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Review

X-linked adrenoleukodystrophy: Clinical, biochemical and pathogenetic aspects

Johannes Berger^{a,*}, Jutta Gärtner^b^a Center for Brain Research, Medical University of Vienna, Spitalgasse 4, A-1090 Vienna, Austria^b Department of Pediatrics and Pediatric Neurology, Georg August University Göttingen, Robert Koch Strasse 40, D-37075 Göttingen, Germany

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Abstract

X-linked adrenoleukodystrophy (X-ALD) is a clinically heterogeneous disorder ranging from the severe childhood cerebral form to asymptomatic persons. The overall incidence is 1:16,800 including hemizygotes as well as heterozygotes. The principal molecular defect is due to inborn mutations in the *ABCD1* gene encoding the adrenoleukodystrophy protein (ALDP), a transporter in the peroxisome membrane. ALDP is involved in the transport of substrates from the cytoplasm into the peroxisomal lumen. ALDP defects lead to characteristic accumulation of saturated very long-chain fatty acids, the diagnostic disease marker. The pathogenesis is unclear. Different molecular mechanisms seem to induce inflammatory demyelination, neurodegeneration and adrenocortical insufficiency involving the primary *ABCD1* defect, environmental factors and modifier genes. Important information has been derived from the X-ALD mouse models; species differences however complicate the interpretation of results. So far, bone marrow transplantation is the only effective long-term treatment for childhood cerebral X-ALD, however, only when performed at an early-stage of disease. Urgently needed novel therapeutic strategies are under consideration ranging from dietary approaches to gene therapy.

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1. Introduction

A case described by Haberfeld and Spieler in 1910 most likely represents the first published case of X-linked adrenoleukodystrophy (X-ALD; McKusick 300100) [1]. The older brother of the described patient died of a similar disease and was probably the only X-ALD case of the three patients described by Paul Schilder in 1913 [2]. Siemerling and Creutzfeldt were the first to describe the combination of adrenocortical atrophy, cerebral demyelination and lymphocytic infiltration in a case of what is now considered the first unequivocal report of X-ALD [3]. In 1963 X-linkage was proposed based on pedigree analysis [4]. The name X-linked Adrenoleukodystrophy was introduced by Michael Blaw in 1970 [5]. A key observation of the presence of lipid inclusions in adrenal cells of X-ALD patients by Powers, Schaumburg and coworkers lead to the demonstration of the

characteristic accumulation of very long chain fatty acids (VLCFA) [6]. The development of assays to detect the accumulation of VLCFAs in blood, red blood cells, fibroblasts and amniocytes enabled accurate patient diagnosis and prenatal diagnosis for X-ALD. In 1976, the first case of an adult form of X-ALD was described by Herbert Budka and colleagues in Vienna [7]. The adult form was named adrenomyeloneuropathy (AMN) by Griffin et al. in 1977 [8]. The increasing knowledge of various disease forms for X-ALD lead to the estimation of an incidence of hemizygotes of 1:42,000 in the total population (1:20,000 males). As for most X-linked disorders, it seems inappropriate to refer to X-ALD as an X-chromosomal recessive disorder. It should be referred to simply as X-linked [9] since at least half the women who are heterozygous for X-ALD develop an adrenomyeloneuropathy-like syndrome in middle or later age [10]. Thus, the relevant figure for the incidence of X-ALD (hemizygotes plus heterozygotes) is 1:16,800 [11]. Thus, X-ALD is both the most frequent monogenetically inherited demyelinating disorder and the most frequent peroxisomal disorder.

* Corresponding author. Tel.: +43 1 4277 62812; fax: +43 1 4277 9628.

E-mail address: johannes.berger@meduniwien.ac.at (J. Berger).

2. The clinical picture of X-ALD

The current clinical classification includes a wide range of phenotypic manifestations and has as criteria the age of onset, the organs involved, and the rate of progression of neurological symptoms [12,13]. There are at least six distinct types ranging in decreasing order of severity from the childhood cerebral form to asymptomatic persons. The various clinical phenotypes commonly occur within the same kindred.

2.1. Childhood cerebral (31–35%)

The childhood cerebral form is the most severe phenotype. Patients seem unaffected until the age of 2 to 10 years, when there is onset of adrenal insufficiency and progressive neurological dysfunction. Frequent initial symptoms include emotional lability, hyperactive behaviour, school failure, impaired auditory discrimination and difficulties in vision. After onset of symptoms the course is rapidly progressive, leading to an apparently vegetative state within 2 to 4 years and to death at varying intervals thereafter.

2.2. Adolescent and adult cerebral (6–12%)

Patients with the adolescent cerebral form usually develop initial symptoms between ages 11 and 21. Clinical symptoms and deterioration resemble those of the childhood cerebral form.

The adult cerebral form occurs in patients with an age of onset beyond 21 years. The clinical symptoms and the rate of progression resemble those of the childhood cerebral form. These patients are often initially misdiagnosed as having schizophrenia or other psychiatric disorders.

2.3. Adrenomyeloneuropathy (40–46%; pure AMN 20–23%; cerebral AMN 20–23%)

The age at onset of AMN is the second to fourth decade of life. The disease mainly involves the spinal cord and presents with slowly progressive stiffness and weakness of legs, impaired vibration sense, sphincter disturbances and impotence. Adrenal insufficiency is present in two thirds of patients. Cerebral changes develop in approximately half of the patients, and then the course of the illness resembles that of the other cerebral ALD forms. AMN is often misdiagnosed as multiple sclerosis or familial spastic paraparesis.

2.4. Addison-only and asymptomatic (diminishes with age; common <4 years; very rare >40 years)

10 to 20% of X-ALD patients have primary adrenal insufficiency without evidence of nervous system involvement. These patients are at high risk of eventually developing AMN. At present, the oldest described Addison-only patient is 78 years old [12].

Some patients with the genetic defect are free of adrenal insufficiency and neurologic disability despite the presence of highly elevated saturated very long chain fatty acid levels.

These patients are still at high risk of eventually developing adrenal insufficiency and/or neurologic symptoms. At present, the oldest described asymptomatic males are in the sixties [13].

2.5. Phenotypes in female carriers (increases with age; approximately 50% >40 years)

More than half of women who are heterozygous for X-ALD have neurologic involvement most likely due to non-random X inactivation favouring the mutant allele in heterozygous cells [14]. The mean age of onset is in the fourth decade. Except for milder clinical symptoms and a slower rate of progression, the clinical course of symptomatic heterozygotes resembles that of AMN patients. In the past, most heterozygotes with paraparesis were diagnosed as having multiple sclerosis. Cerebral involvement and adrenal insufficiency are rare.

3. Mutations in the *ABCD1* gene represent the principal inherited defect in X-ALD

The inherited defect in X-ALD was mapped to Xq28 through linkage with the gene G-6-PD [15] and with polymorphic markers [16,17]. By the use of positional cloning the gene responsible for X-ALD was cloned and originally termed adrenoleukodystrophy gene [18]. As this gene encodes a 745 amino acid peroxisomal transmembrane protein with the general structure of an ATP-binding cassette transporter, the gene nomenclature committee re-named the adrenoleukodystrophy gene to *ATP-BindingCassette transporter subfamily D member1* gene (*ABCD1*). The protein name however has remained as adrenoleukodystrophy protein (ALDP).

The *ABCD1* gene covers approximately 19-kb, contains 10 exons and 9 introns [19]. By now 419 different mutant *ABCD1* alleles have been reported and were collected and updated at an X-ALD mutation database ([20]; <http://www.x-ald.nl>). Of these mutations, 221 are missense mutations, 50 nonsense mutations, 24 amino acid insertions and deletions, 109 frame shift mutations and 15 deletions of one or more exons (<http://www.x-ald.nl>). The mutations are distributed equally among the entire coding region of the *ABCD1* gene, however, when only the 221 missense mutations were investigated it became obvious that there is no disease-associated mutation within the first 88 N-terminal amino acids and in the last 45 C-terminal amino acids. Interestingly, in one X-ALD patient two single base pair substitutions in exon 1 have been observed, both causing amino acid exchanges (N13T and K217E). Expression studies revealed that only K217E was ineffective in the restoration of defective β -oxidation in X-ALD fibroblasts [21]. The N13T amino acid exchange, on the other hand, did not affect ALDP function, which is in agreement with the hypothesis that there is a reduced functional importance of the first 66 N-terminal amino acids of ALDP.

There is no general correlation between the type of *ABCD1* gene mutation and the clinical phenotype. This statement is based on four main observations: (i) all clinical phenotypes of X-ALD are known to occur within the same nuclear family (e.g. [22]); (ii) mutations that are known to cause a complete loss of

ALDP, such as large deletions, can be associated with all different clinical phenotypes, even with very late onset of AMN [23]; (iii) the identical dinucleotide deletion in exon 5 can lead to the entire clinical spectrum of X-ALD [23,24]; (iv) monozygotic twins have been described with clearly different clinical phenotypes [25]. However, all these arguments do not exclude the possibility that residual ALDP activity might prevent the development of the inflammatory cerebral form in X-ALD patients, thus leading to a milder phenotype. In other ABC transporters, such as the p-glycoprotein multidrug resistance transporter, some mutations result in a reduced transport rate. Residual functional activity is also suggested for the *ABCA4* (*ABCR*) gene in age-related macular degeneration and the late-onset form of Stargardt disease [26]. Thus, although there is no general genotype–phenotype correlation such a correlation might exist in exceptional cases.

In addition to the functional *ABCD1* gene on Xq28 several autosomal non-processed pseudogenes are present on several different chromosomes. PCR analysis of a human monochromosomal mapping panel with exon 9/10 PCR primers identifies chromosomes 1, 2, 20, 22, and possibly 16 as containing *ABCD1* pseudogenes [23]. Fluorescence in situ hybridization (FISH) analysis using cloned genomic fragments of the autosomal pseudogenes identified homologous sequences at 2p11, 10p11, 16p11, and 22q11 [27] and additionally on 20pter [23]. These pseudogenes on several different chromosomes complicate mutation analysis [28] and illustrate pericentromeric plasticity of non-homologous interchromosomal exchange [27].

4. The Adrenoleukodystrophy protein: structure and topology

Although the *ALD*-gene has been renamed to *ABCD1* the protein name remains as adrenoleukodystrophy protein (ALDP). However, the name *ABCD1* protein is also found in literature.

ALDP structurally represents a half-ABC transporter, with only one hydrophobic transmembrane domain and one hydrophilic nucleotide-binding domain, and presumably has to dimerize in order to become a functional unit [29]. Three other mammalian half-ABC transporters, structurally similar to ALDP, have been identified: the ALD-related protein (ALDR [30,31]), the 70 kDa peroxisomal membrane protein (PMP70 [32,33]), and the PMP70-related protein (P70R [34,35]) with 63, 33, and 25% amino acid identity to ALDP. ALDR, PMP70 and P70R are encoded by *ABCD2*, *ABCD3* and *ABCD4*, respectively. Thus, one important question is whether ALDP dimerizes as a homodimer, or forms a heterodimer with one specific heterodimerization partner, or forms different heterodimers in different tissue-specific cell types in accordance with the availability of individual peroxisomal-half-ABC transporters. With regard to the last point it is important to note that the four peroxisomal ABC transporters show remarkably distinct expression patterns among different cell types; probably most cell types would express at least two different peroxisomal ABC transporters [36–39]. Using the yeast two-hybrid system, Liu and co-workers showed that homo- as well as heterodimerization occurs between the C-terminal halves of ALDP, ALDRP,

and PMP70 [40]. Two X-ALD disease mutations located in the C-terminal half of ALDP (P484R and R591Q) affect both homo- and heterodimerization of ALDP [40]. Co-immunoprecipitation studies in two different laboratories demonstrated homodimerization of ALDP, heterodimerization of ALDP with ALDRP or PMP70 [23,40], and heterodimerization of PMP70 and ALDRP [40]. However, both studies relied on overexpression of the ABC transporters in cell culture which results in artificial conditions. Stronger evidence for the presence of ALDP and PMP70 homodimerization and ALDP/PMP70 heterodimerization comes from the co-immunoprecipitation of ALDP and PMP70 from purified rat liver peroxisomes [41]. However, in a recent study, ALDP-containing protein complexes were characterized by preparative immunoprecipitation and isolation of PMP70-containing complexes by a two-step purification protocol, and in both cases no evidence for the existence of heteromeric interactions between ALDP and PMP70 could be found [42]. These data indicate that at least in mouse liver probably the homodimeric forms of ALDP and PMP70 predominate.

Whether ALDP forms homo- or heterodimers is of great importance, as the number of possible substrates or substrate families strongly increases when all possible combinations are taken into account. For none of the four peroxisomal ABC transporters, the natural substrate is known. However, it seems clear that eukaryotic half-ABC transporters (i) actively transport using the hydrolysis of ATP; (ii) transport the substrate only in one direction (no bidirectional substrate shuttle is known for eukaryotic half-ABC transporters, but cannot be excluded); (iii) transport from the site of the ATP-binding domain to the other side of the membrane. In the context of this general knowledge of the functions of eukaryotic ABC transporters, the knowledge of the topology and the localisation of the peroxisomal ABC transporters become very important. Digitonin disrupts the plasma membrane only, leaving the peroxisomal membrane intact. Using indirect immunofluorescence, catalase is readily detected in cells treated with Triton X-100, but cannot be stained in digitonin-permeabilized cells, because it is localised intraperoxisomally. Using this technique several groups could demonstrate a cytosolic orientation of the C-terminus of ALDP [43,44]. The same has been shown for PMP70 [45,46]. For ALDRP and P70R, no conclusive data are available due to the current lack of suitable antibodies, and in addition, overexpression of tagged proteins might lead to mistargeting and misorientation. However, for ALDP and PMP70, we strongly hypothesise that they transport substrates from the cytoplasm to the peroxisomal lumen, which is an important puzzle piece in the attempt to understand the pathology of X-ALD. Efforts are currently ongoing to identify the natural substrate for ALDP and other half ABC transporters.

5. Biochemical abnormality in X-ALD and the putative role of ALDP

The principal biochemical abnormality is the elevated levels of saturated, unbranched, very long-chain fatty acids (VLCFA), particularly tetracosanoic (C24:0) and hexacosanoic acid (C26:0). The accumulation of VLCFA can be found in all

tissues, body fluids and cultured cells (for review see [13]). The accumulation of VLCFA is the only biochemical alteration known to be present in all clinical variants of X-ALD, including presymptomatic individuals. In heterozygous females, the accumulation of VLCFAs is reduced compared with the hemizygotes and could lead to false negative carrier status in blood investigations (see diagnosis of X-ALD).

As X-ALD is a peroxisomal disorder, the link between the peroxisomal defect, namely the loss of the ability to transport a substrate from the cytoplasm to the peroxisomal lumen, and the accumulation of VLCFA must be elucidated in order to understand the pathology of X-ALD. There is a “direct” (old) and an “indirect” (new) hypothesis for the molecular mechanism that links the peroxisomes to the characteristic accumulation of VLCFA in X-ALD.

The “direct” (old) hypothesis is based on the fact that the degradation of VLCFA is performed inside the peroxisomes with a separate set of enzymes located within the peroxisomal matrix and that this peroxisomal β -oxidation is decreased to about 30% of normal in cultured human fibroblasts of X-ALD patients. Thus, it was generally assumed that the primary cause of elevated VLCFA is decreased peroxisomal β -oxidation associated with reduced activity of the peroxisomal enzyme, very long-chain acyl-CoA synthetase, which converts VLCFA to their CoA thioesters [47,48]. Originally the primary defect in X-ALD was thought to be within the gene of a peroxisomal very long-chain acyl-CoA synthetase. However, as ALDP, a peroxisomal transporter, and not a very long-chain acyl-CoA synthetase, was identified as the primary cause of X-ALD by positional cloning it was hypothesised that ALDP transports either the VLCFA, the VLCFA-CoA, CoA, or even the very long-chain acyl-CoA synthetase itself into the peroxisomes or to stabilise the very long-chain acyl-CoA synthetase (reviewed in [13]). Thus, the very long-chain acyl-CoA synthetase remains central to this hypothesis. This led to the identification of a peroxisomal very long-chain acyl-CoA synthetase in rat [49], mouse [50] and human [51] tissue. However, as this enzyme is mainly expressed in liver and kidney but only to a very minor extent in X-ALD target tissues like brain and adrenal cortex, and as no clear alteration of this enzyme in X-ALD could be observed, it was suggested that possibly another very long-chain acyl-CoA synthetase exists [50–52]. Thus, several additional very long-chain acyl-CoA synthetases were isolated, leading to the identification of an entire novel family of very long-chain acyl-CoA synthetase. However, none of these additionally isolated enzymes were localised within the peroxisome or were altered in X-ALD fibroblasts or tissues [53–56]. Only recently, it was observed that the mRNA level of Bubblegum, another newly identified member of the very long-chain acyl-CoA synthetase family [57–62], is decreased in *normal* appearing white matter of X-ALD brain in correlation with the severity of the disease [63]. But as Bubblegum is not a peroxisomal protein, this correlation probably cannot be the direct link between the peroxisomes and the accumulation of VLCFA.

The “indirect” (new) hypothesis assumes that the link between the peroxisomes and the accumulation of VLCFA is independent of the peroxisomal β -oxidation. In recent years,

important evidence has accumulated to strongly support this “new” hypothesis. The first observation was that the peroxisomal β -oxidation could be restored in cultured embryonic *Abcd1*-deficient mouse fibroblasts using trichostatin A treatment. This restoration of peroxisomal β -oxidation was independent of ALDP and ALDRP [64]. In addition, we have observed that protease inhibitor treatment of human cultured fibroblasts of X-ALD patients restored the peroxisomal β -oxidation but did not stabilise ALDP (J. Berger, unpublished data). This protease inhibitor mediated restoration of the peroxisomal β -oxidation could be observed in X-ALD fibroblasts with a broad spectrum of different ALDP mutations, however was not able to restore peroxisomal β -oxidation in fibroblasts of patients with Zellweger syndrome (J. Berger, unpublished data). The next crucial observation was again one by Kirby Smith and co-workers, which demonstrated that the peroxisomal β -oxidation is not altered in tissues of *Abcd1*-deficient mice, in spite of the accumulation of VLCFA [65]. This has been observed in brain, adrenal, heart, liver, liver peroxisomes and kidney [65], but also in an independent laboratory in skeletal muscle [66]. Thus, there is an obvious difference between cultured fibroblasts (human and mouse) and tissues with respect to the impairment of the peroxisomal β -oxidation in ALDP-deficiency. Further strong evidence for the independent regulation of peroxisomal β -oxidation and VLCFA accumulation comes from a study in which Smith and co-workers demonstrated that very long-chain acyl-CoA synthetases *Acsvl1*-deficient mice, which had a 50% reduction in peroxisomal β -oxidation, did not show any signs of VLCFA accumulation [67]. These data show that tissue levels of VLCFA are not directly correlated with the rate of peroxisomal VLCFA β -oxidation and suggest that ABCD1 may not participate directly in the degradation of VLCFA.

VLCFAs that accumulate in X-ALD are mostly of endogenous origin and are derived only to a minor extent from the diet [68]. An enhanced fatty acyl-chain elongation has been observed in fibroblasts of peroxisome biogenesis disorders and X-ALD [69,70]. With improved modern technology it was recently demonstrated that the elongation system of both saturated and mono-unsaturated VLCFAs is enhanced in fibroblasts from patients with peroxisome biogenesis disorders and X-ALD [71]. In this article the authors speculated that the enhanced elongation does not result from impaired peroxisomal β -oxidation alone, but is due to the additional effect of unchecked chain elongation [71]. Thus, the unchecked chain elongation might be downstream of the VLCFA accumulation.

Cholesterol lowering normalized VLCFA in fibroblasts of X-ALD patients [72,120]. In addition, we have observed that a high cholesterol level does increase VLCFA in control and X-ALD fibroblasts (J. Berger, unpublished data). Thus in some cell types altered cholesterol metabolism might be in the line of events finally resulting in the accumulation of VLCFAs in X-ALD.

6. Pathology of X-ALD

The molecular basis and biochemical impairment of none of the clinical variants of X-ALD is understood. However, it is of

key importance to note that the pathology of the cerebral forms of X-ALD differs fundamentally from that in pure AMN (reviewed in [13,73,76]).

6.1. Pathology of cerebral ALD

The cerebral forms are associated with huge inflammatory demyelinating lesions that favour the parieto-occipital regions of the cerebral white matter. The most numerous participants in this destruction of myelin and oligodendrocytes were lymphocytes, reactive astrocytes and macrophages. While the lymphocytes were found primarily just within the demyelinating edge, many reactive astrocytes and macrophages were found in the morphologically normal or mildly affected adjacent white matter. At the early demyelinating edge tumor necrosis factor (TNF)-alpha and interleukin (IL)-1 have been described in reactive astrocytes and macrophages [74]. Many major histocompatibility complex (MHC) class II- and transforming growth factor (TGF)-beta positive microglia have been observed [75]. Importantly, CD1 molecules, which play major roles in MHC-unrestricted lipid antigen presentation, were described [75]. B cells and plasma cells were infrequent [74]. Many lymphocytes have been described as CD8 cytotoxic T cells with the alpha/beta TCR also infiltrating morphologically unaffected white matter [75]. In addition, cytolysis of oligodendrocytes, rather than apoptosis, was described as the major mode of oligodendrocytic death in cerebral ALD [75]. The important question whether the inflammatory reaction is secondary to initial dysmyelination or the primary cause of demyelination is not clear. On one hand small white matter lesions with PAS-positive macrophages and negligible or non-existent reactive astrocytic or lymphocytic responses have been found far from the classical inflammatory demyelinating lesion [73]. These lesions have been interpreted as dysmyelinating and due to the biochemical abnormalities in myelin; they have been assumed to be the initial or inciting lesion for the secondary phase of severe inflammatory demyelination [73]. On the other hand the complex lipids with VLCFAs or VLCFA-PLP could constitute an antigen possibly recognized by the CD1 pathway, which would also be a plausible candidate to set off the inflammatory demyelination. The immune response seems not to follow MHC-restricted pathways and it does not fit well into either Th1 or Th2 profiles. Therefore, the MHC-unrelated lipid antigen-presenting CD1 pathway is attractive [76].

Besides the CNS, peripheral nerve tissue, testis and adrenal gland are pathologically involved in AMN and in the inflammatory cerebral form. However, the inflammation has never been observed outside the CNS. Thus, the VLCFA containing lipid antigen (if it plays a role) must be CNS specific, and possibilities include CNS specific gangliosides or CNS specific lipoproteins such as proteolipid protein (PLP).

6.2. Pathology of AMN

Pure AMN is mainly a distal axonopathy [77] and the inflammatory response in “pure” AMN is, by definition, absent or very mild. The spinal lesions consisted of equivalent losses of

axons and myelin sheaths, most commonly in the gracile and corticospinal tracts. The most severe losses were usually observed in the cervical gracile tracts and the lumbar lateral corticospinal tracts. Thus, the most distal parts of the axons furthest from the nutritive parent cell body are affected and this is referred to as a “dying-back” pattern [78]. Peripheral nerve lesions are variable and mild compared with the myelopathy and the largest myelinated fibers appeared to be the most severely affected. Investigations of the dorsal root ganglia showed no apparent neuronal loss, necrosis or apoptosis, nor obvious atrophy [79,80]. Morphometric studies, however, did reveal neuronal atrophy with a decrease in the number of large neurons and a corresponding increase in neurons less than 2,000 μm^2 [80]. Many mitochondria in AMN neurons demonstrate lipid inclusions at the ultrastructural level, raising the possibility that, in addition to the peroxisomal defect, impaired mitochondrial function may contribute to the myelopathy through a failure of ATP-dependent axonal transport in AMN spinal tracts with consequent “dying-back” axonal degeneration [73,76].

6.3. Pathology of adrenals

ALDP is only present in the adrenal cortex and not in the adrenal medulla. This is in good agreement with the pathological findings of lamellae and lamellar-lipid profiles shown to contain VLCFA esterified to cholesterol in adrenocortical cells [81]. The saturated fatty acids were proposed to be toxic to the adrenocortex resulting in apoptotic cell death [82].

6.4. Pathology of testis

In the testis of X-ALD males, lamellae and lamellar-lipid profiles are present in interstitial cells of Leydig and their precursors and can be seen at an ultrastructural level, in addition there can be some Leydig cell loss. Degenerative changes in seminiferous tubules in AMN appear indistinguishable from those of adult cerebral ALD [81, 83].

7. Environmental factors and modifier genes in X-ALD

As described above, several studies have established that there is no general correlation of *ABCD1* gene mutations and the heterogeneous clinical phenotypes in X-ALD. Thus, the reason for the development of different clinical forms of X-ALD could be environmental, genetic or both. The findings of strikingly different clinical phenotypes among monogenetic twins strongly argue for the role of additional environmental factors as the initial trigger of inflammation in X-ALD [25]. A viral infection for example could possibly act as an initial trigger to initiate inflammation in the presence of VLCFA containing complex lipids or lipoproteins. In addition, genetic segregation analysis provide support for the hypothesis that at least one autosomal gene plays a role in the clinical manifestation of X-ALD [84–86]. Several candidate modifier genes that might influence the clinical manifestation of X-ALD after the inheritance of the primary ALDP defect are known. Good candidates are the peroxisomal ABC transporters *ABCD2*, *ABCD3* or *ABCD4*,

the *CD1* genes or the HLA-haplotype, just to name a few. Many studies on several candidate genes have been performed. However, as environmental factors overrule genetic factors large sample sets of very homogeneous phenotypes of each clinical variant of X-ALD must be investigated to not oversee the linkage.

In addition to a modifier locus for the difference between cerebral forms and “pure AMN”, modifier genes might play a role in determining the age of onset for the inflammation in the cerebral form but also in determining the age of onset of axonopathy. Mutations in neurotrophic factors such as CNTF or BDNF are candidates.

Thus, although X-ALD is a clearly monogenic inherited disorder, the environmental and genetic determination of disease might present as a very heterogeneous and complex aetiology.

8. Mouse models for X-ALD

Three independently derived *Abcd1*-deficient mouse lines have been generated as a model for X-ALD [87–89]. Similar targeting constructs were used for homologous recombination resulting in null mutations or in a non-functional truncated ALDP (Fig. 1). All three X-ALD mouse strains have a normal life span and up to the age of 1 year exhibit comparable phenotypes showing accumulation of VLCFA, in particular in brain and adrenals, similar to the human disorder, but appear neurologically intact. Abnormal neurological and behavioural

phenotypes as well as slower nerve conduction and myelin and axonal anomalies in the spinal cord and sciatic nerve start around 15 months of age [90]. None of the three mouse models showed any pathological signs in the CNS, neither demyelination nor inflammation. However, due to the axonal degeneration in the spinal cord and the accumulation of VLCFA in all tissues so far investigated these mice can be seen as a model for pure AMN.

Due to the accumulation of VLCFA, these mouse models are valuable tools for evaluation of the efficacy of therapies aimed at normalizing the VLCFA level in target tissue of X-ALD. To elucidate the effects of potential candidates for novel therapeutic treatments, ALDP-deficient mice were dietary treated successfully with 4-PBA, giving a good rationale for further therapeutic considerations [91]. Additional dietary treatment strategies using fibrates, rolipram and statins did not result in any VLCFA lowering effect [92–95]. Known and unknown differences between man and mouse must be taken into account when interpreting the results.

In particular, the absence of inflammatory response in the X-ALD mouse model might provide a clue to the phenotypic divergence in this disease. Thus, the differences in the *CD1* locus between mouse and man could, for example, be the reason for the absence of inflammation in the mouse model. On the other hand, Powers and co-workers have shown strong signs of oxidative stress in human brain sections but not in the mouse model that also might contribute to the observed difference [96]. In addition the mouse model was useful to demonstrate that the accumulation of VLCFA *per se* does not

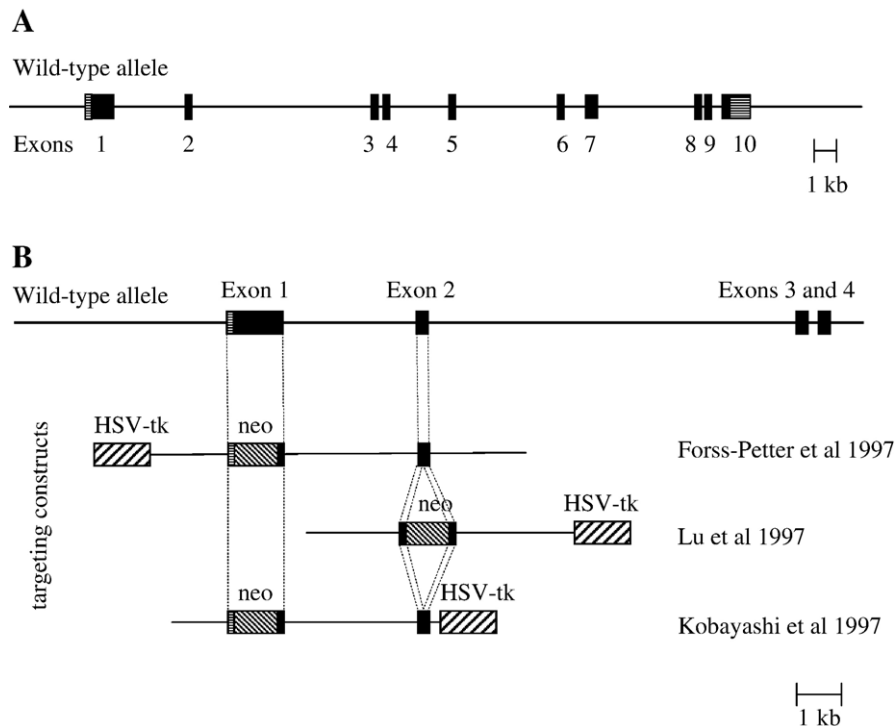


Fig. 1. Targeting constructs used for the generation of three independently derived X-ALD mouse models. (A) Cartoon of the genomic organisation of the wild-type *Abcd1* allele, showing the 10 exons and 9 introns. (B) Two of these mouse lines (Forss-Petter et al. 1997; Kobayashi et al. 1997) were generated by almost identical strategies, whereby an 1140 bp *XhoI* fragment containing the translation start site and most of exon 1 were deleted and replaced by a neomycin resistance (*neo*) gene, resulting in a *null* mutation. For the third X-ALD mouse [88], a silent mutation was introduced at codon 322 in exon 2, creating a novel *XhoI* restriction site for insertion of the *neo* gene. This disruption of exon 2 causes a frameshift in the *Abcd1* gene and premature termination of ALDP (77 codons downstream from the created *XhoI* site). (This figure was also provided for a recent review [129]).

cause mitochondrial abnormalities and, vice versa, mitochondrial abnormalities are not responsible for the accumulation of VLCFA [66]. This finding however should not lead to the conclusion that mitochondrial abnormalities do not play a role in the pathology of X-ALD, but they indicate that there must be other links between peroxisomal dysfunction and the accumulation of VLCFA than the mitochondrial dysfunction as has been recently suggested [65]. Some questions such as investigations of minor deviations in the blood cholesterol level between X-ALD patients and controls cannot be performed due to the high variability of the cholesterol level in humans and the low patient numbers in each age group. In this case the pure inbred ALDP-deficient mice provide an excellent tool for such investigations. Abnormally high blood cholesterol levels could be demonstrated in the X-ALD mouse model [72]. The mouse model could also be used to demonstrate redundant function of the closest homolog of ALDP, ALDRP, which can prevent both VLCFA accumulation and the neurodegenerative features after transgenic overexpression in ALDP deficient mice using an actin promoter [97].

9. What have we learned from yeast?

In contrast to the four mammalian peroxisomal ABC-transporters (ALDP, ALDRP, PMP70 and P70R) only two peroxisomal ABC-transporters *pxa1* (also known as *pat2* or *pall1*) and *pxa2* (also known as *pat1* or *pal2*) are present in *Saccharomyces cerevisiae* [98–100]. The Δ *pxa1* and Δ *pxa2* deletion mutants are unable to grow on oleate (C18:1) as the sole carbon source, and are very likely to function as heterodimers [100,101]. Studies using permeabilized yeast spheroplasts established that Pxa2p was required for the peroxisomal transport of C18:1-CoA, a long-chain acyl-CoA ester, but not for import of C8:0-CoA [102]. Different peroxisomal functions in yeast and man as well as the inability to clearly identify which ABCD gene is the human ortholog of *pxa1* and *pxa2* complicate the attempt to transfer findings from yeast to men. In addition, it may be possible that neither *pxa1* nor *pxa2* is the yeast ortholog of ALDP, meaning that yeast might lack a true ortholog of ALDP.

10. Diagnostic tools

The initial diagnosis of X-ALD relies on the clinical presentation, brain imaging and biochemical analyses of VLCFA [13].

10.1. Clinical presentation

The most common initial clinical symptoms suggestive of X-ALD are in the order of decreasing frequency behavioural changes, intellectual deterioration, impaired vision, impaired hearing, speech difficulty, handwriting changes, gait abnormality, seizures and limb weakness.

10.2. Imaging techniques

Magnetic resonance imaging scans of the brain are obtained as part of evaluation of clinically suggestive patients. Those

with a cerebral form of the disease show characteristic white matter lesions. In the majority of cases, these lesions are symmetric and involve the corpus callosum and the periventricular parietooccipital white matter (Fig. 2).

10.3. Biochemical assays

The impairment of peroxisomal β -oxidation and the accumulation of saturated very long chain fatty acids in tissues and body fluids of patients are pathognomonic for X-ALD. Analyses of the plasma VLCFA levels including lignoceric acid (C_{24:0}), hexacosanoic acid (C_{26:0}) and their ratios to behenic acid (C_{22:0}) are used to confirm the diagnosis in patients suspected to suffer from the disease. In contrast, according to Moser and co-workers [13] only 80% of obligate heterozygous women had increased concentrations of plasma saturated VLCFA. In addition, the majority of male patients have clinical or laboratory evidence of adrenal insufficiency and the adrenal function should be assessed in all cases.

10.4. Mutation analyses

Since the discovery of the *ALD* gene in 1993 [18] the diagnosis can be confirmed by molecular analyses. Although it does not add further information on the clinical course of an individual patient it is surely helpful to identify female carriers and ensure accurate genetic counselling.

10.5. Prenatal diagnosis

Saturated very long chain fatty acids are readily quantified in foetal material including amniotic fluid, cultured amniotic fluid cells and chorionic villus samples. These biochemical analyses have been successfully performed in more than two hundred

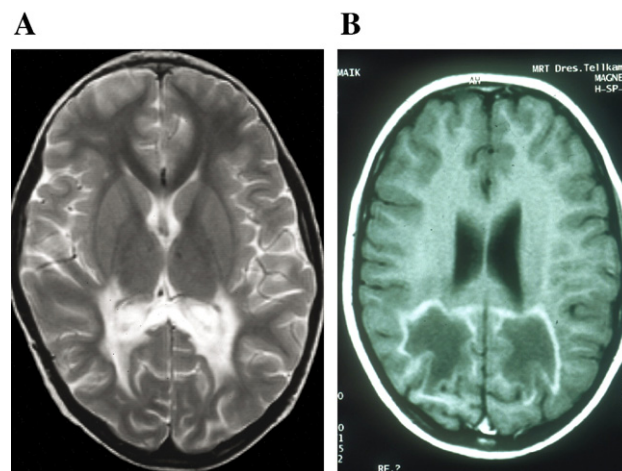


Fig. 2. (A) T₂-weighted magnetic resonance imaging scan of the brain in a 6-year-old boy with childhood cerebral form of X-linked adrenoleukodystrophy. The characteristic demyelinating lesions are symmetric and localised in the occipito-parietal lobes. (B) T₁-weighted magnetic resonance imaging scan of the brain in an 8-year-old boy with childhood cerebral form of X-linked adrenoleukodystrophy. Gadolinium enhancement of inflammatory areas adjacent to demyelination.

pregnancies. However, misdiagnoses have been reported. Two affected male foetuses had normal chorionic villus cell saturated VLCFA profiles [103,104]. DNA based mutation detection techniques have now provided a quite facile and very accurate tool to identify the genotype of a foetus at risk.

11. Therapies in X-ALD

The increasing activity in the field of molecular genetics and the better understanding of disease pathogenesis promote the attempts at devising effective therapies. Nevertheless, at present, steroid replacement for adrenal insufficiency is the only effective and readily available therapy. In addition, bone marrow transplantation is an effective long-term treatment, but only for selected childhood cerebral X-ALD patients in early disease stages. In contrast, traditional pharmacological approaches including Lorenzo's oil and immunosuppression are of little, if any benefit. Other specific therapies are under evaluation including gene replacement and pharmacological gene therapy.

11.1. Symptomatic therapy

Although symptomatic therapy does not correct the basic genetic defect, the patient's current status often ameliorates. In the early disease stage characterized by subtle intellectual and behavioural changes, patients mainly need the assistance of parents, teachers and psychologists. As the disease progresses, major concerns are an increased muscle tone, changes in the sleep wake cycle and bulbar muscle dysfunction. In most patients adequate nutrition has to be maintained by gastrostomy feeding.

11.2. Dietary therapy

A dietary therapy designed to restrict the intake of very long chain fatty acids was initiated in 1980, after the observation that orally administered labelled hexacosanoic acid accumulated in the brain of a terminally ill patient with childhood cerebral ALD [105]. However, this diet failed to lower plasma concentrations of saturated very long chain fatty acids. The normalization of the plasma concentration of saturated very long chain fatty acids was achieved when the dietary restriction was combined with the oral supplementation of glyceryl trioleate (GTO) and glyceryl trierucate (GTE), presumably by inhibiting the endogenous fatty acid elongation system [106]. The 4:1 mixture of GTO and GTE oils is often referred to as "Lorenzo's oil" in recognition of the patient Lorenzo Odone whose parents initiated the development of this therapeutic approach. Therapeutic trials with GTO-GTE oil have been conducted world wide involving more than 500 patients. So far, the results have been disappointing. The diet failed to halt the neurologic progression and did not improve the endocrine dysfunction in patients with childhood cerebral ALD, AMN or symptomatic heterozygous women [107–111]. Studies for asymptomatic patients with in respect to natural disease courses long enough follow-up periods are in progress [112].

11.3. Immunosuppression and other drug therapies

The extent and severity of white matter changes in the cerebral form of disease seem to correlate with the brain inflammatory response mediated by as yet unknown cytokines or immune mechanisms. All therapeutic trials conducted so far to modify the inflammatory response did not reveal a relevant clinical benefit from beta interferon, cyclophosphamide, cyclosporin, immunoglobulins, pentoxifylline, and thalidomide [113,114].

11.4. Bone marrow transplantation

Colonisation of the brain by cells of the monocyte macrophage system provides the rationale for the use of bone marrow transplantation in X-linked ALD [115–117]. The bone marrow derived hematopoietic cells can enter the central nervous system. They can form perivascular macrophages and may undergo transformation to microglial cells. Thus the donor cells serve as an exogenous source of corrective factors; they may contain the ability to degrade VLCFA and/or to provide the favourable modifier substance to prevent cerebral disease forms and perhaps even AMN. The permanent engraftment of the bone marrow cells provides a continuously renewable source of corrective factors that may halt the brain pathology in X-linked ALD. Because cerebral X-ALD is a progressive disorder, all patients with demyelination can be expected to exhibit further injury to myelin before stabilisation occurs after bone marrow transplantation [118]. Bone marrow transplantation has been used to treat more than 126 ALD patients [118]. The majority of patients who survived did improve or stabilise. Although bone marrow transplantation still has a relatively high mortality risk, at this time it provides the only permanent cure when successful and seems to be an appropriate treatment for those patients who show evidence of very early cerebral involvement and for whom a well matched donor is available.

11.5. Perspective of gene therapy

The therapeutic success of bone marrow transplantation in patients with X-ALD has shown that the disease can be cured by replacing the patient's defective hematopoietic stem cells with genetically normal stem cells from another individual. The often futile search for well matched donors provide the impetus for the development of somatic gene therapy. One possible strategy is bone marrow ablation followed by autologous transplantation with genetically corrected hematopoietic cells of the patient's own bone marrow. Experiments could show that virally mediated transfer of cDNA encoding ALDP restored peroxisomal β -oxidation metabolism in X-ALD patient fibroblasts and hematopoietic stem cells [119] and a clinical trial just started.

11.6. Cholesterol lowering drugs

Modulation of cellular cholesterol by either cholesterol depletion [120] or treatment with the cholesterol-lowering

drug lovastatin [121] normalizes VLCFA accumulation in human X-ALD fibroblasts. Thus, a relationship between degradation of VLCFA and cholesterol levels is evident. Lovastatin, which reduces cellular cholesterol by inhibiting HMG-CoA reductase, the key enzyme in cholesterol biosynthesis, seemed promising for therapy of X-ALD because it decreased plasma VLCFA in X-ALD patients [122,123]. However, in a comparable clinical study using Simvastatin the VLCFA could not be normalized in patients plasma [124]. Differences in study design or the use of a different statin might account for the different outcome. The X-ALD mouse model have provided further evidences that ALDP-deficiency and VLCFA are linked to cholesterol but species differences complicate evaluating cholesterol-lowering drugs in X-ALD mice [72]. Further investigations are currently in progress.

11.7. Pharmacological gene therapy

The *ABCD2*-encoded ALDRP is the most closely related peroxisomal ABC-transporter, sharing 66% amino acid identity with ALDP. Upon overexpression ALDRP can functionally compensate for ALDP deficiency in X-ALD fibroblasts and *Abcd1*-deficient mice [93,97]. The endogenous level of ALDRP however is not sufficient to prevent X-ALD. Therefore, pharmacological stimulation of *ABCD2* expression has been targeted as an alternative therapeutic strategy for X-ALD [125] requiring detailed knowledge about how the *ABCD2* gene is transcriptionally regulated. A complex regulation involving several nuclear receptors including liver X receptor (LXR), sterol regulatory element binding protein (SREBP), thyroid hormone receptor (TR), retinoid X receptor (RXR) has been elucidated possibly providing the bases for pharmacological intervention in selected disease related cell types in future [125–128].

12. Concluding remarks

During nearly 100 years of research on clinical, pathological, biochemical and genetic characteristics of X-ALD many twists and turns have occurred in models suggested to explain the molecular mechanisms underlying X-ALD phenotypes. Nevertheless, the main patient demand for effective and all-available treatment still remains to be resolved. Therefore, current research should focus on the identification of the natural substrate for ALDP, as well as on a better knowledge of the molecular mechanisms causing cerebral inflammation and axonal degeneration to move ahead the development of these urgently needed novel effective therapies.

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