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Deciphering the modifiers for phenotypic variability of X-linked adrenoleukodystrophy

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Abstract

X-linked adrenoleukodystrophy (X-ALD), an inborn error of peroxisomal β -oxidation, is caused by defects in the ATP Binding Cassette Subfamily D Member 1 (ABCD1) gene. X-ALD patients may be asymptomatic or present with several clinical phenotypes varying from severe to mild, severe cerebral adrenoleukodystrophy to mild adrenomyeloneuropathy (AMN). Although most female heterozygotes present with AMN-like symptoms after 60 years of age, occasional cases of females with the cerebral form have been reported. Phenotypic variability has been described within the same kindreds and even among monozygotic twins. There is no association between the nature of ABCD1 mutation and the clinical phenotypes, and the molecular basis of phenotypic variability in X-ALD is yet to be resolved. Various genetic, epigenetic, and environmental influences are speculated to modify the disease onset and severity. In this review, we summarize the observations made in various studies investigating the potential modifying factors regulating the clinical manifestation of X-ALD, which could help understand the pathogenesis of the disease and develop suitable therapeutic strategies.

Key Words: X-adrenoleukodystrophy; Cerebral adrenoleukodystrophy; Adrenomyeloneuropathy; Phenotypic variation; Modifiers

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ALD), presents with different clinical phenotypes. The molecular basis for the phenotypic variation has yet to be resolved and is considered to be influenced by genetic, epigenetic, cellular, or environmental factors. We herein discuss the various modifying factors, which can potentially alter the phenotypic presentation of X-ALD.

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INTRODUCTION

Monogenic disorders are primarily caused by a single defective gene, but mutations in a single gene can result in a disease with varying clinical phenotypes. X-linked adrenoleukodystrophy (X-ALD), caused by mutations in the *ABCD1* gene, is one such monogenic disorder affecting peroxisomal β -oxidation. *ABCD1*, mapped on Xq28, comprises of 10 exons^[1] and codes for a 75kDa peroxisomal membrane protein called the ABCD1 protein or adrenoleukodystrophy protein (ALDP)^[2]. ALDP is highly expressed in specific cell types like oligodendrocytes, astrocytes, microglial cells, adrenocortical cells, and endothelial cells in the brain, adrenal glands, testis, and kidney, liver, lung, and placenta^[1,3,4]. ALDP transports very-long-chain fatty acids (VLCFAs), activated by coenzyme-A, into the peroxisomes, for β oxidation. A defect in the *ABCD1* gene results in the synthesis of a dysfunctional ALDP protein, unable to transport VLCFA across the peroxisomal membrane. This leads to the buildup of VLCFA, mainly hexacosanoic and tetracosanoic acids, in various body tissues, primarily the brain, spinal cord, adrenal cortex, testis, and plasma^[1]. The elevated plasma concentration of VLCFA acts as a diagnostic marker for this disorder.

The exact role of VLCFA in the pathogenesis of X-ALD remains unclear, and no correlation has been established between the concentration of VLCFA and the different phenotypes of X-ALD. The abnormally accumulated VLCFA can disrupt the integrity of the plasma membranes through interdigitating between the leaflets of the lipid bilayer and can induce lipotoxicity, endoplasmic reticulum stress, mitochondrial dysfunction, and oxidative stress leading to apoptosis favoring the process of cerebral demyelination in the brain^[5-8].

CLINICAL SPECTRUM OF X-ALD

X-ALD patients have a diverse clinical presentation. They may be asymptomatic or present with the rapidly progressive forms after 3 years of age^[1]. The main types of presentation in male patients are: (1) Cerebral ALD (CALD), the cerebral demyelinating form; (2) Adrenomyeloneuropathy (AMN), with spinal cord demyelination and axonal degeneration; and (3) Addison-like phenotype due to adrenocortical insufficiency.

Cerebral ALD affects males and is typified by progressive inflammatory cerebral demyelination leading to neurodegeneration. It includes the childhood cerebral form (CCALD) - appearing in mid-childhood (4-8 years), adolescent cerebral form or adolescent CALD (10-20 years), and adult cerebral form or adult CALD (> 20 years). Children with CCALD present with behavioral problems and a decline in school performance due to impairment of auditory discrimination and spatial orientation, thereby affecting writing and speech. Rarely, seizures may be the initial manifestation. As the disease progresses, there are further signs of damage to the brain white matter, including spastic quadriplegia, dysphagia, and visual loss leading to a vegetative condition. Adolescent CALD manifests between 10 and 20 years of age with clinical features of cerebral involvement. In adult CALD, psychiatric symptoms, seizures, spastic paraparesis, and dementia develop in males over the age of 20.

The second most common phenotype, AMN, is usually characterized as a gradually developing, non-inflammatory axonopathy, mainly affecting males over 20 years of age. AMN is sub-divided as "pure AMN" and "AMN-cerebral." In patients with pure

AMN, there is spinal cord involvement resulting in gait disturbances and bladder dysfunction, whereas patients with AMN-cerebral form show clinical features of cerebral inflammation besides the symptoms of pure AMN^[9]. The transformation of pure AMN to the cerebral form of AMN is not clearly understood.

A significant proportion of male patients with X-ALD develop adrenocortical insufficiency, which may occur either after the appearance of neurological symptoms or decades ahead. A majority of males present with adrenocortical insufficiency in association with features of CALD or AMN^[1]. Rare cases have shown to manifest adrenocortical insufficiency without cerebral demyelination and are characterized as “Addison only” type of X-ALD^[1,10].

In 20%-50% of heterozygotes or female carriers of X-ALD, symptoms similar to AMN, typically consisting of gait disturbance, dysuria, and urgency, occur after 40 years. There are also reports of female carriers with CALD and adrenal insufficiency^[11]. For instance, Hershkovitz *et al*^[12] reported a case of CALD in a girl of age, 8.9 years, where the genetic analysis was indicative of heterozygosity with a deletion at Xq27.2-tel. Similarly, Chen *et al*^[13] reported CALD and adrenal insufficiency in a 38-year-old Chinese woman. The possible explanation for the symptomatic state observed in certain heterozygotes could be skewed X-chromosome inactivation, resulting in the expression of the chromosome carrying the faulty *ABCD1* gene^[14]. Studies have found a significant association between the degree of skewness and the severity of neurological deficits^[14,15]. However, factors favoring this event remain unidentified.

PHENOTYPIC VARIABILITY IN THE SAME PEDIGREE

The various clinical types of X-ALD frequently appear in the same kindreds and nuclear families carrying the same mutation in the *ABCD1* gene. In half of the kindreds, both CALD and AMN are found^[16]. Diverse phenotypes and clinical features have been seen in mother and son, monozygotic twins, heterozygous siblings, and affected members of several generations of families^[17-19]. For instance, a study reported a family with X-ALD where the proband was diagnosed with the CCALD. In contrast, his two other siblings and maternal uncle were diagnosed with the adolescent form of CALD, Addison's only phenotype of X-ALD, and AMN. Mutational analysis found a hemizygous mutation of *c.1780C>G* in the *ABCD1* gene in all three siblings^[20].

A Brazilian study reports two siblings with CCALD presenting with different clinical features at diagnosis. Both parents had the p.Trp132Ter mutation in *ABCD1*. Addison's disease phenotype was found in their maternal grandfather^[21]. Similarly, different clinical phenotypes have been reported in a Tunisian family with p.Gln316Pro mutation in *ABCD1*^[22].

In an early study of 15 Dutch kindreds, van Geel and coworkers^[23] found only CALD in 20%, only AMN in 40%, and both CALD and AMN in 40%. Another large study of 178 kindreds found CALD in 30%, AMN in 20%, and both CALD and AMN in 50%^[1].

Korenke *et al*^[19] describe phenotypic variation in monozygotic twins with the same mutation (C2203T) in exon8 of *ABCD1*, where neuroimaging studies were found normal for the first twin, and parietooccipital demyelination was found in the second twin at ten years of age. Sobue *et al*^[24] also report genetically confirmed monozygotic twins who presented with different clinical types of X-ALD. Although myeloneuropathy was present in both twins, widespread brain demyelination with cognitive dysfunction and behavioral symptoms was pronounced in the older twin, while the younger twin presented with adrenal insufficiency.

CAUSE OF PHENOTYPIC VARIABILITY IN X-ALD

Despite various studies, the exact cause of the phenotypic variability is not clear. Over > 800 mutations have been characterized in the *ABCD1* gene, but, based on the observations of various studies, it is clear that there is no association between the genotype and the different phenotypes of X-ALD. Identical defects in the *ABCD1* gene have been found in cases with different types of X-ALD (CCALD, adult CALD, AMN, Addison only, and asymptomatic)^[25,26]. Mutations that can cause deleterious damage to the protein, such as large deletions are reported in severe cerebral forms and milder types such as AMN and asymptomatic cases^[27]. These data support the assumption that factors other than the X-linked locus participate in deciding the phenotype. It is

possible that the specific mutation, along with the influence of individual genetic and environmental factors commonly referred to as “modifiers”, could play a crucial role in penetrance and the disease severity. Exploring these potential modifiers and understanding their roles in defining the phenotype in X-ALD associated with a specific mutation in the *ABCD1* gene is crucial in predicting the disease phenotype.

POTENTIAL MODIFIERS IN X-ALD

A variant at a particular genetic locus may not be adequate to determine the clinical phenotype, severity, and progression in human diseases^[28]. Direct or indirect association of different genetic, epigenetic factors, and environmental factors can change the expressivity, penetrance, and severity of a disease progression (Figure 1). The relative involvement of multiple modifiers to the disease phenotype may generate a combined impact on the phenotypic expression, and the combination of these modifiers may differ among individuals. Identifying these modifying factors and establishing a collective association with different clinical phenotypes is very challenging, but maybe crucial for appropriate management of the disease.

Modifier genes

Phenotypic variability of a disease can be explained by the influence of other genes apart from the gene involved in the disease, and these genes are called “modifier genes”^[29]. Modifier genes can affect the expression or function of another gene. The final impact of these genes on clinical variability could depend on their collective interaction and the interplay of other epigenetic or environmental factors. Genetic segregation analysis of a considerable number of families with X-ALD and analysis of concordant and discordant siblings indicates that a modifying gene, with an allele frequency of approximately 0.5, could be the main determining factor for phenotypic differences^[16,30]. Numerous studies have been directed towards identifying potential modifier genes that control the clinical variability of X-ALD. The foremost challenge for many studies is the small sample size for detecting the genetic association. These studies have attempted to investigate a modifier role in various genes involved in the metabolism of VLCFA, inflammatory pathways, methionine metabolism, and bile acid metabolism (Table 1). However, no studies have attempted to elucidate an interactive association of different genes with different phenotypes associated with the primary mutation in the *ABCD1* gene.

Genes involved in peroxisomal metabolism of VLCFA: The molecular defect in X-ALD is a deficiency of the ALDP protein due to which there is a defective passage of VLCFA into the peroxisome. In the peroxisomal matrix, saturated and unbranched VLCFA are metabolized by enzymes of the β -oxidation pathway^[31]. In patients with X-ALD, VLCFA, particularly C26:0, collect in various tissues and are incorporated into different complex lipids. Excess levels of VLCFAs and VLCFA-containing lipids are considered as biochemical triggers playing a central part in the development of X-ALD.

The superfamily of ATP-binding cassette transporters, which ALDP belongs to, also includes ALDRP, PMP70, and ABCD4 coded by *ABCD2*, *ABCD3*, and *ABCD4* genes. Experimental data suggest that *ABCD2* and *ABCD3* genes, when over-expressed, can supplement the biochemical defect in ALD fibroblasts^[32]. However, Asheuer *et al*^[33] demonstrated that the concentrations of *ABCD2* transcripts were similar in the unaffected white brain matter in different ALD phenotypes suggesting that difference in *ABCD2*-gene expression was not likely to contribute to the vulnerability for cerebral demyelination. In contrast, the expression of *ABCD4* genes correlated with the predisposition for brain demyelination and showed a trend of an association with CCALD, AMN-cerebral, and pure AMN phenotypes. Two other independent association studies have reported that ALD phenotypes are not associated with the *ABCD2* genotype^[34]. A Japanese study found no significant association of SNPs in *ABCD2*, *ABCD3*, and *ABCD4*, and ALD phenotypes, except for five single nucleotide polymorphisms in *ABCD4*, were less commonly found in AMN patients than in controls, but no significant association with CCALD (Table 1). However, a repetition of this study of five SNPs on another group of French ALD patients found no significant link with CCALD or pure AMN^[35].

Accumulation of VLCFA could also result from the excessive lengthening of long-chain fatty acids to VLCFA in the cell^[36]. This increased elongation can be due to enhanced expression of elongases and/or imbalance in the degradation and synthesis

Table 1 List of potential modifier genes for X-linked adrenoleukodystrophy investigated in various studies

Series No.	Gene name	Variants studied	No. of cases	Inference	Ref.
Genes associated with VLCFA metabolism					
I	ABCD2	rs11172566	117	No significance	Maier <i>et al</i> ^[34]
		rs11172661		No significance	
I	ABCD2	A/T(5'UTR)	280	No significance	Matsukawa <i>et al</i> ^[35]
		M94V		No significance	
II	ABCD3	rs4148058	280	No significance	Matsukawa <i>et al</i> ^[35]
		rs2147794		No significance	
		rs16946		No significance	
		rs681187		No significance	
		rs662813		No significance	
		rs337592		No significance	
III	ABCD4	rs17182959	280	No significance	Matsukawa <i>et al</i> ^[35]
		rs17158118		No significance	
		rs17782508		No significance	
		rs2301345		No significance	
		rs4148077		No significance	
		rs4148078		No significance	
		rs3742801		No significance	
IV	Cytochrome P450 4F subfamily (CYP4F2)	rs21086622	152	Minor allele A associated with CALD (<i>P</i> = 0.036)	van Engen <i>et al</i> ^[38]
		rs3093207		No significance	
		rs1272		No significance	
		rs3093200		No significance	
		rs3093194		No significance	
		rs3093166		No significance	
		rs4808400		No significance	
		rs3093153		No significance	
		rs3093135		No significance	
		rs3093105		No significance	
Genes associated with methionine metabolism					
I	Cystathionine β-Synthase (CBS)	c.844_845ins68	86	Associated with pure AMN	Linnebank <i>et al</i> ^[46]
I	Cystathionine β-Synthase (CBS)	c.844_845ins68	172	No significance	Semmler <i>et al</i> ^[48]
II	Methionine synthase (MTR)	c.2756A>G	86	No significance	Linnebank <i>et al</i> ^[45]
II	Methionine synthase (MTR)	c.2756A>G	172	No significance	Semmler <i>et al</i> ^[48]
III	Methylenetetrahydrofolate reductase (MTHFR)	c.677C>T	86	No significance	Linnebank <i>et al</i> ^[45]
III	Methylenetetrahydrofolate reductase (MTHFR)	c.677C>T	172	No significance	Semmler <i>et al</i> ^[48]
		c.1298A>C		No significance	
IV	Dihydrofolate reductase (DHFR)	c.594+59del19bp	172	No significance	Semmler

					<i>et al</i> ^[48]
V	5-Methyltetrahydrofolate-Homocysteine Methyltransferase Reductase (<i>MTRR</i>)	c.60A>G	172	No significance	Semmler <i>et al</i> ^[48]
VI	Transcobalamin 2 (TC2)	c. 776C>G	86	GG genotype prevalent in AMN with demyelination compared to pure AMN ($P = 0.001$)	Linnebank <i>et al</i> ^[45]
VI	Transcobalamin 2 (TC2)	c. 776C>G (GG)	172	GG genotype associated with demyelination ($P = 0.036$)	Semmler <i>et al</i> ^[48]
VII	Reduced folate carrier 1 (<i>RFC1</i>)	c.80G>A	172	No significance	Semmler <i>et al</i> ^[48]
Genes associated with inflammation					
I	<i>TNF-α</i>	G- 308A	15	No significance	McGuinness <i>et al</i> ^[64]
II	Cluster of differentiation (<i>CD1</i>)	CD1A-CD1E	139	No significance	Barbier <i>et al</i> ^[44]
III	Human leukocyte antigen (<i>HLA</i>)	HLA-DRB1*16	29	HLA-DRB1*16 associated with X-ALD ($P < 0.02$)	Berger <i>et al</i> ^[40]
		HLA-DRB1*15		No significance	
III	Human leukocyte antigen (<i>HLA</i>)	HLA-DBR1*	70	No significance	Schmidt <i>et al</i> ^[41]
III	Human leukocyte antigen (<i>HLA</i>)	HLA-DRB1*16	106	No significance	McGuinness <i>et al</i> ^[65]
		HLA-DRB1*15			
IV	Interleukin 6 (<i>IL6</i>)		68	No significance	Schmidt <i>et al</i> ^[41]
V	Myelin Oligodendrocyte glycoprotein (<i>MOG</i>)	(TAAA)n	68	226bp (TAAA)n polymorphism associated with the presence of Anti-MOG antibody. ($P < 0.05$).	Schmidt <i>et al</i> ^[41]
V	Myelin oligodendrocyte glycoprotein (<i>MOG</i>)	G15A	44	No significance	Gomez-Lira <i>et al</i> ^[42]
		CTC 5 repeats		No significance	
		G511C		No significance	
		G520A		No significance	
		551+68 A→G		No significance	
		551+77 C→T		No significance	
Other genes					
I	Superoxide oxide dismutase (<i>SOD2</i>)	rs4880		T-allele associated with cerebral involvement in non-CCALD cases	Brose <i>et al</i> ^[49]
		rs2758352		No significance	
		rs2842980		No significance	
		rs2758329		No significance	
II	Apolipoprotein E (<i>APOE</i>)	rs7412 rs429358	83	APOE4 associated with cerebral involvement	Orchard <i>et al</i> ^[52]
III	Cytochrome P450 family 7 subfamily A member 1 (<i>CYP2A1</i>)	rs3824260 (c.-533T>C) rs3808607 (c.-267C>A)	Study carried out on a patient diagnosed with AMN with c.659T>C mutation in ABCD1 gene in patient and mother	CC allele observed in patient whereas CT in mother AA allele observed in patient whereas CA in mother	Platek <i>et al</i> ^[50]
IV	3 β -hydroxysteroid dehydrogenase type 7 (<i>HSD3B7</i>)	rs9938550 (c.748A>G) rs2305880 (c.1068T>C)		GG allele observed in both CC allele observed in patient	
V	Bile acyl-CoA synthetase (<i>SLC27A5</i>)	rs4810274 (c.1668-6T>C)		CC observed in patient	

VI	Aldo-keto reductase family 1 member D1 (<i>AKR1D1</i>)	c.-71G>C	GC observed in patient
VII	Cytochrome P450 Family 27 Subfamily A Member 1 (<i>CYP27A1</i>)	rs397795841 (c.-357dupC)	Homozygous mutation in both

AMN: Adrenomyeloneuropathy; CALD: Cerebral adrenoleukodystrophy; CCALD: Childhood cerebral adrenoleukodystrophy; VLCFA: Very-long-chain fatty acid; X-ALD: X-linked adrenoleukodystrophy.

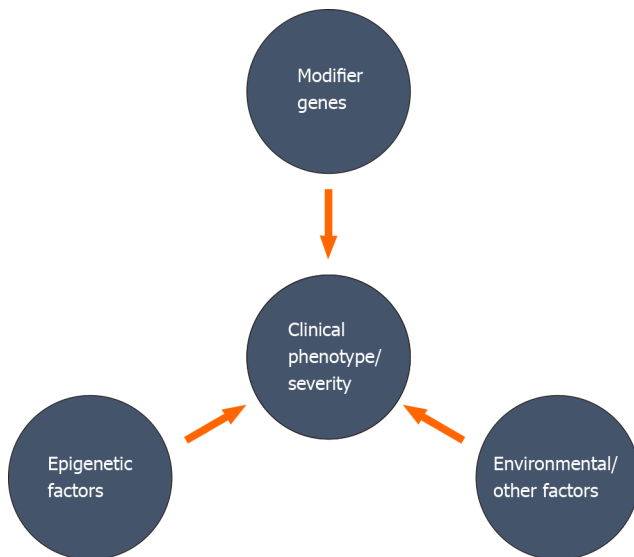


Figure 1 Possible modifiers associated with disease phenotypic variability. Phenotypic heterozygosity observed in a monogenic disorder can be due to the association of modifying factors such as genetic, epigenetic, and/or environmental factors, commonly termed as “modifiers.”

of VLCFA. Ofman *et al*^[37] reported no change in the expression of *ELOVL1* in X-ALD fibroblast, therefore ruling out the possibility of VLCFA accumulation due to increased expression of *ELOVL1*. However, knockdown of *ELOVL1* showed a reduction in C26:0 concentrations in X-ALD fibroblasts, thus indicating that *ELOVL1* could be a possible modifier for X-ALD.

The oxidation of VLCFA starts with its activation by coenzyme A and enzymes with very-long-chain acyl-CoA synthetase (VLACS) activity. In a study of the unaffected brain white matter from X-ALD cases, Asheuer *et al*^[33] have found that the expression of VLACS genes did not correlate with the clinical phenotypes (CALD and AMN phenotypes). On the contrary, lower expression of the *BG1* gene, which codes for a non-peroxisomal synthetase which activates VLCFA to its coenzyme A derivatives, was found in the white matter of ALD patients which correlated with the presence of cerebral demyelination. Hence *BG1* could be considered as a potential modifier gene^[33].

VLCFAs can also undergo ω -oxidation, and are further converted to dicarboxylic acids by the cytochrome P450 system. These reactions may present another route for the metabolism of the accumulated VLCFAs. The gene, *CYP4F2*, codes for a critical enzyme in the ω -oxidation of VLCFA to very long-chain dicarboxylic acids. van Engen *et al*^[38] reported that the *CYP4F2* polymorphism (*CYP4F2* p.433M) increased the chances of acquiring CALD in male Caucasians (Table 1). They further demonstrated the functional impact of the *CYP4F2* p.433M variant on cellular models, which showed reduced *CYP4F2* protein level, led to a reduction in the metabolism of VLCFA through ω -oxidation.

Inflammation-related genes: Brain inflammation and the ensuing progressive inflammatory demyelination is characteristically found in the demyelinating forms of X-ALD. Acute inflammation occurs only in the CNS and not in other tissues of the affected cases^[39]. Variants in the genes playing a role inflammation have been speculated to influence disease variability. The putative modifying genes could participate in an inflammatory response to the buildup of VLCFA or some other related metabolite in the brain. Since the pathology of the cerebral form is akin to that seen in multiple sclerosis (MS), some of the genetic factors involved in triggering inflammation in multiple sclerosis could also participate in the pathogenesis of X-ALD.

Genetic variants of specific major histocompatibility complex class II antigens (*HLA-DRB1*) are reported to be associated with the risk for MS and could be suitable candidate modifiers for X-ALD. Berger *et al*^[40] described a significant relationship between the *HLA-DRB1**16 allele and X-ALD. However, this allele did not show association with CALD, the inflammatory phenotype of X-ALD. The *DRB* genes are involved in the synthesis of peptides receptors playing a central role in the immune system.

Myelin oligodendrocyte glycoprotein (MOG) is the main target for demyelinating autoantibodies in MS. Schmidt *et al*^[41] found increased serum anti-MOG in X-ALD cases and reported that these were linked with *MOG* (TAAA)*n* gene polymorphism but not with clinical types of ALD. Gomez-Lira and coworkers^[42] identified six sequence variants in *MOG* gene: G15A, G511C, G520A, CTC repeats in exon1, 551168A→G and 551177C→T in X-ALD patients, but no frequency difference was observed in cases when compared to controls.

Tumor necrosis factor (TNF-α), a major pro-inflammatory cytokine, is involved in the pathogenesis of many neurological disorders including MS. TNF-α is capable of causing damage to the myelin sheath and oligodendrocytes and has also found to modulate the *MBP* (Myelin basic protein) gene promoter activity, *via* activation of NF-κB transcriptional factor in oligodendrogloma cells^[43]. However, increased TNF-α bioactivity was not found to be associated with any allelic difference in the *TNF-α* gene (G-308A).

Genetic variants of the cytokine, interleukin-6 (*IL-6*), such as the *IL-6* C-allele which is a variable number tandem repeat polymorphism situated on the 3' flanking region of the *IL-6* gene, is reported to be linked to late-onset Alzheimer's and MS, but no association was found with the different clinical phenotypes of X-ALD^[41].

Neuroinflammation in CALD is suspected to be due to the involvement of different classes of lipids enriched in the VLCFA^[2]; thus, the participation of CD1, a lipid antigen-presenting molecule, was speculated. Barbier *et al*^[44] assessed the association between the genetic variants of CD1 molecules (*CD1A-E*) and the presence of neuroinflammation in X-ALD but found no association between them.

Genes associated with methionine metabolism: The pathological characteristic of the cerebral type of X-ALD is CNS demyelination. Demyelination starts in the mid of corpus callosum and advances outwards in both brain hemispheres. This leads to a gradual neurologic decline and death within 3 to 5 years^[3].

The sulfur-containing amino acid, methionine, plays a vital metabolic role in providing methyl group required for DNA methylation, brain myelination, and precursors for the generation of glutathione taurine. S-adenosyl methionine (SAM), the active form of methionine, is a methyl donor. Deficiency of SAM can lead to demyelination in the CNS. Studies have reported variants of methionine metabolism as risk factors causing demyelination in X-ALD patients. Linnebank *et al*^[45] studied the combined risk genotype, *i.e.* the occurrence of a minimum of one distinct genotype of three functional polymorphisms in genes associated with methionine metabolism, 5,10-methylenetetrahydrofolate reductase (*MTHFR*) c.677CT, methionine synthase (*MTR*) c.2756AG, and transcobalamin 2 (*Tc2*) c.776CG, in 86 patients with various phenotypes of X-ALD. These authors reported that CCALD patients tended to have a higher prevalence of the combined risk genotype (46%) in comparison to the group with the benign variant "pure" AMN (33%; *P* = 0.222) due to a higher prevalence of the *MTR* (41% *vs* 22%, *P* = 0.110) and the *Tc2* risk genotype (18% *vs* 14%, *P* = 0.675). Moreover, this genotype was overrepresented in patients with AMN with CNS demyelination (AMN-cerebral) when compared to 49 AMN patients without CNS demyelination ("pure" AMN) and suggested that variations in genes associated with methionine metabolism might influence the phenotypic variability in X-ALD. Cystathionine β-synthase (*CBS*) is another important enzyme in the methionine metabolic pathway, and the *CBS* c.844_845ins68 variant may affect the availability or concentrations of activated methionine and glutathione. Linnebank and colleagues also found that *CBS* c.844_845ins68 insertion allele protected X-ALD patients from cerebral demyelination^[46].

In another study of *CBS* c.844_845ins68, *MTR* c.2756A to G, and *TC2* c.776 C to G in 120 Chinese ALD patients, the frequency of only the GG genotype of the *TC2* c.776 C/G was more in those with brain demyelination than in controls^[47]. *TC2* is the transport carrier protein for cobalamin and methylcobalamin, the active form of cobalamin, a crucial cofactor necessary for the enzymatic activity of methionine synthase.

These results were further confirmed by Semmler *et al*^[48] who genotyped eight polymorphisms in methionine metabolism genes, including *CBS* c.844_845ins68,

MTHFR c.677C>T, *MTR* c.2756A>G and *DHFR* c.594+59del19bp, and found *Tc2* c.776 GG genotype to be more prevalent in X-ALD cases with clinical features of brain demyelination compared to those without demyelination.

Other potential genetic modifiers: Reactive oxygen species (ROS) can trigger oxidative damage to DNA and proteins and ineffective oxidative phosphorylation, and this could result in dying-back axonopathy. Axonal degeneration in the spinal cord is typically observed in the AMN form of X-ALD. The mitochondrial superoxide dismutase (*SOD2*) is responsible for detoxifying ROS and is considered a modifying factor for the development of demyelination in X-ALD. A study reported that *SOD2* variant C47T and GTAC haplotype with reduced activity were associated with adolescent cerebral, adult cerebral X-ALD, and AMN- cerebral patients^[49] (Table 1).

Bile acid metabolism occurs in the peroxisomes. An abnormal bile acid profile and mutations in the genes associated with the metabolism of bile acids such as *CYP7A1*, *CYP27A1*, *CYP7B1*, *HSD3B7*, *AKR1D1*, and *SLC27A52*, has been reported in a Polish AMN patient and these genes have been suggested as potential modifiers of X-ALD^[50] (Table 1). However, more studies are required to confirm this association

Apolipoprotein E, a protein associating with lipid particles and functioning in lipoprotein-mediated lipid transport between organs, has three isoforms APOE2, APOE3 and APOE4 encoded by three alleles situated on a single gene locus. APOE3 protein maintains the blood-brain-barrier integrity (BBB) through the downregulation of cyclophilin A (CypA), a pro-inflammatory protein^[51]. Male X-ALD patients bearing the *APOE4* genotype are reported to have greater cerebral involvement as determined by MRI severity score, lesser neurologic function, and elevated concentrations of matrix metalloproteinase-2 (MMP-2) in the cerebrospinal fluid compared to non-carriers^[52]. The presence of the *APOE4* allele has been suggested to upregulate CypA leading to the activation of MMP-9 and loss of BBB integrity, leading to increased severity of cerebral disease in cerebral ALD^[51].

Influence of epigenetic factors

Epigenetic factors, too, can influence the onset of disease by inducing a subtle change in the gene expression without any notable alteration in the DNA sequence. Epigenetic alterations comprise DNA methylation, post-translational modifications of histones such as methylation, phosphorylation, acetylation, and post-transcriptional regulation by non-coding RNA.

DNA methylation acts as a regulatory mechanism for gene expression, and cell differentiation and various studies have demonstrated the association between change in DNA methylation and disease pathogenesis^[53]. A study by Schlüter *et al*^[54] compared the genome-wide DNA methylation pattern of unaffected frontal brain white matter of patients with CCALD and AMN with cerebral involvement and found hypermethylation of genes that are majorly involved in differentiation of oligodendrocytes including *MBP*, *CNP*, *MOG*, *PLP1* that can result to impaired differentiation of oligodendrocyte precursor cells to remyelinating oligodendrocyte and hypomethylation of genes associated with an immune function such as *IFITM1* and *CD59*. This supports the neuropathological evidence of lack of remyelination and immune activation noted in the cerebral form of X-ALD. This study also showed that combined methylation levels of *SPG20*, *UNC45A*, and *COL9A3* and combined expression levels of *ID4* and *MYRF* could be useful as biomarkers for differentiating CALD from AMN.

Aberrant expression of microRNAs (miRNAs), a group of small non-coding RNAs regulating post-transcriptional gene expression, has been suggested to play a significant part in the development of neuroinflammation and degeneration^[55]. Shah *et al*^[55] found decreased expression of miR-196a and increased expression of *ELOVL*, *IKKα*, *IKKβ*, *MAP4K3*, and *MAP3K2* in cerebral ALD compared to AMN and control fibroblasts, and suggested that the regulation of inflammatory signaling pathway in CALD brain occurs *via* miR-196a.

Other potential modifying factors

Various host or cellular environmental factors may influence disease development in an individual. Oxidative stress is a common phenomenon reported in various neurodegenerative disorders, including X-ALD. Overproduction of free radicals results in lipid peroxidation, whose byproducts can cause deleterious damage to the cells^[56]. Nury *et al*^[57] observed reduced plasma levels of oxidative stress markers such as α-tocopherol, GSH, and docosahexaenoic acid (DHA) in different X-ALD phenotypes. These authors also showed that 7α-hydroxycholesterol, 7β-hydroxycholesterol, 7-ketocholesterol, and 9- and 13-hydroxyoctadecadienoic acids

were produced as a result of oxidative stress. Increased level of 7-ketocholesterol was found to cause overproduction of free radicals, activation of PRAP-1, and caspase 3 and elevated LC3-II/LC3-I and p62 in BV-12 microglia cells, indicating its ability to induce cell death^[53]. A recent study demonstrated 7-ketocholesterol induced activation of PRAP-1 *via* NF- κ B transforms microglial cells from a resting stage to an active stage, ultimately damaging the neurons. As 7-ketocholesterol induces oxidative stress, inflammation, and cell death, high levels could enhance peroxisomal dysfunction in microglial cells, promoting brain damage in the affected patients.

Jang *et al.*^[58] demonstrated the abnormal generation of cholesterol 25-hydroxylase and 25-hydroxycholesterol in CCALD patient-derived cell models and showed that 25-hydroxycholesterol aids the aggregation and activation of NLRP3 inflammasome, a caspase-1-activating multi-protein complex, resulting in increased formation of pro-inflammatory cytokines, IL-1 β , IL-18^[59]. 25-hydroxycholesterol has also been found to induce mitochondrial-dependent apoptosis of cells *via* the stimulation of glycogen synthesis kinase-3 β (GSK-3 β)/LXR pathway in the amyotrophic lateral sclerosis cell model^[60]. This could also account for severe cerebral inflammatory demyelination, the hallmark of CCALD.

Trauma to the head has been speculated to trigger or worsen symptoms in X-ALD^[61], and asymptomatic cases of X-ALD presenting with symptoms after head trauma have been reported. The inflammatory response following a traumatic brain injury, followed by mitochondrial dysfunction, oxidative stress, and disruption of the BBB, has been suggested to activate cerebral inflammatory demyelination resulting in the appearance of symptoms^[62].

The major difference between the different X-ALD phenotypes is the presence or absence of neuroinflammation and cerebral demyelination. The inflammatory response in the brain is believed to begin after the abnormal accumulation of VLCFA. ALDP deficiency has been shown to induce alteration in brain endothelial cells favoring the migration of leukocytes by downregulating the expression of c-Myc, leading to a reduction in the expression of cell surface tight junction proteins CLDN5 and ZO1 and increased expression of cell adhesion molecule ICAM-1 and MMP9^[63]. We speculate that the migration of immune cells into the brain could be a rate-limiting step in the induction of cerebral demyelination. The onset of the migration across the BBB could be precipitated by various environmental triggers, genetic or epigenetic factors and ABCD1 deficiency acting either alone or in concert, marking the onset of brain inflammation leading to cerebral symptoms (Figure 2). Further, the absence of neuroinflammation in “pure AMN” and “Addison’s only” phenotype could also possibly be due to the involvement of modifiers that are protective against the VLCFA toxicity in the brain. For instance, it is commonly known that MMP2 and MMP9 are required for the migration of the immune cells across the endothelial basal membrane and parenchymal border, respectively. However, the delay in the synthesis and secretion of MMP2 and MMP9 could delay the migration process and, ultimately, the disease onset. Genetic factors such as *APOE4*, which is shown to be associated with cerebral involvement in young males, are also associated with increased expression of MMP9 *via* cyclophilin A, leading to BBB leakiness. Thus, a complex interplay between multiple determinants could affect the onset and severity of the disease symptoms.

CONCLUSION

The role of different modifiers influencing disease phenotypes has been described in various metabolic disorders. Therefore, though X-ALD is a monogenic disorder, other genetic factors, along with the environmental triggers, may be responsible for the severity and penetrance of the disease. Although numerous studies have made efforts to understand different genetic, epigenetic, and environmental factors in X-ALD, the exact cause of phenotypic differences in X-ALD patients with the same genotype is not clear. Improved knowledge of these factors will allow identification of patients prone to developing a particular form or clinical type of X-ALD. Besides, detailed elucidation of the association of different potential modifiers with the clinical heterozygosity in X-ALD is crucial for understanding the disease pathogenesis and for developing novel therapeutic strategies. With the introduction of neonatal screening for X-ALD, X-ALD modifiers will become increasingly essential to categorize patients who are likely to develop cerebral demyelination and plan appropriate management of these patients.

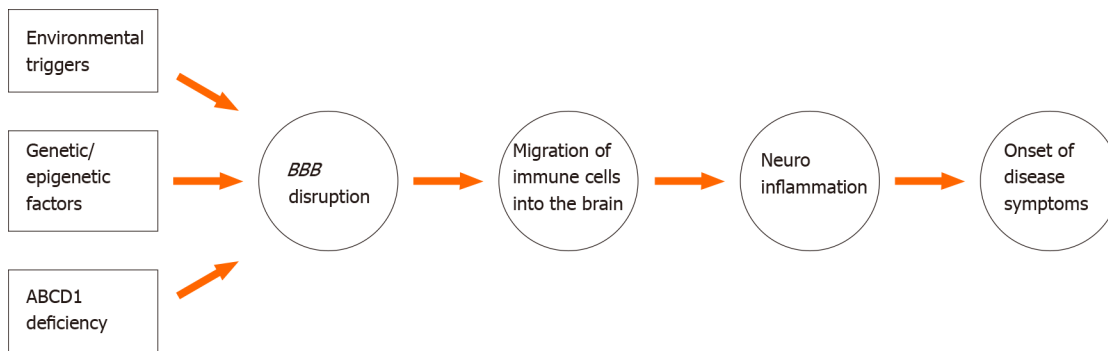


Figure 2 Factors determining the onset of symptoms of cerebral demyelination. Migration of immune cells into the brain could be a rate-limiting step in the appearance of symptoms of cerebral demyelination. Onset of the migration across the blood brain barrier could be precipitated by environmental triggers, genetic or epigenetic factors and *ATP Binding Cassette Subfamily D Member 1 (ABCD1)* deficiency acting either alone or in concert, marking the onset of brain inflammation leading to cerebral symptoms.

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