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REVIEW

Mammalian peroxisomal ABC transporters: from endogenous substrates to pathology and clinical significance

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Peroxisomes are indispensable organelles in higher eukaryotes. They are essential for a number of important metabolic pathways, including fatty acid α- and β-oxidation, and biosynthesis of etherphospholipids and bile acids. However, the peroxisomal membrane forms an impermeable barrier to these metabolites. Therefore, peroxisomes need specific transporter proteins to transfer these metabolites across their membranes. The mammalian peroxisomal membrane harbours three ATP-binding cassette (ABC) transporters. In recent years, significant progress has been made in unravelling the functions of these ABC transporters. There is ample evidence that they are involved in the transport of very long-chain fatty acids, pristanic acid, di- and trihydroxycholestanoic acid, dicarboxylic acids and tetracosahexaenoic acid (C24:6ω3). Surprisingly, only one disease is associated with a deficiency of a peroxisomal ABC transporter. Mutations in the *ABCD1* gene encoding the peroxisomal ABC transporter adrenoleukodystrophy protein are the cause for X-linked adrenoleukodystrophy, an inherited metabolic storage disorder. This review describes the current state of knowledge on the mammalian peroxisomal ABC transporters with a particular focus on their function in metabolite transport.

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Abbreviations

ABC, ATP-binding cassette; AMN, adrenomyeloneuropathy; ALDP, adrenoleukodystrophy protein; ALDRP, adrenoleukodystrophy-related protein; CCALD, childhood cerebral adrenoleukodystrophy; DCA, dicarboxylic acids; DHA, docosahexaenoic acid; DHCA, dihydroxycholestanoic acid; ELOVL, elongation of very-long-chain-fatty acids; EST, expressed sequence tag; PEX, peroxin; PMP70, 70 kDa peroxisomal membrane protein; SNP, single nucleotide polymorphism; SRE, sterol regulatory element; SREBP, sterol regulatory element binding protein; THCA, trihydroxycholestanoic acid; VLCFA, very long-chain fatty acids; X-ALD, X-linked adrenoleukodystrophy

Introduction

Mammalian peroxisomes contain a matrix consisting mainly of soluble proteins that is surrounded by a single membrane. Peroxisomes are present in virtually all eukaryotic cells. They are essential for a number of important metabolic pathways, including fatty acid α- and β-oxidation, biosynthesis of etherphospholipids and bile acids, and the degradation of purines, amino acids and polyamines (Wanders and Waterham, 2006). PXMP2, a channel-forming protein in the mammalian peroxisomal membrane, allows the transmembrane passage of small solute molecules with a molecular mass below 400 Da (Rokka *et al.*, 2009). However, the membrane forms an impermeable barrier for 'bulky' solute molecules (>400 Da) requiring selective transporters for membrane passage (Antonenkov and Hiltunen, 2006; Antonenkov *et al.*, 2010). The mammalian peroxisomal membrane harbours three ATP-binding cassette (ABC) transporters. ABC transporters are transmembrane proteins that bind and hydrolyse ATP and use the energy to drive the transport of various molecules across cellular membranes often against steep concentration gradients (Higgins, 1992). The peroxisomal ABC transporters belong to subclass D of the ABC protein superfamily and are referred to as ABCD1/ALDP (Mosser *et al.*, 1993),

Table 1

Significant features of the ABCD family

	ABCD1	ABCD2	ABCD3	ABCD4
UniGene	Hs.159546	Hs.117852	Hs.700576	Hs.94395
Chromosome	Xq28	12q11-12	1p22-p21	14q24.3
Gene size	19.9 kb	68.8 kb	100.3 kb	17.8 kb
Exons	10	10	23	19
Protein (size)	ALDP (745 aa)	ALDRP (740 aa)	PMP70 (659 aa)	PMP69 (606 aa)
Subcellular localization	Peroxisomal membrane	Peroxisomal membrane	Peroxisomal membrane	ER membrane
Function	VLCFA transport	C24:6ω3 (hypothesized)	pristanic acid DHCA/THCA dicarboxylic acids (hypothesized)	unknown

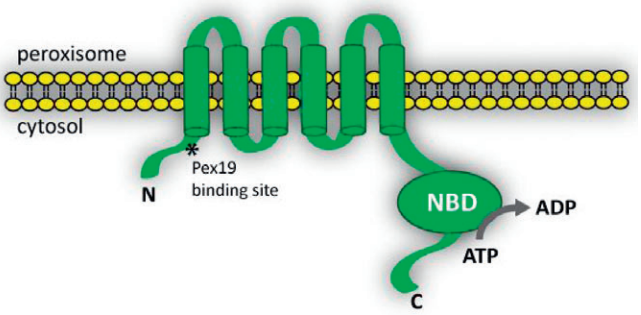

Figure 1

Hypothesized structure of the peroxisomal ABC half-transporters. The nucleotide binding domain (NBD) and Pex19p binding site (*) are indicated.

ABCD2/ALDRP (Lombard-Platet *et al.*, 1996) and ABCD3/PMP70 (Kamijo *et al.*, 1990) (Table 1, Figure 1). The ABCD4 (PMP69 or P70R) protein is also a member of the ABCD family (Holzinger *et al.*, 1997; Shani *et al.*, 1997), but it was recently demonstrated to be localized to the endoplasmic reticulum membrane (Kashiwayama *et al.*, 2009) and will therefore not be discussed in this review. It is hypothesized that the peroxisomal ABCD proteins form dimeric transporters involved in the movement of metabolites across the peroxisomal membrane. Recent publications have provided insight into the nature of the substrates handled by the peroxisomal ABC transporters. This review describes the current state of knowledge on the mammalian peroxisomal ABC transporters.

Pathology and clinical significance

Human diseases

ABCD1. Mutations in the *ABCD1* gene result in X-linked adrenoleukodystrophy (X-ALD: MIM 300100) (Mosser *et al.*, 1993), an inherited metabolic storage disorder. A defect in the peroxisomal ABC transporter, adrenoleukodystrophy protein (ALDP) results in impaired peroxisomal β-oxidation and accumulation of very long-chain fatty acids (VLCFA; >C22:0) in tissues, especially the brain and the adrenal glands (Moser *et al.*, 2001). X-ALD is the most common peroxisomal disorder with a birth incidence of 1:17.000 (Bezman *et al.*, 2001). The disease includes several clinically distinct phenotypes that are categorized by the age of onset, the rate of disease progression and the site of initial pathology (Moser *et al.*, 2001). Childhood cerebral adrenoleukodystrophy (CCALD) affects boys aged 3 to 10 years and is characterized by progressive cerebral demyelination with an inflammatory response in the white matter that results in a vegetative state or death within 2 to 5 years after the onset of symptoms. Adrenomyeloneuropathy (AMN) is more slowly progressive and presents in adulthood (3rd to 4th decade) with progressive myelopathy and peripheral neuropathy. Patients with AMN can also develop secondary cerebral demyelination ('AMN cerebral') (van Geel *et al.*, 2001). Primary adrenocortical insufficiency can occur not only in isolation ('Addison only' phenotype), but also in combination with any of the other phenotypes. The same is true for testicular insufficiency.

Remarkably, there is no genotype–phenotype correlation: in spite of identical mutations, patients can have markedly divergent neurological and neuropathological characteristics (Berger *et al.*, 1994; Kemp *et al.*, 1994). Furthermore, the clinical outcome for the individual patient is currently unpredictable. Mutation analyses have been performed in relatively large series of patients representing various ethnic groups (Ligtenberg *et al.*, 1995; Feigenbaum *et al.*, 1996; Smith *et al.*, 1999; Shimozawa *et al.*, 2010). Mutations have been identified in all X-ALD patients in whom the entire gene was analysed. No promoter mutations have been reported. As of March 2011, the X-ALD database (http://www.x-ald.nl) lists more than 1200 mutations. Of these 1200 mutations, 61% are missense mutations, 22% are frame shifts, 10% are nonsense, 4% are amino acid insertions or deletions and 3% are large deletions of one or more exons. The majority of X-ALD kindreds have a unique mutation; 570 non-recurrent mutations have been identified.

ABCD2. So far, no disease has been associated with mutations in the *ABCD2* gene.

ABCD3. In 1992, two patients with a peroxisomal biogenesis disorder (PBD) referred to as Zellweger syndrome were reported with mutations in the *ABCD3* gene (Gärtner *et al*., 1992). From these findings, the authors concluded that PMP70 plays an important role in peroxisome biogenesis and that mutations in the *ABCD3* gene can cause Zellweger syndrome. Later work revealed that these patients belonged to complementation group 1 (CG1) of the Zellweger syndrome spectrum. Shimozawa *et al*. demonstrated that overexpression of PMP70 cDNA in fibroblasts from seven different PBD-CG1 patients did not restore the assembly of peroxisomes suggesting that the *ABCD3* mutations were unlikely to contribute to the disease phenotype in these patients (Shimozawa *et al*., 1996). Subsequent genetic analysis of the *PEX1* gene by Collins and Gould resulted in the identification of mutations in the *PEX1* gene in both Zellweger patients (Collins and Gould, 1999). It is now well established that Zellweger syndrome is not caused by mutations in *ABCD3* but by a defect in any of at least 12 different *PEX* genes (Ebberink *et al*., 2011). The *PEX* genes encode proteins that are called peroxins and that are involved in various stages of peroxisomal protein import and/or the biogenesis of peroxisomes (Platta and Erdmann, 2007). Thus far, no disease has been associated with mutations in the *ABCD3* gene.

Mouse models

ABCD1. In 1997, three laboratories independently developed a mouse model for X-ALD (Forss-Petter *et al*., 1997; Kobayashi *et al*., 1997; Lu *et al*., 1997). *Abcd1* knockout mice display biochemical abnormalities similar to those found in X-ALD patients. Mutant mice have decreased VLCFA β-oxidation in fibroblasts, hepatocytes (Yamada *et al*., 2000) and tissues (Fourcade *et al*., 2009), and they accumulate VLCFA in organs affected in X-ALD patients including: brain, spinal cord, adrenal glands and testis (Forss-Petter *et al*., 1997; Kobayashi *et al*., 1997; Lu *et al*., 1997). *Abcd1* knockout mice, however, do not develop symptoms of cerebral demyelination. Furthermore, neuropathological analysis of their brains revealed neither inflammatory infiltrates nor white matter alterations (Pujol *et al*., 2002; Dumser *et al*., 2007). Instead, X-ALD mice develop a late onset, progressive neurodegenerative phenotype that resembles the AMN phenotype in patients. Compared to wild-type littermates, the spinal cord of over 18-month-old X-ALD mice revealed increased axonal degeneration and myelin anomalies. Twenty-one-month-old mutant mice show nerve conduction deficits and myelin abnormalities of peripheral (sciatic) nerves and their motor coordination is significantly impaired as demonstrated by the rotarod test (Pujol *et al*., 2002).

ABCD2. An *Abcd2* knockout mouse model has been generated and characterized (Ferrer *et al*., 2005). *Abcd2* knockout mice develop a late-onset cerebellar and sensory ataxia associated with loss of cerebellar Purkinje cells, degeneration of dorsal root ganglia cells and axonal degeneration in the dorsal and lateral columns of the spinal cord (Ferrer *et al*., 2005). In the adrenal gland, *Abcd2*-deficient mice show signs of oxidative damage as demonstrated by an acceleration in the deposition of ceroid, an end-product of oxidative damage (Lu *et al*., 2007).

ABCD3. An *Abcd3*-deficient mouse model has been generated, but unfortunately the findings have not been published in great detail. The only information available comes from abstracts presented at several scientific meetings. *Abcd3* knockout mice are viable. They develop a non-shivering thermogenesis defect that may be related to a disturbance in their fasting fuel homeostasis. In the fed state, *Abcd3* knockout mice show a 10-fold reduction in hepatic glycogen levels compared to wild-type mice (Jimenez-Sanchez *et al*., 1998). As there was no difference between knockouts and wild-type mice in the amount and activity of glycogen synthase, this reduction may reflect an increase in the glycogen utilization in *Abcd3*-deficient mice. Mutant mice also show a striking dicarboxylic aciduria in both the fed and the fasted states (Jimenez-Sanchez *et al*., 1998; Jimenez-Sanchez *et al*., 2000).

Endogenous substrates

ABCD1/ALDP

A defect in ALDP impairs peroxisomal β-oxidation of VLCFA (Singh *et al*., 1984) resulting in elevated plasma and tissue levels of saturated straight chain VLCFA (C24:0 and C26:0) (Moser *et al*., 1999) and monounsaturated VLCFA (C26:1) (Valianpour *et al*., 2003). Complementation studies demonstrated that expression of normal *ABCD1* cDNA in X-ALD patient fibroblasts restored VLCFA β-oxidation (Shinnoh *et al*., 1995) and reduced VLCFA to normal levels (Cartier *et al*., 1995). Based on these findings, it has long been hypothesized that ALDP transports VLCFA across the peroxisomal membrane. Only recently, it has been demonstrated that ALDP indeed transports VLCFacyl-CoA across the peroxisomal membrane (van Roermund *et al*., 2008). In yeast, the peroxisomal ABC transporters, Pxa1p and Pxa2p are the orthologs of the human ABCD proteins. Disruption of either Pxa1p or Pxa2p results in the deficient peroxisomal β-oxidation of long-chain fatty acids such as oleate, but does not impair medium chain fatty acid β-oxidation (Hettema *et al*., 1996). The Pxa1p/Pxa2p heterodimer is involved in the transport of a spectrum of acyl-CoA esters across the peroxisomal membrane (Verleur *et al*., 1997; van Roermund *et al*., 2008). Expression of ALDP in the *pxa1 pxa2Δ* double mutant rescued the mutant phenotype which implies that ALDP is involved in the transport of acyl-CoA esters across the peroxisomal membrane (van Roermund *et al*., 2008). Using fibroblasts from X-ALD patients, Ofman *et al*. demonstrated that a deficiency in ALDP results in a strong elevation in the levels of VLCFacyl-CoA esters which points to a VLCFacyl-CoA transport deficiency in X-ALD (Ofman *et al*., 2010). These data establish that ALDP most likely transports VLCFacyl-CoA into peroxisomes (Figure 2) and that ALDP deficiency in X-ALD has two major effects: it impairs peroxisomal VLCFA β-oxidation and it raises cytosolic VLCFacyl-CoA levels (Ofman *et al*., 2010). These VLCFacyl-CoA esters are substrate for further elongation to even longer fatty acids by ELOVL1, the human C26 specific elongase (Kemp and Wanders, 2010; Ofman *et al*., 2010).

ABCD2/ALDRP

Several lines of evidence point to a function for ALDRP in the synthesis of the poly-unsaturated fatty acid docosohexaenoic

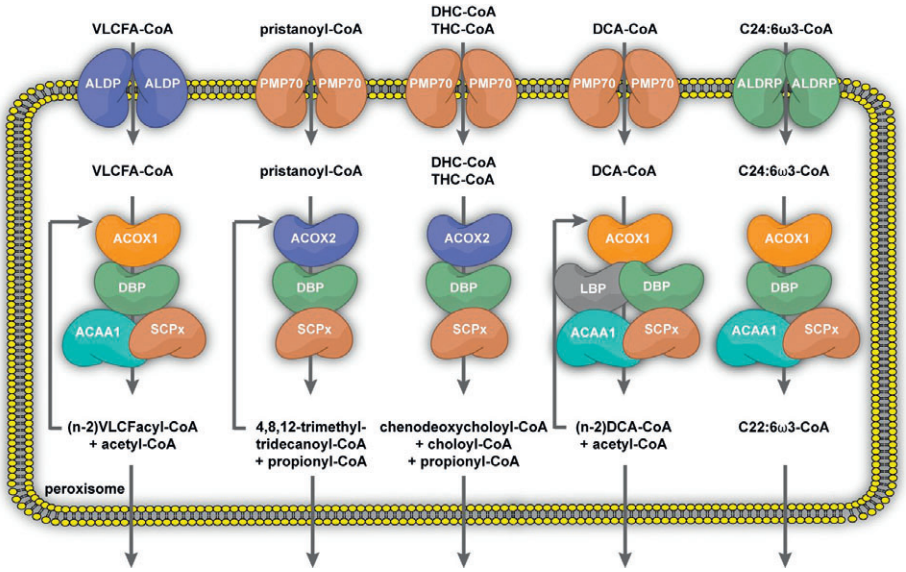

Figure 2

Schematic overview of the function of the mammalian peroxisomal ABC transporters in the import of VLCFA, pristanic acid, di- and trihydroxycholestanoic acid (DHCA and THCA), dicarboxylic acid (DCA) and tetracosahexaenoic acid (C24:6ω3) into the peroxisome. Peroxisomes contain the full enzymatic machinery to β-oxidize fatty acids, although not to completion. The enzymes involved include: two acyl-CoA oxidases (ACOX1 and ACOX2), catalysing the first step, two bifunctional proteins (LBP and DBP), catalysing the second and third step, and two peroxisomal thiolases (ACAA1 and SCPx), catalysing the last step. For complete oxidation, the end-products must be shuttled to mitochondria. There are two mechanisms by which the end-products of peroxisomal β-oxidation can be shuttled to mitochondria. The first mechanism involves four steps: (i) hydrolysis of the different CoA-esters to produce short-chain fatty acids; (ii) transfer across the peroxisomal and mitochondrial membrane probably in protonated form; (iii) activation to the corresponding CoA-esters in the mitochondrion; and (iv) further oxidation. The second mechanism involves: (i) conversion of the CoA-esters into their corresponding carnitine-esters within peroxisomes; (ii) transfer across the peroxisomal membrane, probably via a yet unidentified carrier; (iii) import into to mitochondrial matrix via the mitochondrial carnitine/acylcarnitine translocase (CACT, SLC25A20); and (iv) further oxidation. In liver peroxisomes, the bile acid intermediates THCA and DHCA undergo one cycle of β-oxidation with choloyl-CoA and chenodeoxycholoyl-CoA as end-products respectively. These two CoA-esters are then conjugated with either taurine or glycine. Subsequently, the taurine- and glycine-esters are transported out of the peroxisome into the cytosol by an unknown mechanism, followed by transport across the canalicular membrane via the bile salt efflux pump, that is, BSEP (*ABCB11*), to end up in bile. Figure adapted and modified from Wanders *et al.* (2010).

acid (DHA, C22:6ω3) which requires chain-shortening of C24:6ω3 by peroxisomal β-oxidation (Figure 2). *Abcd2* knockout mice have reduced levels of C22:6ω3 in primary neurons (Fourcade *et al.*, 2009). Experiments measuring peroxisomal β-oxidation capacity using cortical brain slices of wild-type and *Abcd2*-deficient mice revealed reduced C24:6ω3 β-oxidation capacity in *Abcd2* knockout mice (Fourcade *et al.*, 2009). Recent studies in yeast confirmed these data. Expression of human ALDRP in the *pxa1 pxa2Δ* double knockout yeast resulted in the increased capacity of peroxisomes to β-oxidize C24:6ω3 (van Roermund *et al.*, 2011).

ABCD3/PMP70

PMP70 appears to be involved in the transport of 2-methylacyl-CoA esters including pristanoyl-CoA, dihydroxycholestanoyl-CoA (DHC-CoA) and trihydroxycholestanoyl-CoA (THC-CoA) and of medium chain dicarboxylic acids presumably as CoA-esters (Jimenez-Sanchez *et al.*, 2000; Wanders *et al.*, 2001) (Figure 2). When *Abcd3* knockout mice were fed a phytol-supplemented chow, their plasma phytanic and pristanic acid levels were 10-times higher than in wild-type mice (Jimenez-Sanchez *et al.*, 2000). Compared to control fibroblasts, fibroblasts derived from the *Abcd3* knockout mouse showed a 50% reduction in the peroxisomal phytanic and pristanic acid oxidation capacity (Silva-Zolezzi *et al.*, 2004). *Abcd3* knockout mice accumulate the bile acid precursors THCA (trihydroxycholestanoic acid) and DHCA (dihydroxycholestanoic acid) in plasma (P. Vreken *et al.*, unpublished work). Furthermore, analysis of urinary organic acids showed a striking medium chain dicarboxylic aciduria that increased during fasting (Jimenez-Sanchez *et al.*, 2000).

Functional redundancy among peroxisomal ABC transporters

While it becomes increasingly evident that each of the peroxisomal ABC transporters has a unique function, both *in vitro* and *in vivo* studies have clearly established at least a partial functional redundancy among these transporters. First, over-expression of PMP70 in X-ALD patient fibroblasts partially corrected peroxisomal VLCFA β-oxidation capacity (Kemp *et al.*, 1998). Second, over-expression of ALDRP in human and mouse X-ALD fibroblasts completely restored VLCFA β-oxidation and normalized VLCFA levels (Kemp

et al., 1998; Netik *et al.*, 1999). Third, although *Abcd1* knockout mice have elevated VLCFA levels and develop clinical signs at the age of 18–20 months (Pujol *et al.*, 2002), overexpression of ALDRP in *Abcd1* knockout mice resulted in complete correction of VLCFA levels in the adrenal gland and spinal cord and prevented the onset of a clinical phenotype (Pujol *et al.*, 2004). Finally, comparisons of the ability of ALDP and ALDR to restore β-oxidation of different fatty acids to the yeast *pxa1 pxa2Δ* mutant provide direct evidence for distinct but overlapping substrate specificities (van Roermund *et al.*, 2011).

Tissue distribution

Expression during brain development

During mouse and rat brain development the peroxisomal ABCD proteins show different spatial and temporal expression patterns at the mRNA level. ALDP is expressed in all areas of the embryonic brain. ALDP is most abundant at birth and then its expression gradually decreases during maturation of the brain. The expression of ALDRP is low in the embryonic brain, but it increases strongly after the first post-natal week. PMP70 expression is already high in the embryonic brain and it increases in the post-natal period with a peak in the hippocampus and the cerebellum during the second and third post-natal week (Pollard *et al.*, 1995; Berger *et al.*, 1999).

The UniGene expressed sequence tag (EST) profile database (UniGene, http://www.ncbi.nlm.nih.gov/unigene/) shows a breakdown of ESTs by body sites and developmental stage. This database was used to obtain additional insight into the developmental and tissue expression patterns of ALDP, ALDRP and PMP70. Figure 3 shows the expression pattern during mouse development. PMP70 is expressed at each developmental stage. Interestingly, the expression of ALDP, ALDRP and PMP70 is high in the foetus; then drops in neonates and gradually increases again with aging.

ALDP. Overall, the expression of ALDP is most abundant in the adrenal gland, heart, intestine, kidney, liver, lung, placenta and testis (Berger *et al.*, 1999; Langmann *et al.*, 2003; Hoftberger *et al.*, 2007). In the adult mouse and human brain, ALDP is expressed in astrocytes, microglial cells and a subpopulation of oligodendrocytes located in the corpus callosum, internal capsules and anterior commissure (Fouquet *et al.*, 1997; Troffer-Charlier *et al.*, 1998). ALDP is not expressed in neurons (Fouquet *et al.*, 1997), but it is expressed in Schwann cells (P. Aubourg, pers. comm.). In mouse and human adrenal gland, ALDP is expressed in the zona reticularis and fasciculata of the adrenal cortex (Troffer-Charlier *et al.*, 1998; Hoftberger *et al.*, 2007). At the cellular level, human ALDP is selectively expressed in specific cell types: that is, hypothalamus and basal nucleus of Meynert of the brain, distal tubules of the kidney, sweat glands, hair follicles and fibroblasts of the skin, ganglion cells and epithelium of the colon, Sertoli and Leydig cells of the testis, and adrenocorticotropin-producing cells of the pituitary gland (Hoftberger *et al.*, 2007).

ALDRP. ALDRP is most abundant in adrenal gland, brain, heart, liver, lung and skeletal muscle (Lombard-Platet *et al.*, 1996; Holzinger *et al.*, 1997; Berger *et al.*, 1999). In mouse, ALDRP is strongly expressed in the brain, especially in astrocytes, oligodendrocytes and microglia (Troffer-Charlier *et al.*, 1998). In human brain, ALDRP is weakly expressed in neurons, astrocytes and microglia and is undetectable in oligodendrocytes (Flavigny *et al.*, 1999; Aubourg and Dubois-Dalcq, 2000). In the mouse adrenal gland, ALDRP is expressed in the medulla (Troffer-Charlier *et al.*, 1998).

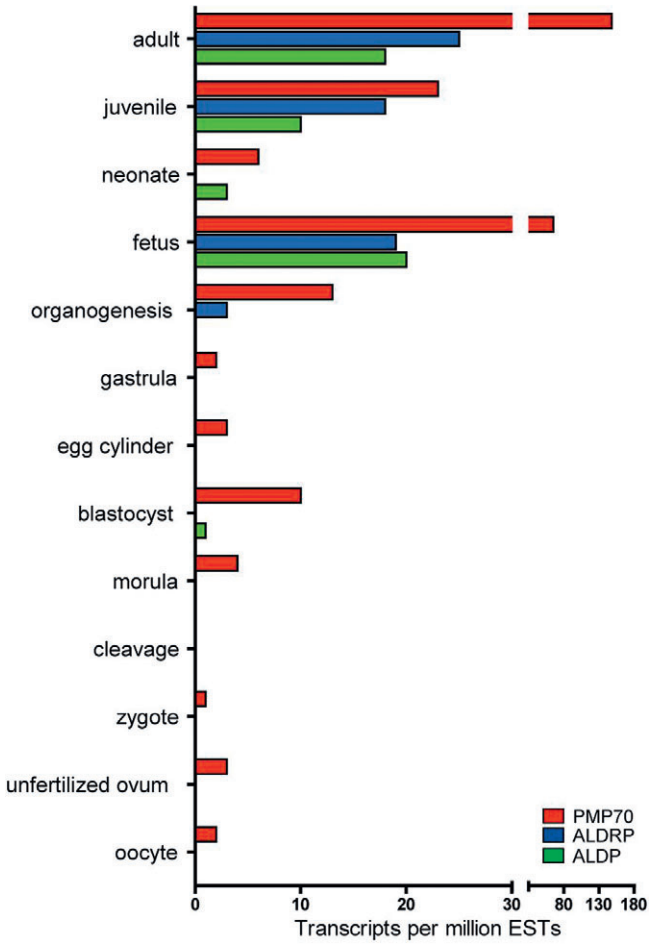

Figure 3

Expression pattern of peroxisomal ABC transporters at different developmental stages in mouse. Data were obtained from the UniGene EST profile database.

PMP70. In mouse, PMP70 shows a more ubiquitous expression pattern with highest expression in liver and kidney (Berger *et al.*, 1999). In the mouse brain, PMP70 expression is detected in both neurons and glial cells (Pollard *et al.*, 1995). The data from the UniGene EST profile database indicate that PMP70 is highly expressed in human thymus, testis and brain (Figure 4).

In Figure 4, the tissue expression pattern of the peroxisomal ABC transporters in mouse and human is compared. Except for mammary gland, testis and pancreas, the expression pattern of ALDP, ALDRP and PMP70 is comparable between human and mouse. Human pancreas shows a high

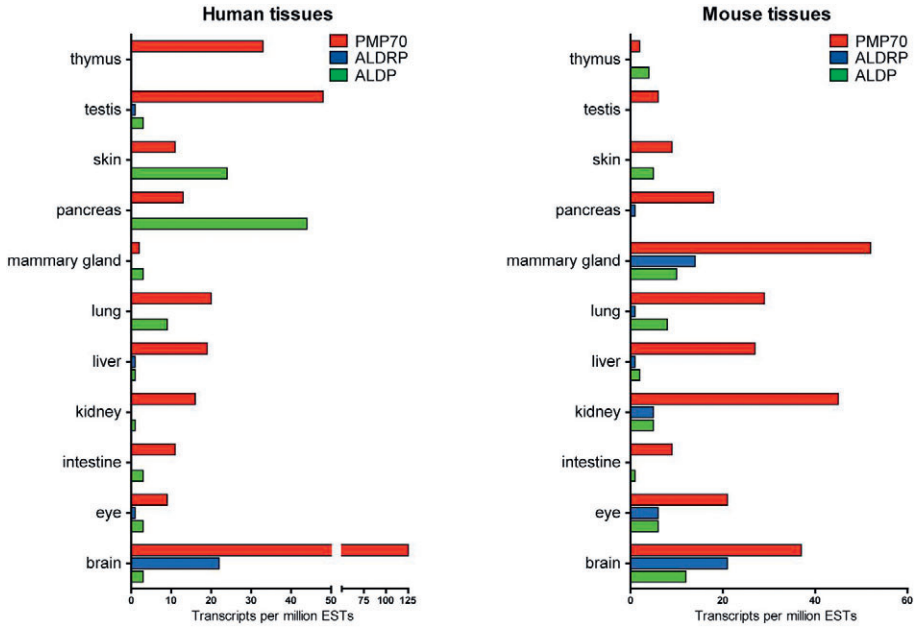

Figure 4

Comparison of the tissue-specific expression of the human and mouse peroxisomal ABC transporters as deduced from the UniGene EST profile database. Only those human and mouse tissues are included for which the total EST number in the pool is higher than 70.000.

ALDP expression, whereas ALDP is not detectable in mouse pancreas. Interestingly, in mouse mammary gland all three transporters are expressed at a high level while in human mammary gland their expression is very low. The species-specific expression patterns of peroxisomal ABC transporters in the mammary gland may suggest that lipid metabolism is differentially regulated between mouse and human.

Subcellular distribution and trafficking

Peroxisomal membrane proteins are encoded by nuclear genes, synthesized on free cytosolic ribosomes and then inserted post-translationally into the peroxisomal membrane (Fujiki *et al.*, 1984). Peroxisomal membrane proteins can be divided into two classes: class I proteins are targeted to peroxisomes via a Pex19p-dependent pathway and class II proteins are targeted to peroxisomes independently of Pex19p (Jones *et al.*, 2004). The trafficking of newly translated peroxisomal ABCD proteins to the peroxisomal membrane is mediated by Pex19p (Gloeckner *et al.*, 2000). Pex19p is a hydrophilic and acidic protein that is bimodally distributed between the cytoplasm and the peroxisomal membrane (Sacksteder *et al.*, 2000). Pex19p functions as a receptor and chaperone, preventing the aggregation and degradation of peroxisomal membrane proteins (Jones *et al.*, 2004; Shibata *et al.*, 2004; Kashiwayama *et al.*, 2005). *In vitro* translation of PMP70 in the presence of purified Pex19p resulted in soluble PMP70 that could be co-immunoprecipitated with Pex19p. However, in the absence of Pex19p, PMP70 formed aggregates during translation (Kashiwayama *et al.*, 2005). The importance of Pex19p for proper peroxisomal targeting was nicely demonstrated by mislocalization experiments with Pex19p. Intentional mislocalization of Pex19p to the nucleus by the introduction of a nuclear targeting signal resulted in the nuclear accumulation of newly synthesized peroxisomal membrane proteins (Sacksteder *et al.*, 2000). Posttranslational modification of Pex19p by farnesylation plays a critical role for the function of Pex19p (Rucktäschel *et al.*, 2009). Farnesylation induces a structural change in Pex19p resulting in a 10-fold increase in the affinity for peroxisomal membrane proteins (Rucktäschel *et al.*, 2009). The sites responsible for Pex19p binding and peroxisome targeting of ALDP, ALDRP and PMP70 have been characterized in some detail. A Pex19p binding site consisting of a 14-amino-acid conserved consensus motif which consists of a cluster of basic and possibly hydrophobic amino acids: [F(F/L)X(R/Q/K)(L/F)(L/I/K)XLLKIL(F/I/V)P] has been identified in the amino-terminal part close to the first transmembrane segment. ABCD proteins that lack this Pex19p binding site are not targeted to the peroxisome (Landgraf *et al.*, 2003) (Figure 1). Deletion of the Pex19p binding site in ALDP (amino acid residues 71–84) abolished peroxisomal targeting. In addition, introduction of disease-causing mutations in the Pex19p binding site of ALDP affected peroxisomal targeting. For example, the missense mutation p.Leu78Phe significantly reduced the targeting efficiency and an in-frame deletion of three amino acids (p.Arg80_Leu82del) resulted in the mislocalization of ALDP to the nucleus, cytosol and mitochondria (Landgraf *et al.*, 2003). The finding that the missense mutation p.Tyr174Cys, which is located in the region between the transmembrane segments 2 and 3 of ALDP, resulted in mistargeting of ALDP to other organelles indicates that this region is also important for the targeting of ALDP to the peroxisome (Takahashi *et al.*, 2007). In ALDRP, a potential Pex19p binding site was identified (Halbach *et al.*, 2005). It encompasses amino acids 84–97

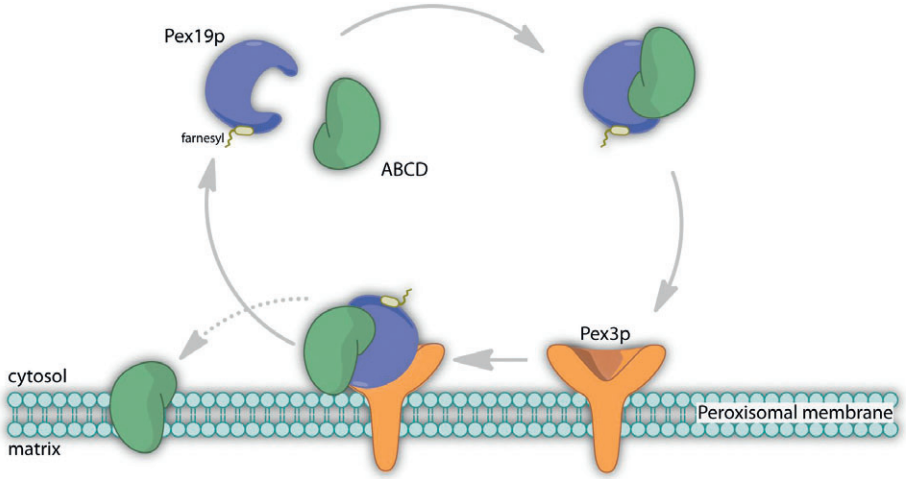

Figure 5

Biogenesis of peroxisomal ABC transporters. After being synthesized on free cytosolic ribosomes, peroxisomal ABC transporters interact with Pex19p through the Pex19p binding motif located in the NH2-terminal region. The interaction is strengthened by sequences located downstream in the transmembrane domain. Pex19p keeps the peroxisomal ABC transporter in a soluble and proper conformation in the cytosol. Then the ABCD-Pex19p complex docks at the peroxisomal membrane via association with its docking factor Pex3p. Finally, the peroxisomal ABC transporter is inserted into the peroxisomal membrane and Pex19p shuttles back to the cytosol to initiate another import cycle. For simplicity only the ABCD monomer is depicted.

which are localized in the proximity of the putative first transmembrane segment. Unfortunately, no experimental data are available yet to support the functionality of this Pex19p binding site. In PMP70, there is evidence for the existence of multiple Pex19p binding sites. The first binding site is located in the proximity of the putative first transmembrane segment (amino acids 63–76) and the second is located around the putative 5th and 6th transmembrane segment (Biermanns and Gartner, 2001; Halbach *et al.*, 2005; Kashiwayama *et al.*, 2005; Kashiwayama *et al.*, 2007). A PMP70 deletion mutant lacking the first 60 amino acids was still correctly targeted to the peroxisome (Biermanns and Gartner, 2001). Recently, Schueller *et al.* crystallized the folded carboxyl-terminal part of Pex19p and demonstrated that it contains a structured α-helical domain that binds to the Pex19p-binding site present in ALDP, ALDRP and PMP70 (Schueller *et al.*, 2010). Upon binding to Pex19p, the ABCD-Pex19p complex docks at the peroxisomal membrane through association with its docking factor Pex3p (Snyder *et al.*, 1999). After Pex3p captures the amino-terminal part of Pex19p, the carboxyl-terminal half of Pex19p, containing the peroxisomal ABC protein, hangs down towards the peroxisomal membrane (Sato *et al.*, 2010). Finally, the ABCD protein is integrated into the peroxisomal membrane. Following the release of its cargo, Pex19p is released into the cytosol to initiate another import cycle (Figure 5). Considering the fact that Pex19p binding sites are present in all three peroxisomal ABCD transporters, it is safe to speculate that the mechanism of peroxisomal targeting and membrane insertion will be the same for ALDP, ALDRP and PMP70.

Recently, it was demonstrated that different types of lipid rafts exist in the peroxisomal membrane (Woudenberg *et al.*, 2010). The authors studied the association of peroxisomal membrane proteins with lipid rafts and provided evidence that ALDP and PMP70 exist in spatially separated lipid rafts. Interestingly, lovastatin-induced cholesterol depletion led to dissociation of peroxisomal membrane proteins from lipid rafts and impaired the sorting of newly synthesized ALDP, but not of PMP70. This indicates that cholesterol depletion results in selective effects on the sorting of peroxisomal proteins, which is most likely a direct effect of the disruption of peroxisomal lipid rafts. Several ABC transporters, including ABCB1, ABCB11, ABCC1, ABCC2 and ABCG2, were shown to reside in lipid rafts and to have their substrate transporting activity regulated by the lipid environment (Klappe *et al.*, 2009; Paulusma *et al.*, 2009). For example, *in vitro* depletion of cholesterol from mouse liver plasma membranes using methyl-β-cyclodextrin demonstrated a near linear relation between cholesterol content of the membranes and ABCB11/ABCC2 transport activity (Paulusma *et al.*, 2009). This indicates that the specific membrane environment in a lipid raft in terms of physical properties, for example membrane fluidity, can influence their transporter function. It seems plausible that the activity of the peroxisomal ABC transporters could also be controlled by their association with lipid rafts.

These findings may have implications for X-ALD patients. In 1998, it was reported that lovastatin reduces VLCFA levels in plasma of patients with X-ALD (Singh *et al.*, 1998). Recently, it was demonstrated that the lovastatin-induced reduction in plasma VLCFA levels is non-specific and linked to a reduction in low-density lipoprotein particles (Engelen *et al.*, 2010). Still, many patients with X-ALD use lovastatin worldwide hoping that it may have a positive effect on their clinical outcome. Ironically, the recent data published by Woudenberg *et al.* suggest that lovastatin-induced cholesterol depletion might reduce ALDP function by affecting the peroxisomal localization of residual ALDP in patients with X-ALD (Woudenberg *et al.*, 2010). This indicates that

lovastatin treatment might ultimately result in a worsening of clinical symptoms.

Regulation

Influences on gene expression of the transporter

ALDP. So far, there is no evidence to suggest that ALDP expression is inducible. Available data indicate that the *ABCD1* gene is, in contrast to the *ABCD2* and *ABCD3* genes (see below) insensitive towards peroxisome proliferators (Albet *et al.*, 1997; Berger *et al.*, 1999; Netik *et al.*, 1999).

ALDRP. Expression of the *ABCD2* gene can be increased in a peroxisome proliferator-activated receptor (PPAR)-dependent and a PPAR-independent manner.

PPAR-dependent. Treatment of *Abcd1* knockout mice with fenofibrate resulted in a 10-fold increase in the levels of ALDRP in the liver (Albet *et al.*, 1997; Netik *et al.*, 1999). To investigate whether fenofibrate-induced expression of mouse ALDRP involves PPARα, Fourcade *et al.* analysed the expression of ALDRP in fenofibrate-treated wild-type and *Pparα* knockout mice (Fourcade *et al.*, 2001). The expression of ALDRP increased upon fenofibrate treatment in wild-type but not in *Pparα* knockout mice. PPARα forms a heterodimer with RXRα and binds to DNA sequences termed PPREs (peroxisome proliferator response elements) in the promoter region of target genes. Analysis of the promoter of the mouse *Abcd2* gene revealed the presence of several candidate PPREs. However, none of these elements turned out to be functional (Fourcade *et al.*, 2001; Rampler *et al.*, 2003). Thus, although fibrate-mediated ALDRP induction is PPARα-dependent, the underlying mechanism appears to be indirect. Additional data indicated that the sterol regulatory element binding protein (SREBP) pathway may be involved as a functional sterol regulatory element (SRE) is present in the promoters of both the mouse *Abcd2* and the human *ABCD2* gene (Rampler *et al.*, 2003; Weinhofer *et al.*, 2005). Indeed, culturing fibroblasts from X-ALD patients in medium with lipoprotein-deficient fetal calf serum resulted in increased ALDRP expression (Weinhofer *et al.*, 2002). Furthermore, treatment of mice with fenofibrate resulted in a PPARα-dependent increase in the *SREBP2* mRNA level in mouse liver (Rampler *et al.*, 2003) (Reviewed in Berger *et al.*, 2010).

PPAR-independent. PPAR-independent compounds that affect the expression of the *ABCD2* gene include thyroid hormone (T3) and 4-phenylbutyrate (4PBA). T3 treatment increased ALDRP expression in rat oligodendroglial (CG4) cells and in fibroblasts from X-ALD patients or *Abcd1* knockout mice. Furthermore, T3 treatment corrected VLCFA levels in X-ALD fibroblasts (Fourcade *et al.*, 2003). However, not all cell types tested responded to T3 treatment. For example, *Abcd2* expression was unaffected in primary rat astrocytes. A similar cell type specific response was found *in vivo*: T3 treatment of rats resulted in increased ALDRP expression in liver, but not in brain (Fourcade *et al.*, 2003).

4PBA is a fatty acid mimetic that is FDA approved for the treatment of urea cycle disorders. Treatment of fibroblasts from X-ALD patients or *Abcd1* knockout mice with 4PBA resulted in correction of the peroxisomal VLCFA β-oxidation, reduction of VLCFA levels, increased expression of the *ABCD2* gene and peroxisome proliferation (Kemp *et al.*, 1998). Gondcaille *et al.* compared the effect of 4PBA and fibrates on ALDRP expression in rat fibroblasts and glial cells. While treatment with fibrates did not affect ALDRP expression, treatment with 4PBA resulted in increased expression of ALDRP (Gondcaille *et al.*, 2005). Additional experiments showed that exposure of fibroblasts from wild-type and *Pparα* knockout mice to 4PBA resulted in a dose-dependent induction of ALDRP expression (Gondcaille *et al.*, 2005). 4PBA-mediated *Abcd2* activation could be mediated by the inhibition of histone deacetylase 1 (HDAC1), as 4PBA is a weak inhibitor of HDAC1 with an IC_{50} in the millimolar range (de Ruijter *et al.*, 2003). The promoter of the rat *Abcd2* gene contains a GC box and a CCAAT box that binds to HDAC1. Deletion of these elements abolished both the binding of HDAC1 to the *Abcd2* gene promoter and the effect of 4PBA on ALDRP expression (Gondcaille *et al.*, 2005).

PMP70. Expression of PMP70 can be enhanced by exposure to PPARα ligands including plasticizers like di(2-ethylhexyl) phthalate (DEHP) (Kamijo *et al.*, 1990), and fibrates, such as ciprofibrate and fenofibrate (Causeret *et al.*, 1993; Albet *et al.*, 1997; Fourcade *et al.*, 2001). Treatment of mice with fenofibrate resulted in a 10-fold increase in PMP70 in liver and intestine (Albet *et al.*, 1997; Berger *et al.*, 1999; Netik *et al.*, 1999). No increase in PMP70 expression was observed in *Pparα* knockout mice (Fourcade *et al.*, 2001). In monkeys, a 15 day treatment with ciprofibrate resulted in a 17-fold increase in liver PMP70 expression (Colton *et al.*, 2004). It should be noted that the human *ABCD3* gene lacks an apparent PPRE (Gärtner *et al.*, 1998). Whether the promoter of the *ABCD3* gene contains a functional SRE as has been found in the promoter of the *ABCD2* gene remains to be investigated.

Biochemistry and genetics

Topology and dimerization of the peroxisomal ABC transporters

ALDP, ALDRP and PMP70 are ABC half-transporters located in the peroxisomal membrane (Figure 1). The amino-terminal part contains a transmembrane domain predicted to consist of six α-helices. The carboxy-terminal part contains a single hydrophilic ATP-binding domain. ALDP and PMP70 are both integral peroxisomal membrane proteins with the nucleotide binding fold located towards the cytoplasmic surface of the peroxisomal membrane. Protease treatment of peroxisomes revealed that the ATP-binding domain of PMP70 is exposed to the cytosol (Kamijo *et al.*, 1990). Watkins *et al.* used an antibody against the 18 carboxy-terminal amino acids of ALDP to determine the topology of ALDP within the peroxisomal membrane. Under conditions where cells were permeabilized with digitonin (which leaves the peroxisomal membrane intact), ALDP could be stained. These data show that the ATP-binding domain of ALDP is located in the

cytosol (Watkins *et al.*, 1995). Similar studies to definitively establish the topology of ALDRP have not been performed due to the absence of a suitable antibody against ALDRP. However, as ALDP and ALDRP share 66% identity and 88% homology it is very likely that the topology of ALDRP is comparable to that of ALDP and PMP70.

A functional ABC full-transporter is composed of two transmembrane domains and two ABCs. Hence, for complete functionality, dimerization of two half-transporters is a prerequisite. With respect to dimerization of the peroxisomal ABC proteins, conflicting observations have been reported. Heterodimerization is at least theoretically possible in tissues where different transporters are co-expressed, but it is precluded in others which only or predominantly express one of the ABCD proteins (Figure 4). *In vitro* investigations using either co-immunoprecipitation assays or the yeast two-hybrid system revealed that ALDP forms both homodimers and heterodimers with ALDRP and PMP70 (Liu *et al.*, 1999; Smith *et al.*, 1999; Tanaka *et al.*, 2002). These data are in sharp contrast with more recent *in vivo* studies. For example, Guimaraes *et al.* performed co-immunoprecipitation experiments using mouse liver peroxisomes to investigate protein–protein interactions. Protein blot analysis showed that neither PMP70 nor ALDRP co-immunoprecipitated with ALDP (Guimaraes *et al.*, 2004). This indicates that ALDP primarily functions as a homodimeric protein. It should be noted, however, that the high abundance of PMP70 and ALDP homodimers in the peroxisomal membrane may make it difficult to detect ALDP-PMP70 heterodimers which could still be present at lower abundance. The preferential formation of homodimers was also demonstrated by Hillebrand *et al.* who used FRET microscopy and showed that in intact living cells ALDP and PMP70 form homodimers as well as ALDP/PMP70 heterodimers, but that ALDP homodimers predominate (Hillebrand *et al.*, 2007). Taken together, these data indicate that the peroxisomal ABCD proteins have a strong preference for the formation of homodimers, but that heterodimers are formed as well, although at a lower abundance.

ATP-binding and ATPase activity

Tanaka *et al.* used photoaffinity labelling of peroxisomes and demonstrated that both PMP70 and ALDP bind ATP in the absence of Mg^{2+}. In the presence of Mg^{2+}, the bound ATP is hydrolysed to ADP and dissociates from PMP70 and ALDP (Tanaka *et al.*, 2002). Two X-ALD causing missense mutations located in the ATP-binding domain of ALDP resulted in either decreased ATP-binding capacity (p.Ser606Leu) or reduced ATPase activity (p.Gly512Ser), when analysed in the context of recombinant nucleotide binding domains (Roerig *et al.*, 2001). Using limited-trypsin digestion, Kashiwayama *et al.* investigated the effect of ATP-binding and hydrolysis on the conformation of PMP70. The binding and hydrolysis of ATP was found to induce conformational changes in PMP70 close to the boundary between the transmembrane and nucleotide binding domains and the helical domain between the Walker A and B motifs (Kashiwayama *et al.*, 2002). Similarly, limited digestion by factor Xa was used to demonstrate substrate-induced conformational change for ALDP (Guimaraes *et al.*, 2005). Although these studies, together with evidence from heterologous expression (van Roermund *et al.*, 2008; van Roermund *et al.*, 2011) are consistent with the action of peroxisomal ABC transporters as ATP-driven pumps, unequivocal demonstration of ATP-dependent fatty acid metabolite transport has not yet been published for any of the proteins.

Phosphorylation

Phosphorylation is one of the most common mechanisms of post-translational protein regulation in the cell (Cohen, 2001). Phosphorylation provides cells with a simple, low energy, fast and efficient way to change transporter function. Many members of the ABC superfamily of transporters are phosphorylated (Stolarczyk *et al.*, 2011). For example, the peptide transport activity of the heterodimeric ABC transporter associated with antigen processing (TAP; ABCB2/ABCB3) is inhibited by phosphorylation (Li *et al.*, 2000). Conversely, phosphorylation by Pim-1 kinase is required for the activity of both P-glycoprotein (Pgp; ABCB1) and breast cancer resistance protein (ABCG2) (Xie *et al.*, 2008; Xie *et al.*, 2010). In the case of Pgp, phosphorylation serves to prevent proteolytic and proteasomal degradation and promotes translocation to the cell surface. Interestingly, Tanaka *et al.* reported that both ALDP and PMP70 are subject to phosphorylation *in vivo* and that this phosphorylation may affect the function of the proteins (Tanaka *et al.*, 2002). ALDP can be phosphorylated at amino acid Ser_{733} (Dephoure *et al.*, 2008), and PMP70 at amino acid Tyr_{145} (Imami *et al.*, 2008). There is no evidence yet with respect to a possible phosphorylation of ALDRP. The physiological significance of phosphorylation of the peroxisomal ABC proteins remains to be investigated, but it may modulate their transport activity or perhaps their turnover. For example, mutations affecting the phosphorylation of the yeast ABC transporter (Ste6) change the regulation of ubiquitination and trafficking to the yeast vacuole (Kölling, 2002). No X-ALD patients have been identified with a mutation affecting amino acid residue Ser_{733} of ALDP (http://www.x-ald.nl). It would nevertheless be interesting to study the effect of phosphorylation on the function and stability of ALDP. Using site-directed mutagenesis, the amino acid residue Ser_{733} of ALDP could easily be modified and the effect of phosphorylation on the function of ALDP could be assessed by measuring the effect on protein stability and VLCFA β-oxidation.

Significant single nucleotide polymorphisms

ABCD1

Two frequent single nucleotide polymorphisms (SNPs) are present in the untranslated regions (UTR) of the *ABCD1* mRNA: rs4148030 (T-allele frequency 7% and C-allele frequency 93%) is located in the 5′ UTR and rs2229539 (G-allele frequency 31% and C-allele frequency 69%) in the 3′ UTR. No experimental data are available with respect to the effect of these SNPs on for example mRNA stability or the activity of the *ABCD1* promoter. Two frequent SNPs have been identified in the coding region of the *ABCD1* gene: p.Leu516Leu (rs41314153, A-allele frequency 10% and G-allele frequency 90%) and p.Phe673Phe (T-allele frequency 4% and C-allele frequency 96%). Although both SNPs are silent polymorphisms it remains unclear whether they have an effect on mRNA stability.

As mentioned above, in the section 'Pathology and clinical significance', X-ALD is characterized by the absence of a genotype–phenotype correlation. Patients in the same family may express different phenotypes (Berger *et al.*, 1994; Kemp *et al.*, 1994). This raises the possibility that other genetic and/or environmental factors are involved in the clinical presentations of X-ALD. Raymond *et al.* identified five X-ALD patients who developed inflammatory demyelinative lesions following head trauma (Raymond *et al.*, 2010). This indicates that head trauma may be an environmental factor leading to the onset of cerebral ALD. Segregation analysis suggests that the phenotypic variability is due to at least one autosomal modifier gene (Smith *et al.*, 1991; Maestri and Beaty, 1992). As summarized below, several investigations have focused on the role of SNPs in the *ABCD2* and *ABCD3* genes in the phenotypic expression of X-ALD.

ABCD2

Over-expression of ALDRP can compensate for the loss of ALDP both *in vitro* (Kemp *et al.*, 1998) and *in vivo* in *Abcd1* knockout mice (Pujol *et al.*, 2004). These demonstrations of the functional equivalence of ALDP and ALDRP made the *ABCD2* gene a putative X-ALD modifier gene. Maier and colleagues investigated whether SNPs in the *ABCD2* gene may play a role in the phenotypic variability of X-ALD. Sequence and segregation analysis of the *ABCD2* gene in a large kindred showed that identical *ABCD2* alleles were inherited in brothers affected by either a mild phenotype (such as Addison-only and AMN) or a severe phenotype (such as CCALD). These data exclude the *ABCD2* locus as a major modifier locus for X-ALD (Maier *et al.*, 2008). Matsukawa *et al.* came to the same conclusion. These authors conducted association studies using two additional SNPs in the *ABCD2* gene and found no significant differences in the allele frequencies between AMN patients and patients with cerebral adrenoleukodystrophy (Matsukawa *et al.*, 2010).

ABCD3

In addition to *ABCD2*, Matsukawa *et al.* also analysed nine SNPs in the *ABCD3* gene for a possible role in the phenotypic expression of X-ALD (Matsukawa *et al.*, 2010). This study revealed no significant association between genetic variants in the *ABCD3* gene and the X-ALD phenotype.

Non-processed autosomal pseudogenes

Between 5–10 million years ago, during the course of higher primate evolution, a 9.7 kb DNA segment encompassing exons 7 though 10 of the *ABCD1* gene was duplicated from the X-chromosome to specific locations near the pericentromeric regions of chromosomes 2 (2p11), 10 (10p11), 16 (16p11) and 22 (22q11) (Eichler *et al.*, 1997; Smith *et al.*, 1999). Comparative sequence analysis of this fragment showed that these four paralogs share 92–96% nucleotide identity with the *ABCD1* gene (Eichler *et al.*, 1997). Due to the very high homology, caution should be exercised when performing mutation analysis of the *ABCD1* gene using genomic DNA. In 1999, Corinne Boehm *et al.* developed and validated a robust genomic DNA-based diagnostic test for X-ALD. Xq28, *ABCD1* gene specific primers were designed that allow accurate mutation analysis without interference of the pseudogenes (Boehm *et al.*, 1999).

The human genome does not contain pseudogenes for either the *ABCD2* gene or the *ABCD3* gene.

Conclusion

As this review points out, the mammalian peroxisomal ABC transporters are essential for peroxisomal metabolism. In recent years, significant progress has been made in elucidating the substrates for ALDP, ALDRP and PMP70. This knowledge will lead to a better understanding of cell physiology. It is puzzling, however, that there is only one disease known to be caused by a deficiency of a peroxisomal ABC transporter. The existing mouse models could be helpful in generating a clinical profile of putative patients with a defect in either ALDRP or PMP70.

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Conflict of interest

The authors declare that they have no conflict of interest.

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