

The genetic landscape of X-linked adrenoleukodystrophy: inheritance, mutations, modifier genes, and diagnosis

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Abstract: X-linked adrenoleukodystrophy (X-ALD) is caused by mutations in the *ABCD1* gene encoding a peroxisomal ABC transporter. In this review, we compare estimates of incidence derived from different populations in order to provide an overview of the worldwide incidence of X-ALD. X-ALD presents with heterogeneous phenotypes ranging from adrenomyeloneuropathy (AMN) to inflammatory demyelinating cerebral ALD (CALD). A large number of different mutations has been described, providing a unique opportunity for analysis of functional domains within ABC transporters. Yet the molecular basis for the heterogeneity of clinical symptoms is still largely unresolved, as no correlation between genotype and phenotype exists in X-ALD. Beyond *ABCD1*, environmental triggers and other genetic factors have been suggested as modifiers of the disease course. Here, we summarize the findings of numerous reports that aimed at identifying modifier genes in X-ALD and discuss potential problems and future approaches to address this issue. Different options for prenatal diagnosis are summarized, and potential pitfalls when applying next-generation sequencing approaches are discussed. Recently, the measurement of very long-chain fatty acids in lysophosphatidylcholine for the identification of peroxisomal disorders was included in newborn screening programs.

Keywords: X-ALD, AMN, mutations, incidence, prenatal diagnosis, newborn screening

Introduction

X-linked adrenoleukodystrophy (X-ALD) is a monogenic disease caused by mutations in the *ABCD1* gene located on Xq28.¹ Clinically, X-ALD can present with a wide range of phenotypic manifestations.^{2,3} Two predominant phenotypes prevail: adrenomyeloneuropathy (AMN) and the cerebral form of X-ALD (CALD). AMN is characterized by a slowly progressive axonopathy and first symptoms in males usually appear between 20 and 30 years of age, while affected females may develop some symptoms of AMN with an average onset between 40 and 50 years. In contrast, CALD usually only affects males and presents with rapidly progressive inflammatory demyelination in the brain, leading to rapid cognitive and neurological decline.^{3,4} The mutation in *ABCD1* is necessary but not sufficient for CALD to occur, as additional genetic or environmental factors are required to trigger this devastating brain inflammation.

The *ABCD1* gene codes for the peroxisomal transporter ATP-binding cassette subfamily D member 1 (*ABCD1*, formerly *ALDP*), which mediates the import of very long-chain fatty acid (VLCFA) CoA esters across the peroxisomal membrane.^{5,6} The dysfunction of *ABCD1* results in impaired degradation of VLCFAs in peroxisomes and consequently leads to their accumulation in various lipid species in tissues and body fluids.⁷ While this accumulation of VLCFAs is thought to directly contribute

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to the demyelinating pathology in AMN, the molecular mechanism by which VLCFAs are involved in the onset or progression of inflammation in CALD is still not completely understood. Among the biomaterial available for diagnosis, VLCFAs accumulate in plasma, leucocytes, and fibroblasts from X-ALD patients independent of phenotype. Thus, an elevated level of VLCFAs represents the standard biomarker for diagnosis of X-ALD, but does not predict the phenotype or progression of disease.

While the lifetime risk for male X-ALD patients to develop CALD is estimated to be at least 60%, the peak occurrence is between 5 and 12 years of age. In cases where brain inflammation is detected early, it can be arrested by hematopoietic stem cell transplantation (HSCT).⁸ In cases where no appropriate donor is available, autologous hematopoietic stem cell gene therapy may be an option and is currently being actively investigated.⁹ However, HSCT is thought to only arrest the inflammatory demyelination and not impact the non-inflammatory axonopathy.¹⁰ Therefore, it is not a therapeutic option for AMN patients without inflammatory involvement. Thus, no curative therapy is currently available for AMN, and treatment is limited to symptomatic therapy of adrenal or gonadal insufficiency, neuropathic pain, and spasticity.²

Inheritance and frequency of X-ALD

X-ALD is a monogenic, X-linked disorder and the risk of affected parents for transmitting the affected allele can be clearly predicted. While sons of males with X-ALD will

never inherit the affected X chromosome from their father, their daughters will automatically be carriers of the disease allele. For heterozygous female carriers, the probability for transmission of the affected gene is 50% for both sons and daughters. The exceptions are parents of patients with de novo mutations and rare cases of gonosomal or gonadal mosaicism, where different rates of risk for transmission must be expected.¹¹

While the majority of patients typically inherit the defective *ABCD1* allele from one parent, between 4.1% and 19% of X-ALD cases have been reported to carry mutations acquired de novo (see “de novo mutations in *ABCD1*”).

Incidence and prevalence of X-ALD

The estimation of rates for incidence or prevalence of X-ALD cases faces various problems. These include clinical heterogeneity with late onset of symptoms (yet undetected), small numbers of subjects and differences in the extent of pedigree characterization. Furthermore, the lack of centralized recording in some countries and underdiagnosis of mildly affected male patients as well as heterozygous females can lead to an underestimation of frequency. It should be mentioned that the comprehensive inclusion of females heterozygous for X-ALD relies on genetic testing, as normal plasma VLCFA levels are found in at least 15%.^{12,13} Incidence estimates from different countries in Europe, North and South America, Asia, and Australasia are summarized in Table 1. For the estimation of incidence rates, basically two different methods were used: either the numbers of newly diagnosed patients within a certain time

Table 1 Summary of studies reporting incidence rates for X-linked adrenoleukodystrophy in different populations

Population	n	Incidence	Study design and limitations	Reference
Norway	39	1:61,000 (m + f)*	Retrospective nationwide survey covering the years 1956–1995 ^{###}	Horn et al ¹⁴
Brazil	13	1:35,000	Cases identified by a local center during a 5-year period [#]	Jardim et al ¹⁵
Japan	286	1:30,000–1:50,000	Nationwide questionnaire survey with 50% response rate; values represent an extrapolated incidence rate	Takemoto et al ¹⁶
USA	282 ^a	1:21,000 1:16,800 (m + f)	Represents the most comprehensive data as the majority of cases is expected to be covered by the main US centers included in this study covering a 3-year period ^{b#}	Bezman et al ¹⁷
Italy	57	1:28,000	Cases identified nationwide during a 6-year period [#]	Di Biase et al ¹⁸
Australasia	36	1:27,000 ^c	Cases identified by a multinational center during a 6-year period [#]	Kirk et al ¹⁹
Germany	44	1:68,000 ^{d*}	Retrospective nationwide survey covering the years 1978–1984 ^{###}	Heim et al ²⁰
Netherlands	77	1:100,000*	Retrospective nationwide survey covering the years 1910–1990 ^{###}	van Geel et al ²¹
France	87	1:100,000*	Retrospective nationwide survey covering the years 1956–1986 ^{###}	Sereni et al ²²

Notes: The incidences represent cases of male patients with X-linked adrenoleukodystrophy per male live births or, when indicated (m + f), the combined numbers of male and female cases per total live births. *Values represent minimum estimations due to methodological issues, eg, because periods before the onset of widespread establishment of very long-chain fatty acid measurements were included. Therefore, the true number of X-linked adrenoleukodystrophy cases must be expected to be higher; [#]values were calculated by dividing the number of diagnosed cases within a certain time period by the number of births during the same period; ^{###}values were calculated by retrospectively referring diagnosed patients to the respective birth years; ^anumber of patients was calculated by multiplication of the incidence rate with the birth rate reported in the study; ^bprevious estimates derived from overlapping sample sets were not included in this table; ^cthe incidence rate was obtained by use of data obtained during the last 6 years of the study for which the ascertainment rate was supposedly higher. For the whole study duration of 15 years a minimum estimate of 1:60,000 was found; ^dthe value was calculated by referring the 44 patients reported by the study to be born between 1978 and 1984 to 3,007,589 male births in Germany (© Statistisches Bundesamt, Wiesbaden 2015 destatis.de) during the same period; the study mentions 0.8 cases/100,000 total births.

period were compared with the birth rates in the same period or the established patients were assigned to their respective birth years. While the former design faces the problem that during periods when routine diagnosis became readily available, the number of annually diagnosed cases might have exceeded the annual rate of affected newborns, the latter design must be expected to underestimate actual numbers, as before the widespread availability of VLCFA detection, a portion of cases was likely missed.

In summary, the data given in Table 1 suggest the average incidence rates for X-ALD in male patients to be between 1:20,000 and 1:30,000. Further, the study of Horn et al for the first time also directly addressed the number of affected females and found a combined incidence rate of 1:61,000 for males plus females in the Norwegian population.¹⁴ However, some underestimation must be assumed in this study. In contrast, the combined incidence rate of 1:16,800 reported by the thorough study of Bezman et al represents an extrapolation based on male incidence rates and therefore also includes unidentified and unaffected female carriers.¹⁷ In addition to incidence rates, prevalence rates of around 1:200,000 for males and 1:100,000 for females were estimated by thorough investigations carried out for the Dutch and the Norwegian populations.^{14,21} However, the implementation of newborn screening programs for X-ALD will provide a more complete coverage. Preliminary results already suggest a similar incidence rate of around 1:30,000. Overall, the data suggest that no major differences in incidence rates occur between countries across the world.

Phenotypes and their relative frequencies

While AMN represents the default manifestation of X-ALD, only a subset of patients is affected by CALD. It was recently reported that all male and possibly even most female patients will develop at least some symptoms of AMN late in life and thus AMN has a near 100% lifetime penetrance.^{12,14,23} The onset of AMN usually occurs between the second and fourth decade of life. A recent prospective cross-sectional cohort study including 46 heterozygous X-ALD females reported that 18% of women under the age of 40 years had symptoms but in the age group 60 years or older, 88% were symptomatic.¹² Similarly, Horn et al found that no heterozygous females older than 50 years were free of neurological symptoms.²⁴

While previously the lifetime risk for a newborn male X-ALD patient to develop the inflammatory form of X-ALD was estimated to be around 60%,^{3,25,26} two recent reports

suggest an even higher risk to develop CALD.^{14,27} The risk of developing the inflammatory form of X-ALD between 5 and 12 years of age is around 35%–40% and decreases during adulthood. However, while CALD was previously considered to occur only rarely after the age of 45 years, recent reports suggest a substantial risk to develop CALD even at higher ages.^{27–29} The majority of male X-ALD patients develops primary adrenocortical insufficiency (Addison's disease) which can be the first and often sole symptom in young male X-ALD patients.³⁰ These patients will develop AMN later in life and are at risk of developing CALD.

No relevant genotype–phenotype correlation exists in X-ALD

Based on several different observations, it is now well established that no generalized correlation exists between certain types of mutations in *ABCD1* and the two main clinical phenotypes, ie, AMN and CALD, observed in X-ALD patients. Firstly, even large deletions or frameshift mutations leading to the complete absence of protein can lead to the milder AMN phenotype.³¹ Secondly, within individual kindreds, phenotypes of the entire clinical spectrum of X-ALD have been described.^{15,32,33} Thirdly, even in monozygotic twins, different clinical phenotypes have been observed.³⁴ However, it cannot be excluded that specific mutations may lead to *ABCD1* protein with some residual activity, which could potentially influence the severity of the phenotype.^{35,36} Further, it is possible that certain mutations exclusively lead to specific phenotypes or affect the age of onset of first AMN symptoms, but this question has not yet been studied in detail. The main reasons for that are the low number of patients with identical mutations as well as the current lack of knowledge concerning the influence of environmental factors because this might confound characteristic phenotypes of particular mutations.

Diagnosis of X-ALD

Typically, when a diagnosis of X-ALD is suspected based on clinical presentation or magnetic resonance imaging abnormalities, biochemical testing for elevated plasma VLCFA levels is performed. VLCFAs are elevated in all male X-ALD patients regardless of age, disease duration, metabolic status, or clinical symptoms. The most frequently used diagnostic parameter is the concentration of total C26:0 (saturated fatty acid with 26 carbon atoms) after hydrolysis.³⁷ Alternatively, the ratio of C26:0/C22:0 or C24:0/C22:0 can be used, because C22:0 remains

unchanged or is even slightly reduced in plasma samples of X-ALD patients.^{38,39}

While the measurement of total C26:0 is highly specific in male patients, measurement of VLCFAs can only detect about 85% of heterozygous female carriers.¹³ Thus, additional mutation analysis of the *ABCD1* gene is necessary for proper identification of heterozygous X-ALD females.

One possible diagnostic pitfall is the fact that plasma VLCFA levels strongly depend on dietary intake of VLCFAs, for example, by the consumption of peanuts.⁴⁰ To circumvent this problem, cultured skin fibroblasts were previously used to confirm the diagnosis. Nowadays, other patient-derived cells, like blood leucocytes, provide a less invasive alternative for confirmation of the diagnosis.⁴¹ Furthermore, VLCFAs are not only increased in X-ALD patients, but also in other peroxisomal disorders such as peroxisomal biogenesis defects of the Zellweger syndrome spectrum or single enzyme defects of peroxisomal beta-oxidation (acyl-CoA oxidase deficiency and D-bifunctional protein deficiency). Therefore, mild, atypical, or late-onset variants of these diseases must be considered in the differential diagnosis of VLCFA accumulation.^{42–45} Although other peroxisomal disorders can usually be excluded by measuring phytanic acid and plasmalogen levels,⁴⁶ confirmation of the X-ALD diagnosis by *ABCD1* mutation analysis is advisable, especially in patients with atypical symptoms or when HSCT is considered.

Mutation detection and *ABCD1* pseudogenes

Beside the identification of female carriers and confirmation of the diagnosis in male patients, analysis of mutations in the *ABCD1* gene is also essential to characterize X-ALD kindreds and for prenatal diagnosis.

Mutation detection is typically done by analysis of the genomic area of the *ABCD1* gene, which contains ten exons and is located at chromosome position Xq28, spanning a genomic region of around 20 kb.¹ However, the sequence-based analysis of the *ABCD1* gene is complicated by the presence of several nonfunctional pseudogenes on different chromosomes. While various pseudogenes were originally attributed to chromosomes 1, 2, 10, 16, 20, and 22 by polymerase chain reaction analysis of monochromosomal mapping panels and by the use of fluorescence in situ hybridization (FISH), five different pseudogenes are now assigned to chromosomal regions 2p11 (two copies), 10p11, 16p11, and 22q11.^{31,47} These all consist of exons 7–10 of the *ABCD1* gene and are derived from one ancestral duplication, probably

to chromosome 2, which was followed by further duplications in a process termed pericentromeric plasticity.⁴⁷ As these pseudogenes show a high degree of sequence homology to the original *ABCD1* gene, special care must be taken in the analysis of genomic DNA. Therefore, primers specific for the *ABCD1* gene have been designed for conventional sequence analysis.⁴⁸

In cases where no mutations can be detected by DNA sequence analysis, methods such as quantitative polymerase chain reaction, multiple ligation-dependent probe amplification (MLPA), or Southern blot analysis have been used for detection of large deletions, duplications, or chromosomal rearrangements.

Next-generation sequencing

The fast development of high-throughput methods, like exome sequencing or whole genome sequencing, provides another opportunity to identify X-ALD, especially in cases presenting with an atypical disease course, or when occurring as comorbidities of unrelated severe conditions, which would previously have been missed.^{49,50} However, care must be taken in the interpretation of such sequencing results, as depending on the methods used for target enrichment, sequencing, and data analysis, pseudogenes may give rise to false positives. On the other hand, cases might be missed due to too stringent filtering during data analysis.⁵¹

One example of difficulties concerning the analysis of data from next-generation sequencing is demonstrated by a report in which the mutation c.1823 G>A in exon 8 of the *ABCD1* gene was discussed as a non-deleterious single nucleotide polymorphism variant because it was identified in a patient who was obviously not suffering from X-ALD.⁵² However, in a previous study, this mutation was clearly shown to cause X-ALD, as it was found in the *ABCD1* coding sequence in different patients.⁵³ Therefore, the most plausible explanation for the incorrect detection of the mutation in the former study⁵² is that the same variant is present in an *ABCD1* pseudogene located on chromosome 2 and was by mistake attributed to the real *ABCD1* gene.

Prenatal diagnosis

As X-ALD is a monogenic disorder, the risk of affected parents passing on the mutated allele can be clearly predicted and consequently genetic counseling should be offered. Prenatal testing is preferably done by genetic analyses, but biochemical methods are also available for cases where the mutation in the *ABCD1* gene has not been established.⁵⁴ Genetic testing can be performed either by chorionic villus

sampling at 11–13 weeks of pregnancy or by amniocentesis at 15–18 weeks' gestation. Usually, the karyotype of the fetus is determined first, and if male, fetal DNA is analyzed for the previously established familial disease-causing mutation in the *ABCD1* gene.⁵⁵ However, depending on local regulations, different strategies are pursued.⁵⁶

In the past, the determination of VLCFA levels in cultured amniocytes or cultured chorionic villus cells was the standard method for prenatal diagnosis of X-ALD.⁵⁷ However, cases have been reported for which normal VLCFA levels were obtained in affected fetuses and thus led to false negative diagnosis.^{58,59} Therefore, this method should only be considered when genetic analysis is not possible.

Preimplantation genetic diagnosis may be a further option during in vitro fertilization, depending on the particular national regulations. Different strategies have been described for selection of unaffected embryos by analysis of single blastomeres. First, sexing of embryos was used to select female embryos over possibly heavily affected males in a case of established female X-ALD carriership.⁶⁰ In a different sex selection approach, male embryos derived from a father diagnosed with X-ALD and a healthy mother were selected since these are all unaffected.⁶¹

In a more advanced approach, direct detection of the affected allele by multiple displacement amplification of DNA from a single blastomere followed by analysis of microsatellite markers has been used to ensure unaffected offspring.^{62,63}

Finally, a recent report describes the selection of healthy embryos in combination with HLA matching, which gives rise to the possibility of HSCT in affected siblings for whom no matching donor was available before.^{64,65} However, such an approach depends on applicable regulations and ethical considerations in different countries.

Newborn screening

Many cases of X-ALD are diagnosed early due to extended family screening programs in some but not all countries. Sadly, in the absence of a family history, a situation which is inevitable due to the relatively high rate of *ABCD1* mutations occurring de novo (see section on de novo mutations in *ABCD1*), many patients are detected relatively late and in an advanced stage when therapy is limited. Therefore, the development of newborn screening for X-ALD is a major advancement, as this can ensure timely diagnosis and monitoring of patients. For instance, HSCT is only effective in CALD when performed at an early disease stage, because cerebral inflammation still progresses for 12–18 months

after the procedure.^{8,66} In addition, in patients with subclinical adrenal insufficiency, monitoring and early initiation of hormone replacement therapy can avoid adrenal crises and life-threatening events.^{30,67}

In recent years, substantial progress has been made in the development of suitable methods for high-throughput biochemical screening for X-ALD and other metabolic disorders. Determination of C26:0 lysophosphatidylcholine (C26:0-LPC) from dried blood spots has turned out to be most promising and slightly different methods based on tandem mass spectrometry (MS/MS) were reported by different laboratories.^{68–71}

The feasibility of C26:0-LPC determination has been proven by two large pilot studies based on either liquid chromatography MS/MS⁷² or flow injection analysis MS/MS.⁷³ Indeed, since the beginning of 2014, X-ALD is included in the New York State newborn screening program,⁷⁴ and first preliminary estimates suggest a comparable incidence rate for X-ALD as described before. Further newborn screening legislation has been passed in the states of California, Connecticut, and Massachusetts.

In addition to the detection of male X-ALD patients, determination of C26:0-LPC will also identify the majority of female X-ALD carriers as well as patients affected by different peroxisomal biogenesis disorders or single enzyme deficiencies of the peroxisomal beta-oxidation pathway.^{72,74}

Mutations in *ABCD1*

Due to the X-chromosomal localization of *ABCD1* already a single affected allele can lead to disease. Therefore, a tremendous number of different disease-causing mutations have been described in X-ALD. A comprehensive overview of all described mutations can be found in the X-ALD database (<http://www.x-ald.nl>)⁷⁵

A large portion of the described mutations lead to an absence of *ABCD1* protein. While mutations such as deletions, frameshifts, and nonsense mutations generate truncated proteins, missense mutations often lead to unstable proteins as judged by immunofluorescence microscopy. However, more thorough investigations based on Western blot analysis indicate that many mutated *ABCD1* isoforms with single amino acid substitutions are not completely absent, as initially suggested, but are present at strongly reduced levels, which are not detected by standard immunofluorescence microscopy.⁷⁶ Some variations in *ABCD1*, such as the “missense mutations” N13T and H97L, do not cause disease but rather represent polymorphisms, which do not affect protein function.^{53,77}

Distribution of mutations

To date, 695 non-recurrent mutations have been described in the X-ALD database, out of which 343 represent missense mutations. Figure 1 shows the distribution of the 209 different amino acids affected by disease-causing missense mutations throughout the ABCD1 protein in comparison with the evolutionary conservation across seven mammalian species.

These missense mutations are rather equally distributed throughout the ABCD1 protein with the exception of three regions that are devoid of disease-causing mutations. The unaffected region in the middle of the protein (AA 430–470) acts as a linker between the transmembrane and the ATP-binding domains of ABCD1, probably allowing a considerable degree of structural flexibility. The very N-terminal part of the protein precedes the PEX19 binding site, which is required for proper peroxisomal targeting of ABCD1.⁷⁸ Based on the nearly complete absence of mutations within amino acids 1–73, together with the relatively low level of evolutionary conservation, it might be speculated that this region is not absolutely essential for ABCD1 protein function. In a case of defective translation initiation due to a 26-base pair deletion, it was suggested that, despite the presence of an out-of-frame start codon, an alternative internal translation initiation at amino acid position 66 leads to a low level of truncated ABCD1_{66–745} protein. Indeed, all cases of translation initiation mutations in *ABCD1* reported to date have presented with the AMN phenotype and none with CALD.^{35,79,80}

Interestingly, no disease-causing mutations have been found after amino acid position 693, although evolutionary conservation remains high up to position 721, suggesting that this region of ABCD1 is not directly involved in substrate transport. This idea is further supported by the finding that amino acids 694–745 are not necessary for ABCD1 function, as truncated ABCD1_{1–693} was also able to restore beta-oxidation of VLCFAs in X-ALD fibroblasts.³¹ However, two different studies found the very C-terminal region to play a major role in homodimerization and heterodimerization of ABCD1.^{81,82}

De novo mutations in *ABCD1*

In a considerable number of cases, X-ALD is not inherited but the respective mutations occur spontaneously. Initially, de novo mutation rates of around 5% were described.^{17,83} Similarly, a comprehensive study based on molecular characterization of 489 families found only 4.1% of index cases to carry de novo mutations in the *ABCD1* gene.¹¹ In addition, ten cases of de novo mutations, including three cases of gonosomal or gonadal mosaicism, were described in mothers of index cases in this study. However, a recent population-based Norwegian study reported a higher de novo mutation rate of at least 19%.¹⁴ It remains to be established whether these diverging results reflect real differences, are in part caused by the unavoidably small sample size of the Norwegian study, or by possible underestimation in the study by Wang et al due to underdiagnosis.¹¹ The latter could be

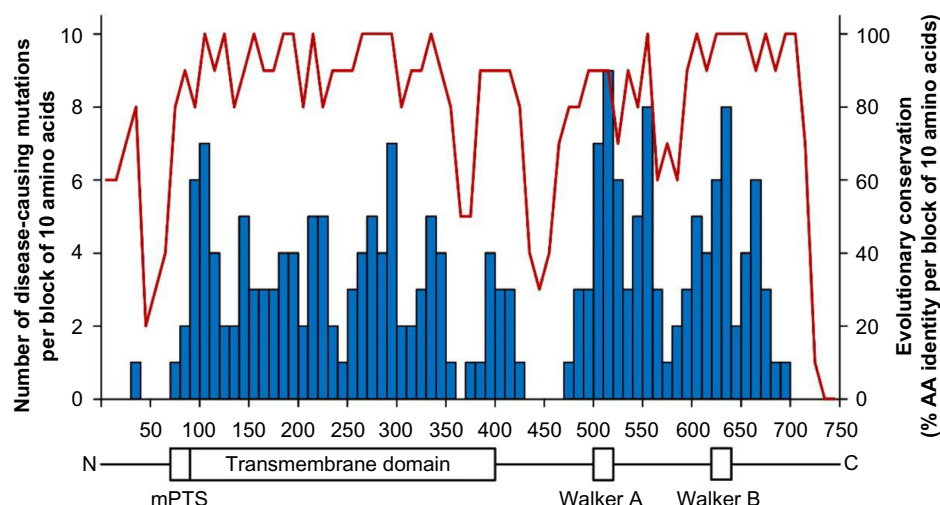


Figure 1 Distribution of disease-causing amino acid mutations in X-linked adrenoleukodystrophy (X-ALD) in relation to the evolutionary conservation across the ABCD1 protein. **Notes:** The distribution of the 209 amino acids (AA) currently known to be affected by disease-causing (X-ALD) missense mutations is presented as the number of mutations per ten residues (blue bars). The extent of evolutionary conservation was compared over seven mammalian species and is plotted as % of conserved AA per ten-residue intervals (red line). Data for ABCD1 mutations in X-ALD were obtained from the X-ALD database (<http://www.x-ald.nl>). The conservation was determined by protein sequence alignment of the ABCD1 orthologs from seven mammalian species covering different branches of this class (human, mouse lemur, mouse, cat, pig, elephant, armadillo) using the multiple sequence alignment program Clustal Omega (<http://www.ebi.ac.uk/Tools/>).¹²⁷ **Abbreviation:** mPTS, membrane peroxisomal targeting signal.

caused by the fact that spontaneously occurring de novo cases, in particular when presenting with mild phenotypes, might not be recognized as X-ALD patients and therefore were not included in the study set.

However, the initiation of systematic newborn screening for X-ALD will allow systematic identification of at least all male patients and will help to elucidate the rate of de novo mutations. However, it remains to be shown whether newborn screenings can help identify a high number of female index cases, as only about 85% of female carriers can be detected by newborn screening.

Aspects of negative dominance in X-ALD

All four members of the ABCD protein family are half transporters, which have to dimerize in order to form functional units. Some *ABCD1* mutations lead to stable and correctly localized proteins but without transporter activity.⁷⁵ If such a mutated ABCD1 protein dimerizes with a functional monomer, it might exert a dominant-negative effect on its dimerization partner, thus resulting in a nonfunctional transporter.⁸⁴ Negative dominance could potentially play a role in autologous gene therapy, when introduced functional ABCD1 might form dimers with the endogenous mutated, but stable protein. However, due to high levels of expression of the therapeutic gene, driven by a strong viral promoter, presumably resulting in excess of functional ABCD1, this is probably of minor concern for most cell types.

Similarly, in therapeutic approaches relying on compensatory pharmacological induction of *ABCD2*, the function of putative ABCD1/ABCD2 heterodimers could be affected by dominant-negative ABCD1. However, given that homodimers of ABCD1 as well as ABCD2 seem to prevail in vivo,^{82,85} a serious impact of dominant-negative effects on pharmacological gene therapy is unlikely. Owing to X-inactivation, negative dominance should not have any harmful consequences at all for heterozygous carriers.

X-inactivation and clinical symptoms in females

As adrenoleukodystrophy is X-linked, heterozygous X-ALD females were regarded to be more or less unaffected and their disabilities often remained unrecognized and untreated for long times.⁸⁶ Indeed, the fatal cerebral phenotype of the disease is extremely rare in females and probably only occurs when both alleles are affected.^{4,87,88}

However, the majority of female carriers have elevated plasma VLCFA levels, although to a lesser extent than male

patients, and the rate of beta-oxidation of VLCFAs was found to be decreased in fibroblasts.¹²

During recent more detailed studies, it became apparent that a high number, if not all, of the heterozygous females develop at least some symptoms of AMN and that the severity of symptoms increases with age.^{12,24,89} It has been speculated for a long time that skewed X-inactivation in females may account for the disease course and severity of symptoms. However, contradictory results concerning skewed X-inactivation have been reported, possibly due to inadequate neurological characterization and consideration of the age of the patients. Additional studies focusing on the specific symptoms of heterozygous females will be needed to finally resolve this question.^{12,90–95}

Modifier genes

The observation that within one family³² or even among monozygotic twins³⁴ one and the same mutation can lead to different X-ALD phenotypes suggests that, in addition to the primary mutation in the *ABCD1* gene, other environmental or genetic factors contribute to the phenotype. Indeed, segregation analysis based on 3,862 individuals from 89 kindreds suggested the presence of an autosomal modifier locus.⁹⁶

Numerous candidate genes were analyzed either by association and/or segregation studies or by analyses of gene expression levels (Table 2). However, a problem faced by most of these studies is the low number of available subjects.

A second methodological problem in some of the published studies concerned the definition of non-inflammatory subjects. An excellent collective with clinically well defined samples originates from the group led by Patrick Aubourg. In these studies, only male AMN patients older than 45 years of age with a demonstrated absence of brain inflammation by magnetic resonance imaging were included in the non-inflammatory sample set.^{97–100} As the likelihood for initiation of brain inflammation strongly diminishes with age, X-ALD patients without cerebral involvement at 45 years were considered as cases of “pure AMN”, although rare instances of conversion to CALD still cannot be excluded (see section on phenotypes and their relative frequencies).

Upon overexpression, *ABCD2*^{101–104} and, to some extent, *ABCD3*^{5,102,105} can compensate for the lack of ABCD1 in vivo and in vitro, and therefore represent excellent candidates for a modulation of the phenotype. However, two independent association studies comparing *ABCD2* and *ABCD3* polymorphisms with different clinical phenotypes failed to determine significant correlations.^{98,99}

Table 2 Summary of genes investigated as possible modifiers for X-linked adrenoleukodystrophy

Gene locus	Cases*	Study design	Conclusion	Reference
Fatty acid metabolism				
<i>ABCD2</i>	117	CCALD vs AMN	NS	Maier et al ⁹⁸
<i>ABCD2</i>	280	CCALD vs AMN	NS	Matsukawa et al ⁹⁹
<i>ABCD3</i>	280	CCALD vs AMN	NS	Matsukawa et al ⁹⁹
Inflammation-related				
<i>TNFA</i>	15	Ct vs CCALD vs AMN	NS	McGuinness et al ¹¹⁶
<i>MOG</i>	44	Ct vs CCALD vs ACALD vs AMN	NS	Gomez-Lira et al ¹¹⁷
<i>CD1</i>	139	CCALD vs AMN	Initial positive correlations for <i>CD1D</i> were not found to be statistically significant after correction	Barbier et al ⁹⁷
<i>MOG</i>	70	Adult X-ALD ^a	NS	Schmidt et al ¹¹¹
<i>IL6</i>	70	Adult X-ALD ^a	NS	Schmidt et al ¹¹¹
<i>HLA</i>	70	Adult X-ALD ^a	NS	Schmidt et al ¹¹¹
<i>HLA</i>	29	CCALD vs AMN vs ADD	HLA DRB1*16 was found more frequently in X-ALD patients than in controls	Berger et al ¹¹⁰
<i>HLA</i>	106	Ct vs CCALD vs AMN	125 different HLA loci were investigated by serotyping. Initial positive correlations for several loci were not found to be statistically significant after correction	McGuinness et al ¹¹²
<i>SOD2</i>	222	CALD vs AMN	The Ala16Val variant of <i>SOD2</i> is suggested as risk factor for cerebral involvement	Brose et al ¹¹⁸
Vitamin B12 metabolism				
<i>ABCD4</i>	280	CCALD vs AMN	Although some variants were associated with disease severity, this could not be confirmed in different patient collectives	Matsukawa et al ⁹⁹
<i>TCN2</i>	172	CCALD vs ACALD vs AMN	The c.776C>G variant of <i>TCN2</i> was identified as risk factor for cerebral demyelination	Semmler et al ¹⁰⁰ Cao et al ¹⁰⁷ Linnebank et al ¹⁰⁸
<i>DHFR</i>	172	CCALD vs ACALD vs AMN	NS	Semmler et al ¹⁰⁰
<i>CBS</i>	172	CCALD vs ACALD vs AMN	While initially an association was found, ¹⁰⁶ this could not be confirmed in later studies	Semmler et al ¹⁰⁰ Cao et al ¹⁰⁷
<i>MTR</i>	172	CCALD vs ACALD vs AMN	NS	Semmler et al ¹⁰⁰ Cao et al ¹⁰⁷ Linnebank et al ¹⁰⁸
<i>MTHFR</i>	172	CCALD vs ACALD vs AMN	NS	Semmler et al ¹⁰⁰ Linnebank et al ¹⁰⁸
<i>MTRR</i>	172	CCALD vs ACALD vs AMN	NS	Semmler et al ¹⁰⁰
<i>RFC1</i>	172	CCALD vs ACALD vs AMN	NS	Semmler et al ¹⁰⁰

Notes: For genes investigated in more than one study the case number of the most comprehensive study is indicated. ^a70 adult patients with six different phenotypes were investigated: pure AMN (n=31), AMN with tract involvement (n=15), female heterozygotes (n=16), ACALD (n=4), Addison (n=2), and asymptomatic (n=2). *Indicates the number of total X-ALD cases included in the study, independent of particular phenotypes.

Abbreviations: Ct, unaffected controls; CALD, cerebral ALD; CCALD, childhood cerebral ALD; ACALD, adult cerebral ALD; X-ALD, X-linked adrenoleukodystrophy; AMN, adrenomyeloneuropathy without cerebral involvement; ADD, Addison's disease without further symptoms; HLA, human leukocyte antigen; NS, no significant associations found; vs, versus.

Various genes related to vitamin B12 metabolism were investigated for a role as modifiers of the phenotype in X-ALD. While initially a protective role of the *CBS* gene was suggested,¹⁰⁶ this finding could not be confirmed by a more comprehensive study in a different patient collective.¹⁰⁰ However, a polymorphism within the *TCN2* gene was found to be significantly associated with cerebral demyelination in X-ALD.^{100,107,108}

There are some similarities in the pathology between multiple sclerosis and the cerebral inflammatory demyelination in X-ALD. Some triggers of inflammation may be shared by these two diseases. Certain HLA-DRB1 haplotypes within the major histocompatibility complex represent a confirmed

genetic risk factor for multiple sclerosis,¹⁰⁹ and were therefore speculated to be good candidates for modification of the X-ALD phenotype. However, previous studies investigating this association were not able to demonstrate significant associations of certain HLA type II haplotypes with cerebral involvement.¹¹⁰⁻¹¹² Additional studies would be required to resolve this issue. Furthermore, genes involved in fatty acid elongation¹¹³ or omega-oxidation of fatty acids¹¹⁴ represent reasonable modifier gene candidates, but unfortunately association studies concerning these genes have not yet been published. Besides direct association studies, expression levels of possible modifier genes have been investigated. While for *ABCD2*, *ABCD3*, and *ACSVL1* no significant

differences in mRNA expression levels were found in brain tissues when comparing CALD and AMN, an altered expression of *ACSBG1* and *ABCD4* mRNA was observed in normal appearing white matter of CALD patients.¹¹⁵

An obvious, although often unavoidable, problem in carrying out association studies in rare diseases like X-ALD, is the inevitably low number of analyzed subjects. As studies with relatively low sample numbers can only detect genetic associations with a relatively large effect on the disease course, genes with a smaller influence typically remain below the significance threshold level. Therefore, negative results should not necessarily be regarded as exclusion criteria for a possible modifier gene but rather as an indication that it is not a major determinant.

So far, most association studies have focused on the presence or absence of cerebral inflammatory demyelination. However, additional clinical variability, like the age of onset of spinal cord degeneration, may also be influenced by the genetic background of the patients.

In summary, it appears that not one single major factor is responsible for the different clinical phenotypes in X-ALD. More likely, several different genetic as well as environmental factors contribute to some extent to the phenotypic variability in X-ALD.

Substrate specificity of the related peroxisomal ABC transporters

In addition to ABCD1, its two closest homologs, ABCD2 (ALDRP) and ABCD3 (PMP70), are also located in the peroxisomal membrane. It has been reported that, upon overexpression, both of these homologs are in principle able to compensate for dysfunctional ABCD1.^{5,101–103}

However, at the endogenous basal levels, ABCD2 and ABCD3 obviously fail to compensate for ABCD1 deficiency, as they are not able to prevent the clinical manifestations in X-ALD patients. Regarding ABCD2, the main reason for this seems to be the opposite expression pattern of ABCD1 and ABCD2 in many cell types, meaning that in cell types with high levels of ABCD1 expression, ABCD2 is often present at low abundance and vice versa.^{119–121} A further explanation for the lack of compensation *in vivo* may be different affinities toward VLCFAs. Different substrate preferences were suggested for ABCD1-3: straight-chain VLCFAs for ABCD1, unsaturated VLCFAs for ABCD2, and branched-chain fatty acids, dicarboxylic acids, and bile acid precursors for ABCD3.^{122–124} Furthermore, the ABCD family of transporters needs to form dimers in order to be functional. It is still debated whether ABCD1 occurs only in homodimeric units

or whether heterodimers with ABCD2 and ABCD3 are also formed *in vivo*.^{81,82,85,125} As chimeric heterodimers of ABCD1 and ABCD2 were shown *in vitro* to be functional, this would raise the possibility of different substrate preferences for such putative heterodimers.¹²⁶

Conclusion and future outlook

The implementation of newborn screening for X-ALD will ensure that boys affected by X-ALD will not remain undiagnosed, and thus their chance to receive HSCT at an appropriate time will increase. Possibly improved knowledge about different genetic modifiers will in future allow the identification of groups of patients with an increased risk for developing the cerebral form of X-ALD, which could then be monitored more closely. The increased integration of next-generation sequencing into routine clinical practice will enable the detection of additional modifier genes and evaluation of previously suggested modifier genes. To this end, it is important to establish worldwide collaborations for X-ALD research in order to organize systematic collection of data sets for future comprehensive analyses. During the last few years, large consortia, which strongly involve patient organizations, were formed in Europe (European AMN Board) and the USA (ALD Connect). These consortia will allow the establishment of large sample sets for genetic studies and provide high-quality platforms for more thorough metabolic and genetic investigations.

Although X-ALD is a monogenic disorder, the individual genetic background, involving many different genes, may be responsible for the age of onset of AMN symptoms in male as well as in female patients. For the inflammatory demyelination in children and adults, next to genetic modifiers, different environmental factors are likely to be involved as triggers, contributing to the overall complexity of X-ALD. For example, it has been reported that severe head trauma can elicit the rapidly progressive brain inflammation in adult patients. Thus, it is possible that independently of the genetic background, every male X-ALD patient might be at risk of developing the inflammatory form of the disease in response to environmental factors, but the likelihood for this to occur may depend on specific genetic modifiers.

There is some evidence that the amount of VLCFAs may be directly related to the risk of developing inflammatory demyelination.¹¹⁵ Modern lipidomic methods now enable unbiased screening for predictive lipid species and provide an additional possibility for the development of new markers allowing improved prediction of clinical progression.

Detailed knowledge of the involvement of modifying genes, lipids, and environmental factors will not only help us to understand the detailed molecular and pathogenic mechanisms underlying the broad spectrum of clinical heterogeneity in X-ALD, but will also help to develop novel therapeutic strategies and enable a more refined evaluation of therapeutic efficacy in clinical trials for X-ALD.

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Disclosure

The authors report no conflicts of interest in this work.

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