livmarli™) are under clinical trials for PFIC2 patients (148,149) and may constitute combinatory therapeutic strategies for these patients.

4. Genetic variations of ABC transporters: Towards a unified classification

Predicting drug-induced response is an attractive challenge that could be efficiently translated to patients for clinical decision making. However, to achieve this goal we must define a precise classification of gene variants indicating the consequences of the genetic defects on the production of a given protein mutant, its maturation, stabilization at the membrane and function.

4.1. ABCC7/CFTR

Soon after the *CFTR* gene discovery, it was attempted to classify CFTR variants according to the molecular defect. Welsh & Smith first established four classes numbered from I to IV in 1993 (150). Class I is composed of non-synthetized CFTR variants, mostly due to premature stop codons (e.g. R553X, G542X). Class II corresponds to defective CFTR processing (e.g. F508del). Class III CFTR variants (e.g. G551D) have severe altered gating. Class IV CFTR variants (e.g. R117H) show a reduced chloride permeability. The production of class V CFTR proteins is reduced without apparent effect on processing or function (151,152). The classification was further extended in 1999 with the class VI (153) to describe CFTR variants showing a reduced expression due to a rapid removal from the apical membrane (accelerated turn-over) caused by C-terminal truncations. Patients with classes I to III CFTR variants develop severe CF forms due to chloride and bicarbonate impermeabilities of epithelial cell apical membranes, then leading to lung and pancreas damages. On the contrary, patients with CFTR variants belonging to classes IV to VI develop milder forms of the disease due to the partial functioning of the CFTR channels consecutive of either a decreased unitary conductance, less protein or less stable protein (150).

Alternatively, the six classes could also be grouped differently (154). One group includes classes I, II and V for variants affecting plasma membrane expression of CFTR variants. A second group is composed of classes III and IV CFTR variants with abnormal channel activity: gating defect for class III and reduced conductance for class IV. However, it is difficult to precisely analyze whether a variant alters the gating without affecting chloride permeability (as for G551D or P67L variants) or reduces conductance without affecting gating (as for R117H) (154–156). Finally, truncated CFTR variants having normal biosynthetic processing and macroscopic chloride channel function, but reduced biological stability of their mature form, were proposed as a third group (153). Most of the severe CFTR variants belong to classes I, II and III (respectively 22%, 88% and 6% of people with CF who have at least one of these variants), but a majority of CF variants has not yet been functionally characterized. Classification is not an easy task and may change with additional tests. For example, the variant P67L was first classified as a class IV deleterious variant but reexamination of protein maturation and function showed that it is more likely a class II and III variant because the P67L-CFTR channel displays protein misfolding, impaired biogenesis, gating defect but normal conductance (155).

4.2. ABCA3

To date, more than 300 pathogenic variants have been reported in the *ABCA3* gene (NM_001089): they are located all along the protein without any hotspot. Except a few recurrent variants such as R288K, R1474W, A501E, A275V and E292V missense variants (95,157,158), ABCA3 variants are mostly private and rare. Affected individuals can bear homozygous or compound heterozygous variants. In case of homozygous or compound heterozygous nonsense and/or frameshift variants, called null variants (because no protein is produced), the outcome is lethal if no lung transplantation is planned (94). Other variants involving missense, splice site variants or in-frame insertion/deletion can allow a residual ABCA3 function and phenotypes are variable whether the variants are present in homozygous or compound heterozygous states (94,100). Since 2006, a classification subdivides ABCA3 missense variants (accounting for 64% of all identified variants) into two types referring to

their effect on the maturation or the function of the protein: type I variants lead to immature misfolded proteins retained in the endoplasmic reticulum (ER), and type II variants produce mature proteins with a correct localization but a decreased phospholipid transport activity (159). More recently, we identified a new class of ABCA3 variants leading to a stability defect of the mature protein (160). This discovery brings the ABCA3 classification closer to the ones for other ABCs, since four classes can now be distinguished: class I exhibiting no protein, class II with defective protein processing, class III with altered phospholipid transport and class IV with a less stable mature form.

For rare genetic diseases, interpretation of familial genetic variants can be challenging, especially in cases with an atypical clinical presentation. To discriminate between pathogenic or benign consequences of the variants, assign the right diagnosis and adapt clinical care and genetic counseling of patients, it is crucial to assess the pathogenicity of these variants *in vitro*. To date, over the 300 known ABCA3 missense variants, only 12% have been characterized with structure-function analyses in cell model systems. While some research delves into the routing and maturation of proteins within cells, investigating the role of ABCA3, which is anchored to membranes of hard-to-isolate intracellular organelles, presents significant challenges. Nevertheless, methodologies like phospholipid uptake assessment have facilitated the exploration of trafficking and functional activities of mutated ABCA3 proteins. Two papers particularly summarize the various *in vitro* studies allowing assessment of trafficking and functional activity of mutated ABCA3 proteins (161,162). These *in vitro* studies and analyses conducted in recent years enable the examination of the effects of therapeutic molecules or treatments (163,164,103). A noteworthy example is the study on the impact of hydroxychloroquine treatment on cells with ABCA3 variants, highlighting the utility of these models for the preliminary assessment of new treatments (103). Indeed, this study emphasized a strong correlation between *in vivo* and *in vitro* responses to hydroxychloroquine. However, it also noted variable clinical responses based on the specific ABCA3 variants present, underscoring the importance of implementing personalized therapy.

4.3. ABCB4 and ABCB11

More than 500 and 600 distinct genetic variants have been identified in each locus encoding ABCB4 and ABCB11 transporters from patients with hepatobiliary diseases, respectively (158,165,166). Unlike *CFTR*, the genetic variations identified in patients are much less recurrent. They are mostly private and familial, except for the E297G and D482G variants of ABCB11, which are reported in more than 50% of PFIC2 patients across Europe (167,168). More than 80% of *ABCB4* and *ABCB11* genetic variations are missense variants and can affect all domains of the proteins, without any sticking hotspot. Moreover, these variations can have variable effects on the expression, traffic, function, or stability of these transporters (124,169). Therefore, their classification and characterization would allow personalized pharmacotherapy according to the type of variant(s) carried by the patient.

According to Delaunay and colleagues, a first classification of *ABCB4* variations has been proposed: *i)* class I with defects in protein synthesis and expression, mostly caused by nonsense variants ; patients with this type of variants generally have the most severe phenotype; *ii)* class II with impaired maturation and intracellular traffic; the protein mostly remains trapped within the ER; *iii)* class III with impaired PC secretory activity but a correct canalicular membrane localization; *iv)* class IV with stability defects of the mature and active transporter (169). Moreover, a fifth class has been proposed for *ABCB4* variants that do not display any significant defects based on experimental data from *in vitro* studies in cell models (169); however, this later class will not be further considered here since it does not designate defective variants but rather single-nucleotide polymorphisms (SNPs) with no associated defective phenotypes.

For *ABCB11*, Byrne and colleagues have also classified missense variants and SNPs according to the identified transporter deficit (alteration of mRNA splicing, maturation, or protein function) (170). More recently, a classification of patients according to the severity of the phenotype induced by *ABCB11* variations has also been proposed (168). Indeed, the clinical phenotype of patients depends on several factors, which are the number of affected alleles (homozygous or heterozygous) and the type and severity of the variant. Thus, according to this classification, homozygous or compound heterozygous patients in the BSEP1 category have a less severe phenotype and carry at least one copy of the E297G or D482G variants. BSEP2 patients have at least one missense variant other than E297G or D482G. Finally, BSEP3 patients have the most severe phenotype with variants predicted to induce a non-functional or unexpressed protein (168). However, this classification is mostly based on the severity of the disease in patients and not the cellular/molecular defects induced by *ABCB11* variations, as proposed for *ABCB4* (see above); and thus, this aspect would have to be further characterized in order to propose a more accurate classification required for targeted pharmacotherapy.

4.4. A unified classification?

As shown above, numerous variant classifications have been proposed in each field of ABC transporters, and are still evolving according to advances in the understanding of these proteins (124,150). Therefore, developing a consensus categorization that can be adapted to all ABC transporters would be a valuable tool for personalized therapy, especially for patients with a disease-causing variant in an ABC transporter for which no treatment exists yet. Variants in the most studied ABC transporter, *CFTR*, are classified since 1993 into four classes (150). Since then, detailed classes have been added and now seven different classes of *CFTR* variants are proposed. Strikingly, great attention is paid by the community to these classifications promoting numerous discussions (154,171–173). Such classifications provide a detailed description of the different types of variants, which is currently more important for understanding the defective mechanisms of the mutated protein, rather than for developing therapies. Indeed, only five therapeutic approaches (readthrough compounds, correctors, potentiators, antisense oligonucleotides and stabilisers) have been linked to these variant classes, so far (154). This observation highlights the importance of focusing on the five variant classes that have been associated with specific treatments, if we hope to expand genotype-associated treatments to other ABC transporters. Then, since existing classifications for several ABC transporters are very close and all human ABC transporters share structural (and sometimes functional) similarities, it is reasonable to propose a new classification applicable to all ABC transporters. Moreover, recent studies illustrate the impact of CF-developed pharmacotherapies on defective variants of other ABC transporters (124,163,164,174–178).

To propose a consensual and easy-to-implement classification for all ABC transporters variants, we suggest directly jumping to theratyping, with the following classification (Figure 3):

Class 0: Absence of mRNA (non-rescuable = bypass therapy)
Class I: Absence of protein
Class II: Defective maturation/trafficking of the protein
Class III: Defective function/regulation of the protein
Class IV: Unstable mRNA or protein

The concept of theratype emerged in the CF field in 2015 with a review from Gary Cutting's team (179), who emphasized the interest in classifying DNA variants according to the molecular-based treatment to which they respond. While frameshifts and nonsense variants will mainly lead to class 0 and I variants, respectively, these variants can be subjected to nonsense-mediated mRNA decay (NMD). If NMD occurs, it would switch variants from class

I to class 0, or give rise to reduced transcript amounts (class 0/I), we have thus classified frameshifts in class 0 and nonsense variants in class I but it will depend on each variant (Figure 4). It is much more challenging to predict the impact of missense variations on the trafficking, the activity and/or the stability of the transporters. Indeed, some bioinformatics tools exist to predict the pathogenicity of genetic variations, such as Polyphen2 (180), SIFT (181), SNAP2 (182), and PROVEAN (183) (the latter is unfortunately discontinued) but they do not predict the potential defect classes. Thus, in order to unambiguously characterize molecular defects of ABC transporter missense variants, they all have to be investigated, one by one, at the cellular level. This is also why classes II, III and IV variants are largely underestimated due to the lack of *in vitro* characterization of the variants, for which the characterization is mostly descriptive at the genetic/clinical level. More than 300 missense variants are identified for each transporter (CFTR/ABCC7, ABCA3, ABCB4 and ABCB11) but only a few of them have been further investigated and classified (Figure 4). Nowadays, routine diagnostic laboratories use either targeted next-generation sequencing panels or wide-genome methods to identify variants (184). This strategy strongly contributes to enhance the number of variants identified in each *ABC* gene. And even if many variants are first interpreted based on criteria published by the American College of Medical Genetics and Genomics (185), it remains a huge number of variants which need to be further tested by functional assay. Therefore, the need for a better classification at the protein level is even necessary.

Thus, the proposed unified classification system aims to provide a consistent framework for understanding ABC transporter variants, while several limitations and potential biases in existing classification systems must be acknowledged and some of them will not be overcome. Indeed, many variants may exhibit pleiotropic effects or context-dependent behavior, making it challenging to assign them to a single class (Figure 4). Additionally, a significant number of ABC transporter variants remain uncharacterized or incompletely characterized at the functional level, leading to potential misclassification or underestimation of certain classes (Figure 4). Whatever the difficulty is, we believe that proposing a unified classification is noteworthy as it can aid in determining the appropriate therapeutic strategy.

5. Targeted pharmacotherapies for defective ABC transporter variants

As shown in Table 1, there is a wide variety of diseases caused by ABC transporter variants. Those variants affect protein biogenesis at any stage from production to maturation, stabilization and function. Therefore, multiple pharmacological approaches are needed to address these defects. We present below several pharmacotherapeutic strategies for ABC transporter variants, such as readthrough molecules, splicing modulators and antisense oligonucleotides (ASO) for class I; pharmacological and chemical chaperones and proteostasis modulators for class II and potentiators for class III variants.

5.1. Pharmacological strategies for class I ABC transporter variants

5.1.1. Readthrough therapies

A nonsense variant leads to the apparition of an in-frame premature termination codon (PTC) triggering NMD, interrupting translation before a full-length protein is produced, thus no protein is translated. Some treatments termed as readthrough therapies suppress translation termination at PTCs by inserting an amino acid at the site of the PTC, thus allowing in-frame translation of a full-length protein (186). Aminoglycosides, like gentamicin or amikacin, partially restore CFTR activity *in vitro* and *in vivo* (187,188) but show modest readthrough efficacy in patients. The small molecule ataluren (PTC124, Translarna®) allows the ribosome to readthrough mRNA containing such a PTC, resulting in the production of a full-length protein but nevertheless failed phase 3 clinical trials (189). More recently, SRI-41315, a new compound able to restore CFTR expression and function has been identified by screening

molecules with readthrough activity. It potentiates aminoglycoside-mediated readthrough by synergistic action and constitutes a new hope to treat diseases caused by nonsense variants (190). Aminoglycosides (including G418 and gentamicin) have also been shown to rescue class I variants of ABCB11 (191).

5.1.2. Correction of splicing by antisense oligonucleotides (ASO) and modulators

ASO are short synthetic DNA or RNA molecules that target pre-mRNA fragments and modulate splicing process for aberrant forms or restore correct reading frame. The advantage of ASO is to skip some exons that can be mutated and can lead to PTCs. An ASO was designed to target a splicing defect of an ABCB11 variant identified in a PFIC2 patient. The c.76+29T>G variation in this patient resulted in the insertion of 42 bp in the mature mRNA. The ASO successfully excluded the pseudoexon formed, marking the first demonstration of ASO use as a therapeutic strategy for PFIC2 patients with intronic variants (192). A similar approach has been used to target exon 23 of the *CFTR* gene (carrying the variant W1282X) and led to increased expression level of CFTR-Δex23 protein levels and CFTR activity in the presence of CFTR modulators (193,194).

There are three aerosolized ASO (ENaCrx, TPI ASM8 and granulocyte-macrophage colony-stimulating factor – GM-CSF) developed by three different industries (IONIS, Pharmaxis and Savara Inc, respectively) with active clinical assays (see (195)). ENaCrx ASO reduce mucus accumulation in Nedd4L-KO mice (a model with mucus accumulation to copy CF symptom) and avoid the reduction of CFTR function. This ASO completed a phase 1/2a clinical trial for CF subjects on October 2020 (NCT03647228) and for moderate chronic obstructive pulmonary disease (COPD) with chronic bronchitis on December 2022 (NCT04441788). TPI ASM8 is a drug containing two ASO : TOP004 (targeting common β cytokine family of GM-CSF, IL3, IL5) and TOP005 against the human chemokine receptor CCR3 (196). CCR3 and common β-subunit are involved in antigen-induced eosinophil influx which is responsible of inflammation pathway. As shown by Gauvreau and colleagues in 2011, TPI ASM8 allows a dose-dependent decrease of inflammation in *in-vitro* assays (196). Clinical trials have been conducted in asthma subjects (NCT01158898) and could be extended to other pulmonary diseases with associated modulators/correctors (197). The factor GM-CSF, known as a growth factor may act as an inflammation marker too (198) and can induce perturbation on neutrophils and alveoli macrophages by a wrong coordination (199). GM-CSF could be a new potential pharmaceutical target to focus on the degradation of surfactant to reduce the risk of any infections. The Molramostim (human recombinant GM-CSF) are developed for this purpose. This drug is still in phase 2 of clinical trials (NCT03597347). Some small molecules are used to increase the expression of ABC transporters through gene transcription. This is the case of bezafibrate (BF) upregulating mRNA but not the total quantity of protein of ABCB4 in human-derived hepatocytes (200). BF is an agonist of PPARα, a nuclear receptor that increases its transcriptional activity for ABCB4 (201) but this nuclear receptor is not well expressed in human-derived hepatocytes (202). PPARα is necessary to induce protein expression at a very defined localization, or to regulate ABCB4 redistribution at bile canaliculi for example. BF could also be used in a specific tissue-like pseudocanaliculi to increase ABCB4 production, which was confirmed in mice (203).

5.2. Pharmacological approaches for class II ABC transporter variants

These approaches are used to rectify the classic pathway of defective proteins, allowing them to be addressed to the plasma membrane without being degraded (*via* the lysosomal and/or Ub-proteasome pathways) despite the variant. Different approaches can lead to the rescue of defective proteins using pharmacological and chemical chaperones, as well as proteostasis modulators, their combination thereof, or the use of low temperature. These agents are collectively named correctors because they rescue the abnormal trafficking and maturation of

the pathological variant. Correctors are so far classified into 3 groups according to their binding sites on CFTR. Correctors C1 (e.g. VX-809 and VX-661) improve the formation of NBD1-multispanning domain 1 (MSD1), NBD1-MSD2 interfaces (204) and stabilize the interactions between NBD1 and intracellular loops 1 and 4 of CFTR (205,206). Correctors C2 (e.g. Corr-4a) stabilize NBD2 of CFTR and its interface with other protein domains. Correctors C3 (e.g. VX-445) have been shown to rectify interdomain assembly of CFTR by interacting with transmembrane region and to correct the thermodynamic instability of purified NBD1-F508del (207).

5.2.1. Pharmacological chaperones

A pharmacological chaperone (also called pharmacoperone) is a small chemical that binds to the client protein either in early intracellular compartments or at the plasma membrane, forcing the misfolded protein into a conformation close to its native state. Among the first pharmacological chaperones discovered acting on an ABC transporter was CFcor-325 that rescued F508del- R258G-, S945L-, and H949Y-CFTR (maturation and delivery of a functional protein to the cell surface) (208). CFcor-325 could also rescue misprocessed variants of the ABC transporter P-gp. Soon after, the compound VX-809 (also named lumacaftor) was identified through high-throughput screening focused on correction of F508del-CFTR trafficking (209). Orkambi is a combination of the corrector VX-809 and the potentiator VX-770 (also named ivacaftor). Orkambi was the first Food and Drug Administration (FDA)-approved drug for people with CF, 12 years and older (NCT03150719), who have two copies of the F508del CFTR variant. Thereafter, the combination Trikafta/Kaftrio (VX-445 + VX-661 + VX-770, also named elexacaftor + tezacaftor + ivacaftor, respectively) was developed and made available in the USA in 2019 and in Europe in 2020 for F508del-CFTR homozygous or heterozygous 12 years and older individuals. Recent cryo-EM studies have provided insights into the mechanism of action of Trikafta/Kaftrio modulators (ivacaftor will be discussed in section 5.3.1). Tezacaftor acts by preserving CFTR stability within the ER during the early stages of its biosynthesis. This mechanism prevents premature degradation by specifically binding to a hydrophobic pocket located within TMD1. Elexacaftor stabilizes the transmembrane helices 10 and 11, along with a structural feature known as the lasso motif, these three domains being critical for CFTR maturation and function. In addition, elexacaftor stabilizes the interface between TMD2 and NBD1 (207,210). However, until now, the effect of these correctors has not been described on ABCA3, ABCB4 or ABCB11.

Other compounds are in development. For example, Vertex Pharmaceuticals Inc.develops other drugs: VX-152 + VX-661 + VX-770 for the same conditions as Kaftrio (clinical trial phase 2 identifier: NCT02951195). For others, development has been discontinued, such as miglustat and riociguat for F508del homozygous 18 years and older individuals (clinical trial phase 2 identifiers: NCT02325362 and NCT02170025, respectively) (211,212).

The ER-retained I541F missense variant of ABCB4, identified in a PFIC3 patient (Table 1), is rescued by cyclosporin A treatment (213). Cyclosporin A, which is a competitive inhibitor of ABCB1, would behave as a pharmacological chaperone. Cyclosporin A and some analogues have also been shown to rescue membrane targeting of other class II ABCB4 variants (169). However, cyclosporin A cannot be considered as a corrector with a therapeutic potential since it is highly inhibitory of the PC secretory function of ABCB4 (214). Correctors C10 (KM11057), C13 (Corr-4C), and C17 (15jF), as well as the combinations of C3 + C18 (VRT-325 + VRT-534) and C4 + C18 (Corr4a + VRT-534), allowed the rescue of maturation and canalicular localization of four distinct traffic-defective ABCB4 variants, I541F-, I490T, R545H- and L556R-ABCB4. However, such treatments did not permit a rescue of PC secretion activity of these defective ABCB4 variants and were also inhibitory of the activity of wild type ABCB4 (215). *In silico* molecular docking analyses attributed this inhibitory

effect to the potential interaction of these molecules with key residues involved in ABCB4 function (215).

Certain mutated and mistrafficked ABCA3 proteins can be redirected and functionally corrected to wild type levels. This was shown using a phenotypic cell-based assay to identify new drug candidates for ABCA3-specific molecular correction by screening 1,280 FDA-approved small molecules. Among them, cyclosporin A was again identified as a potent corrector, selective for some but not all ABCA3 variants (216). The ABCA3 variants Q215K, A1046E, K1388N or G1421R expressed in A549 cells, abnormally diffusely distributed in the cell, are rescued by the bithiazole correctors C13 and C17 tested among a panel of many CFTR correctors (C2, C4, C13, C14, C17, C18, and VX-809) (163). Correctors C13 and C17 were the most potent compounds to correct these misfolded ABCA3 variants, indicated by processing and intracellular localization restoration. However, the other ABCA3 variant M760R was not rescued by any of the tested CFTR correctors, suggesting different cellular mechanisms lead to ABCA3 misfolding (163). Nevertheless, identification of lead molecules still represents an important step towards pharmacotherapy of ABCA3 misfolding-induced lung disease.

5.2.2. Chemical chaperones

Chemical chaperones are small molecules of two types: osmolytes and hydrophobic chaperones. They are not protein-specific because they are able to promote the correct folding of several ABC transporters but at higher concentrations than pharmacological chaperones (217). They are effective on cellular milieu to induce a better correction of misfolded proteins (218). The most common osmolytes are glycerol, trimethylamine-N-oxide (TMAO) and dimethyl-sulfoxide (DMSO) that can prevent aggregation of partially folded proteins (219). Other chemical chaperones such as 4-phenylbutyrate (4-PBA) and suberoylanilide hydroxamic acid (SAHA) act on reducing ER stress (220) and on heat shock proteins (221). Some ABCA3 mutant proteins are rescued by the chemical chaperone TMAO but neither by DMSO, glycerol, 4-PBA or SAHA (163). 4-PBA was also effective to rescue F508del-CFTR but with minimal clinical benefits for patients (222,223). DMSO and 4-PBA have been reported to rescue variants of several ABC transporters (for a review, see (124)). 4-PBA also rescues defective ABCB4 variants (224) with encouraging results obtained with PFIC2 patients having class II ABCB11 variants (225–227).

5.2.3. Proteostasis modulators

Proteostasis regulators represent a promising group of molecules, although less well characterized compared to pharmacochaperones. They do not act directly on the mutated misfolded proteins but rather target classical proteostatic regulatory pathways like unfolded protein response and the heat shock response, and therefore have the potential to correct the phenotype of several misfolded proteins (228,229). New regulators of folding-trafficking defects for some ABC transporters have been discovered. In the case of CF, such molecules have been identified through screening campaigns as well as by rational approaches to target chaperones and/or glycosylation enzymes involved in proteostasis and have shown promising correction effects *in vitro* (230–232). One of these molecules, roscovitine, also known as Seliciclib© or CYC202, is a 2,6,9-trisubstituted purine that was identified as a potent and selective cyclin-dependent kinase inhibitor (233,234). Roscovitine has undergone numerous studies in many indications up to clinical phase trials in various cancers, rheumatoid arthritis, glaucoma and cystic fibrosis (235). Roscovitine partially corrects F508del-CFTR trafficking and function by promoting the apical membrane location of the mutated variant through a mechanism of action independent of kinase inhibition (236). Roscovitine and its analogues MRT2-235, MRT2-237 and MRT2-243 also corrected the localization at the plasma membrane, maturation and function of three class II ER-retained ABCB4 variants, namely

I541F, I490T and L556R (237). Therefore, such agents should be considered as therapeutic means for severe biliary diseases caused by this class of variants.

The nonsteroidal anti-inflammatory drug ibuprofen (238) and glafenine (239) partially correct F508del-CFTR trafficking. Glafenine rescues the CFTR variants F508del-, G85E- and N1303K via cyclooxygenase 2 inhibition of the arachidonic acid pathway (239). However, glafenine has been clinically discontinued due to hepatoxicity (240).

Other molecules target ABCB4 and ABCB11 variants. This is the case of UDCA (Ursodiol^{TM}), which is a physiological constituent of human bile, even if much less abundant than in bears (221). The beneficial use of this BA could be explained by a range of mechanisms of action, including cholangiocyte protection against the detergent action of endogenous BA, the induction of bile secretion, as well as anti-inflammatory and anti-fibrotic effects (242). At the molecular level, UDCA and its conjugate tauro-ursodeoxycholic acid (TUDCA) activate signaling cascades including the small GTP-binding protein Ras, ERK1/2 (243,244), but also Src kinase and Ras/Raf to enhance ABCB11 insertion into canalicular apical membranes (245,246). Another pathway can be activated by rifampicin and cholestyramine, inducing CYP3A4 expression to stimulate hepatobiliary transporter systems (247). UDCA and rifampicin treatments have independent but complementary effects. However, although efficient, more than two-thirds of PFIC2 patients and around 50% of PFIC3 patients do not respond to UDCA, highlighting the limitation of these treatments (123,147).

The specificity of their action and suitability of targeting these pathways in patients have not been explored extensively. However, given that signaling pathways have long been utilized as therapeutic targets, careful attention to controlling proteostasis might be a potent and safe way to translate these findings to patients.

5.2.4. Low temperature

Denning and colleagues studied the molecular defect of F508del-CFTR and provided the first evidence in favor of the temperature sensitivity of misfolded proteins (71). Indeed, when cells expressing misfolded ABC proteins are cultured 24h at low temperature (27°-30°C), the class II ER-retained ABC variant reaches its final destination. Then, low temperature was also shown to restore processing and subcellular localization of ABCA3, ABCB4 and ABCB11 variants (163,170,248–250). Although the mechanism underlying this effect on misfolded proteins is not completely solved, it might be related to heat-shock proteins and other molecular thermo-sensitive chaperones.

5.3. Pharmacological approach for class III ABC variants

Potentiators have been developed to rescue defective ABC transport function. They are classified as types P1 and P2 according to their distinct binding sites.

5.3.1. P1 potentiators

Kalydeco (ivacaftor/VX-770) is a P1 potentiator approved for treating CF patients with gating variants (251), which correspond to 39 distinct variants (252). Ivacaftor has been shown to rescue the function of more than 15 class III ABCB4 variants (253,254). Ivacaftor also potentiates the function of ABCA3 variants: N568D (from 14% of WT to 114% in presence of ivacaftor of lipid transport activity), F629L (from 12% to 46% of WT) and G667R (2.8 fold increase of lipid transport function with ivacaftor) (164). Ivacaftor also rescues the function of class III ABCB11 variants (T463I and A257V) (175,176). Cryo-EM studies demonstrated that ivacaftor binds to a specific binding site on CFTR at the protein-lipid interface, which involve the transmembrane helices 4, 5 and 8. This binding site corresponds to a region of transmembrane helix 8 that plays a crucial role in channel opening (251). More recently, using photoactivatable probes, the effect of ivacaftor on CFTR has also been shown to be mediated by another binding site located in the intracellular loop connecting NBD1 to TMD2 (255). The extent to which this mechanism of action is shared with other ABC

transporters remains unknown. Since cryo-EM studies demonstrated that ivacaftor binds to the central cavity of ABCB1 (256), which shares important sequence identity with ABCB4, we may speculate a similar interaction of ivacaftor with the phospholipid transporter. However, further studies will be required to fully elucidate the binding process and mode of action of ivacaftor on ABCA3, ABCB4 and ABCB11.
The AbbVie/Galapagos CFTR potentiators -1837, -3067, -2451, P2, P3 and P5 have potential binding sites spread through the ABC transporter sequence, including MSD1, MSD2, NBD1, NBD2 and lasso domain (257,258). Other CFTR potentiators are in clinical assays like QBW251 (Novartis) or CTP-656 (Vertex Pharmaceuticals Inc.) (both in phase 2, NCT02190604 and NCT02971839).

5.3.2. P2 potentiators

P2 potentiators include ASP-11 (arysolfonamide-pyrrolopyridin) and the flavonoid apigenin (259). The latter is also called co-potentiator since it acts in synergy with a P1 potentiator on two distinct binding sites to activate CFTR channels (260). Even if binding sites of P2 potentiator remain to be identified, *in silico* studies on genistein, a CFTR co-potentiator, suggest the binding of genistein to the LSGGQ signature motif at the NBD1-NBD2 dimer interface (261). Genistein was also effective in some ABCA3 variants (164). The P2 potentiator ASP-11 allows an 8-fold increase over VX-770 alone for the CFTR variants N1303K and W1282X; and a 1.5-fold increase for the CFTR variant G551D (259). Recently, the corrector elexacaftor/VX-445, was also shown to increase CFTR activity (262,263). Thus, VX-445 may also be a P2 co-potentiator acting in synergy with VX-770 (P1 potentiator) to restore the activity of class II and III variants. Indeed, VX-445 enables a 2-fold increase of VX-770 effect and in return, the presence of VX-770 allows a 4-fold increase of VX-445 response (264).

6. Conclusions

In this review, we proposed a unified classification of the genetic variants of ABC transporters according to their molecular defects such as expression, traffic to the membrane, transport function and/or stability at the membrane, which may be considered as a general guideline for all ABC transporters' variants. In fact, in each field, classification systems already exist but regardless of the classification used, the main pitfalls are the experimental biases. The *in vitro* assays performed to characterize variants may not fully recapitulate the physiological conditions or cellular contexts in which these transporters operate. This is particularly true for recessive disorders where patients are compound heterozygotes. Altogether, this adds an additional layer of complexity to the results and could potentially introduce biases in the classification. Indeed, with reference to the experimental data, classification is not as straightforward as we might wish. Therefore, standardized experimental protocols and collaborative efforts to share data and harmonize classification criteria could help reduce biases and improve the accuracy of variant classification. By acknowledging these limitations and potential biases, we can better appreciate the challenges involved in developing a robust and universally applicable classification system for ABC transporter variants and identify areas for future improvement.

We also discussed recent progress in the field of targeted pharmacotherapy, to correct specific molecular defects using small molecules, opening the path to treatment repurposing for diseases involving similar deficiencies in other ABC transporters. Like ABCC7/CFTR, therapeutic strategies targeting ABC transporter variants will be able to benefit from the first generation of molecules such as the correctors lumacaftor (VX-809), Trikafta/Kaftrio (VX-445/VX-661/VX-770) or the potentiator ivacaftor (VX-770). While chemical chaperones are

non-specific and of limited clinical interest, proteostasis modulators represent an interesting group of molecules that will need to be further explored before clinical evaluation.

Based on CFTR natural history of variant classification, we can also anticipate that a variant will cumulate several defects and thus be attributed to different classes (265) (Figure 4). This is illustrated by the F508del-CFTR, the leader of the defective processing class II mutants, but then further defined as belonging to three classes (adding a gating defect and an accelerated protein turnover, see Figure 4), when the deciphering of molecular mechanisms was refined. If a variant belongs to multiple classes, the appropriate strategy would be to combine therapeutic approaches adapted for each single defect triggered by the variant. This is illustrated by the clinical success of the triple combination Trikafta/Kaftrio (VX-445/VX-661/VX-770) that associates two correctors of different types and a potent potentiator, as demonstrated *in vitro* and *in vivo* (266,267).

While molecules active on CFTR are currently considered in the field of other ABC transporters involved in monogenic diseases, their mechanisms of action (corrector or potentiator) need to be better understood or at least tested on the ABC transporters of interest. Therefore, it is necessary to develop efficient tests to identify or repurpose molecules through a class-by-class approach using customized high-throughput screening. The challenge with rare and very rare diseases is that the models for high-throughput screening often need to be tailored to individual cases. This makes it difficult to draw broader conclusions. Developing innovative screening methods that can be adapted to individual variants will be essential such as structure-based drug design, which relies on the development of accurate 3D structures of ABC transporters. Our challenge ahead is thus significant but the path has been opened during the last two decades, in particular by studies on CFTR and other proteins, such as G-protein coupled receptors (268). With ongoing collaborative research, we should be able to propose new therapies for pathologies caused by ABC transporter variants, ultimately improving patient outcomes.

Conflict of interest

The authors have no conflict to declare.

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Authors' contributions

P.F., F.B. and T.F.: Conceptualization, Writing - Review & Editing. M.L, M.O. and T.C.: Writing - Original Draft, Visualization. All authors read and approved the final manuscript.

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Legends to figures

Figure 1. Membrane topology of human ABC transporters. The different membrane topologies of human ABC transporters are represented. Note the presence of large extracellular domains for the ABCA subfamily as well as an additional TMD0 at the N-terminus of several transporters of the ABCB and ABCC subfamilies. The soluble ABCE and ABCF subfamilies are not represented here. NBD: nucleotide-binding domain; TMD: transmembrane domain; R: R domain. See text for details.

Figure 2. Structures of ABC transporters discussed in this review. PDB accession codes (https://www.rcsb.org/) are indicated above and correspond to cryo-EM structures that have been resolved in ATP-bound conformation. Gray areas represent membrane bilayers. Dashed lines indicate unresolved parts of the proteins.

Figure 3. Refined global classification of ABC transporter variants based on their cellular defect and potential therapeutic strategies.

Figure 4. Frameshift and nonsense variants are classified as class 0 and I respectively. Missense variants are classified as classes II, III and/or IV, with some selected examples. Multiple possible combinations of ABC variants with some chosen examples overrepresented by CFTR, which is the most studied ABC transporter, so far.

Table 1. ABC transporters and related human diseases.

Gene	Function	Genetic disease (OMIM* numbers and links)
ABCA1	Cholesterol and phospholipid efflux	Tangier disease (#205400)
ABCA3	Phospholipid efflux	Surfactant metabolism dysfunction-3 (#610921) (RDS, ILD)
ABCA4	Import of all-trans-retinal aldehyde across the photoreceptor cell membrane	Stargardt disease (#248200)
ABCA12	Lipid transport via lamellar granules	Recessive congenital ichthyosis (#242500 and #601277)
ABCB2	Antigen processing for MHC-I presentation	HLA class I deficiency (#604571)
ABCB3	Antigen processing for MHC-I presentation	HLA class I deficiency (#604571)
ABCB4	Phosphatidylcholine floppase	PFIC3 (#602347), LPAC (#600803), ICP (#614972)
ABCB7	Export heme from the mitochondria to the cytosol	X-linked sideroblastic anemia with ataxia (#301310)
ABCB11	Bile salt exporter	PFIC2 (#601847), BRIC2 (#605479), ICP
ABCC2	Anionic compounds / Drugs (implicated in multidrug resistance)	Dubin-Johnson syndrome (#237500)
ABCC6	Adenosine-triphosphate cellular efflux (still debated)	Pseudoxanthoma elasticum (#264800)
CFTR (ABCC7)	Chloride and bicarbonate channel	Cystic fibrosis (#219700), congenital bilateral absence of vas deferens (#277180)

ABCC8	Modulator of ATP-sensitive potassium channels and insulin release	Familial hyperinsulinemic hypoglycemia-1 (#256450)
ABCC9	Drug-binding channel-modulating subunit of the extra-pancreatic ATP-sensitive potassium channels	Cantú syndrome (#239850), dilated cardiomyopathy (#608569)
ABCD1	Peroxisomal import of very long chain fatty acids	X-linked adrenoleukodystrophy (#300100)
ABCD3	Peroxisomal import of fatty acids	Congenital bile acid synthesis defect 5 (#616278)
ABCG5	Sterol excretion	Sitosterolemia type 2 (#618666)
ABCG8	Sterol excretion	Sitosterolemia type 1 (#210250)

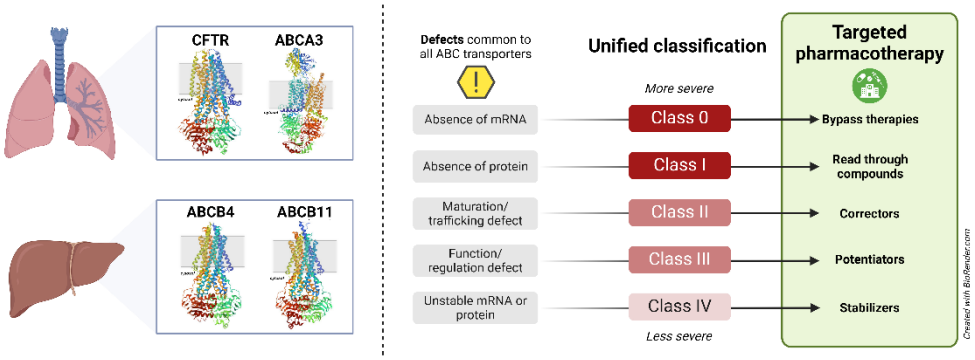
*OMIM: Online Catalog of Human Genes and Genetic Disorders.

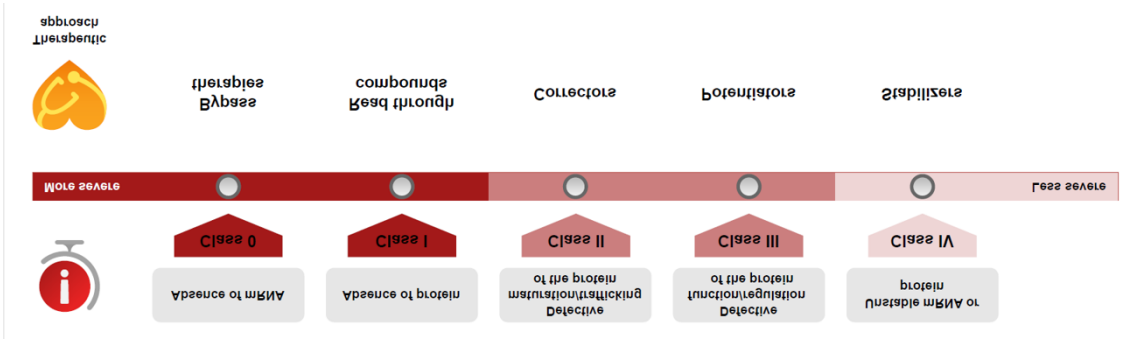
RDS: Respiratory Distress Syndrome; ILD: Interstitial Lung Diseases; HI: harlequin ichthyosis, CIE: congenital ichthyosiform erythroderma, LI: Lamellar Ichthyosis, LPAC: Low Phospholipid-Associated Cholelithiasis syndrome; ICP: Intrahepatic Cholestasis of Pregnancy; PFIC2/3: Familial Progressive Intrahepatic Cholestasis type 2/3.

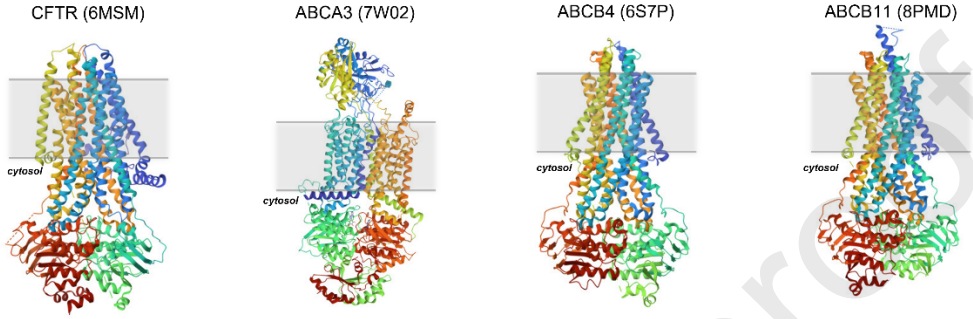
Table 2. ABC transporters discussed in this article and associated diseases.

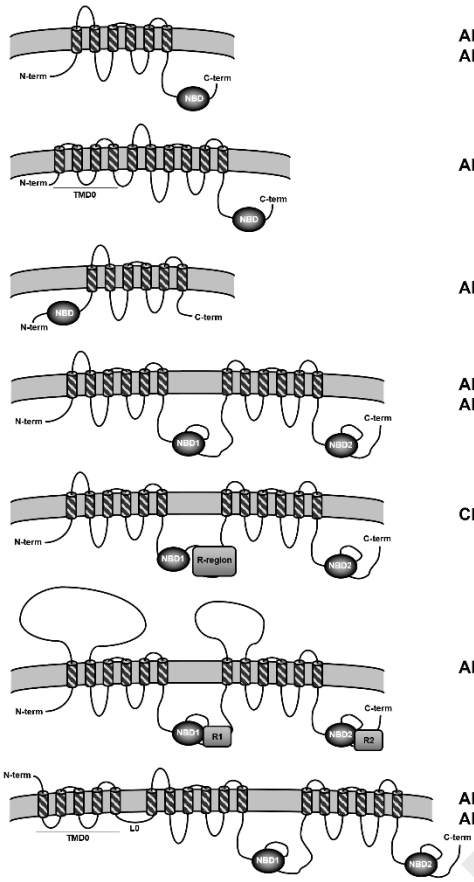
		ABCC7/CFTR	ABCA3	ABCB4/MDR3	ABCB11/BSEP
Gene/Protein	Gene location (number of exons)	7q31.2 (27)	16p13.3 (33)	7q21.12 (34)	2q31.1 (30)
	Genomic sequence (RefSeq)	NG_016465.4	NG_011790.2	NG_007118.3	NG_007374.2
	Main protein expression	Epithelial cells (lung, pancreas)	Pneumocytes type II	Hepatocytes	Hepatocytes
	Protein function	Chloride channel	PC and PG floppase	PC floppase	Bile salt exporter
Associated diseases	Disease names	Cystic fibrosis CBAVD[1] CFTR-related disorders	RDS[2] ILD[3]	PFIC3[4] LPAC[5] syndrome ICP[6]	PFIC2[4] ICP[6] BRIC[7]
	Etiology	Loss of function variants	Loss of function variants	Loss of function variants	Loss of function variants
	Inheritance	Autosomal recessive	Autosomal recessive	Autosomal recessive	Autosomal recessive
	Age of onset	Birth-adulthood	Birth-adulthood	Birth-adulthood	Birth-adulthood

[1]Congenital bilateral agenesis of the vas deferens; [2]Respiratory Distress Syndrome; [3]ILD: Interstitial Lung Diseases; [4]Progressive familial intrahepatic cholestasis types 2/3; [5]Low phospholipid-associated cholelithiasis; [6]Intrahepatic cholestasis of pregnancy; [7]Benign recurrent intrahepatic cholestasis.









ABCB7, ABCB8, ABCB10
ABCD1, ABCD2, ABCD3, ABCD4

ABCB2, ABCB3, ABCB6, ABCB9

ABCG subfamily

ABCB1, ABCB4, ABCB5, ABCB11
ABCC4, ABCC5, ABCC11

CFTR (ABCC7)

ABCA subfamily

ABCC1, ABCC2, ABCC3, ABCC6,
ABCC8, ABCC9, ABCC10