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ABCD1 and X-linked adrenoleukodystrophy: A disease with a markedly variable phenotype showing conserved neurobiology in animal models

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Abstract

X-linked adrenoleukodystrophy (X-ALD) is a phenotypically heterogeneous disorder involving defective peroxisomal β-oxidation of very long-chain fatty acids (VLCFAs), due to mutation in the *ABCD1* gene. X-ALD is the most common peroxisomal in-born error of metabolism and confers a high degree of morbidity and mortality. Remarkably, a subset of patients exhibit a cerebral form with inflammatory invasion of the central nervous system and extensive demyelination, while in others only dying-back axonopathy or even isolated adrenal insufficiency is seen, without genotype–phenotype correlation. X-ALD's biochemical signature is marked elevation of VLCFAs in blood, a finding that has been utilized for massive newborn screening for early diagnosis. Investigational gene therapy approaches hold promises for improved outcomes. However, the pathophysiological mechanisms of the disease remain poorly understood, limiting investigation of targeted therapeutic options. Animal models for the disease recapitulate the biochemical signature of VLCFA accumulation and demonstrate mitochondrially generated reactive oxygen species, oxidative damage, increased glial death, and axonal damage. Most strikingly, however, cerebral invasion of leukocytes and demyelination were not observed in any animal model for X-ALD, reflecting upon pathological processes that are yet to be discovered. This review summarizes the current disease models in animals, the lessons learned from these models, and the gaps that remained to be filled in order to assist in therapeutic investigations for ALD.

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of the article at the publisher's website.

CONFLICT OF INTEREST

The authors have no conflict of interest to report.

Keywords

ABCD1 ; animal model; demyelination; dying-back axonopathy; peroxisome; very long-chain fatty acid; X-linked adrenoleukodystrophy

1 | X-LINKED ADRENOLEUKODYSTROPHY: A PEROXISOMAL DISORDER ENTERING A NEW ERA IN NEWBORN SCREENING

Peroxisomes are essential multifunctional organelles that contribute to numerous anabolic and catabolic pathways such as hydrogen peroxide metabolism, synthesis of bile acids, plasmalogens, and the β-oxidation of very long-chain fatty acids (Cooper, 2000; Islinger et al., 2012). A number of described Mendelian disorders in human are linked to defects in peroxisomal proteins, resulting in multisystem involvement and often premature death. The most commonly inherited peroxisomal disorder is X-linked adrenoleukodystrophy (X-ALD), a progressive disorder that affects myelin and axons in the CNS and the adrenal gland, with a prevalence of affected males and females at 1:17,000 (Moser et al., 2016). The spectrum of the disease includes three distinct phenotypes: (i) *childhood cerebral ALD* (ccALD), which accounts for approximately 35% of the cases, manifested by early childhood onset of inflammatory demyelination, progressive impairment of cognition and behavior, and early death; (ii) *adrenomyeloneuropathy* (AMN), later onset and slowly progressive peripheral axonopathy that results in progressive stiffness and weakness of the legs and seen in 40%–45% of affected individuals, and (iii) *adrenal insufficiency* (10%) in which the adrenal cortex is the only affected organ at presentation (Berger et al., 2014; Raymond et al., 1993). The nature of X-ALD is progressive: males with adrenal insufficiency can later develop either AMN or ccALD, and AMN males can develop cerebral demyelination (Engelen et al., 2012). Moreover, about 65%–80% of females will show signs of AMN by the age of 60 (Engelen, Barbier, et al., 2014; Moser et al., 2016). All cases of X-ALD are due to loss-of-function variants in the gene *ABCD1*, a highly conserved gene located at Xq28 and encodes for ATP-binding cassette (ABC) transporter, subfamily D, named ALDP (or ABCD1) (Moser, 2006). Variants in this gene are diverse: according to the http://adrenoleukodystrophy.info database, more than 2,800 variants in *ABCD1* had been recognized, with the most common pathogenic variant representing only 7% of cases, and more than one in four of the variants are nonrecurrent (unique to one family). There is no established genotype–phenotype correlation between *ABCD1* variants and specific presentation of X-ALD (Matsukawa et al., 2011), even within the same family, and in monozygotic twins, discordant phenotypes are reported (Korenke et al., 1996).

The biochemical fingerprint of X-ALD is the elevation of very long-chain fatty acids (VLCFAs), which are saturated fatty acids comprised of 22-carbon backbone or more, and in particular, the significant elevation of hexacosanoic acid (C26:0) (Watkins et al., 1987). Similar elevations are observed with defects in *ACOX1* gene, encoding the peroxisomal acyl-coenzyme A oxidase 1, which participates in the first step of peroxisomal β-oxidation of VLCFAs and results in a β-oxidation defect leading to pseudoneonatal ALD (El Hajj et al., 2012). ABCD1 is believed to directly import Coenzyme A (CoA)-conjugated VLCFA into the peroxisome; however, the exact mechanism is not completely understood

(Kawaguchi & Morita, 2016). In fibroblasts derived from X-ALD patients, β-oxidation is strongly reduced, an attenuation that can be replicated in normal fibroblasts when incubated with inactivating ABCD1 antibodies (Berger et al., 2014). Yeasts' peroxisomal ABC transporter, a heterodimer of Pxa1p and Pxa2p, was shown to be involved in the transport of a spectrum of acyl-CoA esters, and *ABCD1* cDNA rescued the *pxa1-Δ/pxa2-Δ* double mutant (van Roermund et al., 2008). Furthermore, it was shown that very long-chain acyl-CoA esters are hydrolyzed by the Pxa1p–Pxa2p complex prior to the actual transport (van Roermund et al., 2012). *COMATOSE*, *Arabidopsis thaliana's* analog to ABCD1, was shown to possess intrinsic ATP-dependent thioesterase activity (De Marcos Lousa et al., 2013), which upon its specific inhibition resulted in defective fatty acid degradation. The essentiality of the thioesterase activity has also been demonstrated in ABCD1, when expressed in yeasts (Kawaguchi et al., 2021), suggesting a two-step model in which the VLCFA-CoA binds to the thioesterase domain followed by an ATP-dependent conformational change and substrate hydrolysis, releasing VLCFA into the peroxisome. It is yet, however, to elucidate the involvement of elevated VLCFAs in the pathogenesis of X-ALD. The enrichment of phospholipid bilayers with C26:0 showed a disruptive effect on artificial bilayer structure (Berger et al., 2014); nonetheless, further evidence connecting VLCFAs to myelin disruption or the toxic effect of its accumulation in X-ALD pathogenesis remains to be explored.

In human, there are four different ABC transporter of the subfamily D, encoded by *ABCD1–4*. ABCD1–3 are localized in peroxisomes, and ABCD4 is inserted into the endoplasmic reticulum and targeted to lysosomes (Kashiwayama et al., 2009; Kawaguchi & Morita, 2016). Of interest, the ABCD2 shares some substrates with ABCD1, and double null mice (*Abcd1*$^{-}$/$^{-}$, *Abcd2*$^{-}$/$^{-}$) exacerbated VLCFA accumulation in macrophages when compared to *Abcd1*$^{-}$/$^{-}$ mice (Muneer et al., 2014), and its overexpression rescues VLCFA accumulation observed in *Abcd1*-deficient mice (Pujol et al., 2004). However, human ABCD2 expression in *Pxa1-Δ*/*Pxa2-Δ* yeast double mutant could not restore the elevated polyunsatu-rated VLCFA profile (van Roermund et al., 2011), suggesting that there is a different substrate specificity in yeasts (Kawaguchi & Morita, 2016). Furthermore, the polymorphism of *ABCD2* does not appear to be a significant modifier locus for X-ALD (Maier et al., 2008; Matsukawa et al., 2011). Several other disease modifier genes have been proposed (e.g., inflammation-related genes); however, more studies are required to correlate these modifiers with X-ALD progression (Wiesinger et al., 2015).

The two main phenotypes of X-ALD, ccALD, and AMN, differ substantially in their pathology. Brain tissues of ccALD patients show demyelinated lesions predominantly in the splenium of the corpus callosum and the parieto-occipital white matter (Engelen, Kemp, et al., 2014). The demyelinated lesions comprised of activated microglia, astrocytes, and infiltrating macrophages (Berger et al., 2014; Eichler et al., 2008), along with the heavy presence of perivascular macrophages and lymphocytes (Manz et al., 1980). The elevation of pro-inflammatory cytokines (IL-8, IL-1ra, MCP-1, MIP-1b) was observed in ccALD patients' CSF (Lund et al., 2012) while the stimulation of blood-derived monocytes with lipopolysaccharides (LPS) produced secretion of IL-12 and TNFα (Di Biase et al., 2001), both supporting Th1-mediated inflammatory response. Interestingly, in autopsy cases of ccALD, in adjacent to lesions of active demyelination, perilesional areas

show marked microglia loss that preceded oligodendrocyte decay and myelin breakdown, and showed a pattern suggesting of programmed cell death (Bergner et al., 2019). Moreover, neither demarcated areas of demyelination nor focal remyelination areas were found in brain specimen of ccALD patients, distinguishing X-ALD's pathology from multiple sclerosis (Bergner et al., 2021). Yet, a multi-omic analysis (including genomic, transcriptomic, epigenomic, proteomic, and lipidomic) of six affected patients compared to discordant siblings found no applicable molecular markers indicative of early signs of CNS involvement (Richmond et al., 2020).

In contrast, individuals with AMN are spared of the characteristic large demyelinating lesions in the CNS with minimal to no reactive astrocytosis and lymphocytosis (Powers et al., 1982). The spinal cord shows marked symmetric atrophy prominently in the lateral corticospinal, gracile, and spinocerebellar tracts, with distal (dying-back) axonopathy and myelin loss and Periodic acid–Schiff (PAS)-positive macrophages and gliosis, but does not show any evidence of perivascular lymphocytes or active inflammation (Powers et al., 2000). The accumulation of VLCFA-esters in lamellar-lipid profiles is observed in the adrenal cortex and Leydig cells, both observed on either AMN or adult-onset cerebral ALD without signs of active inflammation and are suspected to lead to adrenocortical failure and testicular atrophy (Powers et al., 1982; Turk et al., 2020). Lipidomics, in combination with transcriptomic, revealed that patients with AMN showed an upregulation of sphingolipid catabolism and triglycerides anabolism when compared to ccALD patients indicating that there is a potential recovery mechanism to avoid a VLCFA toxicity. (Lee et al., 2019).

Given that the accumulation of VLCFA occurs prior to CNS damage, it has been thought that VLCFA toxicity is a driving force of both demyelination and axonopathy. Most studies on the toxicity of VLCFAs have been focused on the free fatty acids themselves (Turk et al., 2020), while in the brain tissues of ccALD patients, phosphatidylcholine, lysophosphatidylcholine, gangliosides, and cholesterol esters are identified, implying that there is more complexity to its pathogenesis (Singh & Pujol, 2010).

ABCD1 is ubiquitously expressed in skeletal myocytes, hepatocytes, the renal and endocrine systems, and in the stroma of connective tissues—fibroblasts, macrophages, and endothelium (Höftberger et al., 2007). However, in the CNS, its expression is limited to microglia, astrocytes, epithelial cells, and subpopulations of oligodendrocytes, while in neurons, it is only expressed in the hypothalamus, the basal nucleus of Meynert, periaqueductal gray matter, and locus coeruleus (Berger et al., 2014). Therefore, a neuroglia-centric approach for the disease's demyelinating process had been promulgated.

The demyelinated lesions of patients with ccALD showed marked apoptosis of oligodendrocytes (Feigenbaum et al., 2000). Moreover, incubation with C26:0 resulted in five to sixfold increase in cell death in 24 hr in rat brain-derived oligodendrocytes but not in neurons (Baarine et al., 2012), and sixfold increase in lipid-induced apoptosis in human fibroblasts (Baarine et al., 2012; van de Beek et al., 2017). Similar toxicity was observed toward astrocytes, in which incubation with VLCFAs resulted in increased apoptosis, strong accumulation of ROS, and in situ depolarization of mitochondria in *Abcd1*-deficient mice (Baarine et al., 2012; Kruska et al., 2015). Oligodendrocyte death occurs via apoptosis and

necrosis; however, it is preceded by the activation of autophagy in oligodendrocytes as a response to oxidative stress, similar to age-related neurodegenerative diseases (Doria et al., 2019). Furthermore, study of the epigenetic signature from intact frontal white matter of ccALD patients revealed hypermethylation of several oligodendrocyte differentiation factors, myelin basic protein (MBP), 2′,3′-cyclic nucleotide 3′ phosphodiesterase (CNP), myelin oligodendrocyte glycoprotein (MOG), and proteolipid protein 1 (PLP1) (Schlüter et al., 2018).

A significant body of evidence points to oxidative stress and damage in glial cells. Oxidative damage in the form of lipoxidation, carbonylation, and glycoxidation was observed in VLCFA-stressed rat-derived oligodendrocytes, *Abcd1* null mice's spinal cord, and *ABCD1*-deficient fibroblasts (Baarine et al., 2012; Fourcade et al., 2008), the latter also showing increased intracellular reactive oxygen species (ROS) when incubated with free VLCFAs (Fourcade et al., 2008). The accumulation of mitochondrial manganese superoxide dismutase and Hsp70 was seen in brain biopsies of ccALD patients (Khan et al., 2010), along with mitochondrial ROS production due to oxidative phosphorylation impairment in *ABCD1*-deficient fibroblasts (López-Erauskin et al., 2013), pointing to oxidative stress of mitochondrial origin. Furthermore, the excess production of ROS was reversed when treating the cells with inhibitors of complex I, II, and V (Fourcade et al., 2014) as did treatment with the antioxidant cocktail (*N*-acetylcysteine, tocopherol derivatives, and lipoic acid) (López-Erauskin et al., 2011). *Abcd1* null mice showed 50% decrease in mitochondrial DNA (Fourcade et al., 2015), rescued by the treatment with Pioglitazone, a peroxisome proliferator-activated receptor-gamma (PPARγ) agonist known to induce mitochondrial DNA replication, fatty acid oxidation, and thermogenesis (Morató et al., 2013).

Astrocytes from CNS tissue of ccALD patients exhibit a ubiquitous increase in leukotrienes (LTB4 and CysLT) which may act as a chemoattractant for leukocytes (Khan et al., 2010). Additionally, mouse-derived *Abcd1*-deficient immortalized astrocytes stimulated by LPS exhibit increased expression of pro-inflammatory cytokines (IL6, CCL2, CXCL10) and thus may be more responsive to innate immune stimuli (Morita et al., 2021). The oxidative stress associated with VLCFA accumulation creates oxidized cholesteryl species (hydroxyoctadecadienoic and hydroxycholesterols) (Baarine et al., 2012), which may contribute to the pathogenesis of the disease (Turk et al., 2020). Postmortem studies of ccALD patients showed marked increase in cholesterol esters of VLCFA in areas of early demyelination with microglia and lymphocyte infiltration (considered active zones), as compared to areas of intact myelin in which no cholesterol ester elevation is seen (Theda et al., 1992). It has been thought that VLCFA accumulation in microglia leads to the accumulation of esterified cholesterols by inducing a transcription of acyl-CoA cholesterol acyltransferase 1 (ACAT) as a stress response, while its counterpart, cholesterol hydrolases, has reduced catalytic activity for VLCFAs (Ogino & Suzuki, 1981; Turk et al., 2020). VLCFAs, on the contrary, induce the expression of cholesterol 25-hydrolase, which in turn lead to the overproduction of IL1β and increased caspase 1 activity, an effector of the NLRP3 inflammasome (Jang et al., 2016). The toxicity of VLCFA comes not only from VLCFA-cholesterol complex: Cortical injection of lysophosphatidylcholine–C24:0 complex resulted in apoptosis and microglial activation (Eichler et al., 2008). More clues regarding the cytotoxic effect of VLCFAs may arise from a recently developed *Abcd1:Abcd2* double

null murine microglial model, showing accumulation of lipid inclusions similar to those observed in brain macrophages of ccALD patients (Raas et al., 2019). However, it remains unclear whether the accumulation of VLCFAs directly induces oxidative stress and how either process triggers an immune response.

Treatment options are limited and include symptom-based therapies and hematopoietic stem cell transplant (HSCT), which halts the progression of ccALD (Fernandes et al., 2018), if performed before the manifestation of significant symptoms (Kühl et al., 2018). This latter requirement for early diagnosis is key for the success of HSCT therapy and is only accomplished by early recognition, frequent neuroimaging until changes become evident (expressed as Loes score). Newborn screens for X-ALD are changing the landscape of early recognition which has been the main challenge limiting the effectiveness of HSCT. A more recent approach utilizes an infusion of autologous $CD34^+$ cells transduced lentiviral vector including the reference *ABCD1* cDNA (Eichler et al., 2017). The specific mechanism by which HSCT assists in the stabilization of the disorder is unclear but is thought to mitigate the inflammatory response directed by $ABCD1^-$ microglia (see below) (Kühl et al., 2018). Posttransplant VLCFA level decreases slowly (years) to heterozygote level; however, the levels do not correlate with neurological outcome (Kato et al., 2019).

The elevation of VLCFAs is unrelated to dietary intake and is thought to be the result of the endogenous synthesis by the two paralogs ELOVL fatty acid elongase 1 and 6 (Berger et al., 2014). While research for the elucidation of the effect of VLCFA accumulation on the CNS is ongoing, clinicians had recognized the advantage of this early disease marker when trying to offer the time-sensitive transplant. In February 2016, the secretary of the US Department of Health and Human Services (HHS) recommended to include C26:0-lysophosphatidylcholine (C26:0-LPC) levels in newborn screening (NBS) based on results from New York State (Kemper et al., 2017). $ABCD1^-$ hemizygote males have about 5.5-fold increase in C26:0 level compared with healthy newborns, while carrier females have almost threefold increase (Raymond et al., 1993). While these numbers provide a clear cutoff for newborn screen laboratories, a population-wide overlap exists, and positive results trigger molecular sequencing for confirmation. Justification for NBS is based on established cutoffs for serum C26:0-LPC and the opportunity for early diagnosis and treatment that would otherwise not be beneficial if offered after the development of clinical symptoms. However, diagnostic dilemmas still occur: (i) other peroxisomal disorders can manifest with an elevation of C26:0-LPC on NBS and HSCT may not be suitable for them; (ii) since *ABCD1* gene lacks mutation “hotspots,” full sequencing is performed when the elevation of C26:0-LPC is obtained on NBS. Considerations include the possibility of exposure to serial sedated MRIs (Mallack et al., 2021) who would not ultimately go onto have ccALD, and (iii) due to lack of genotype–phenotype correlation nor to the VLCFA concentration, positive NBS with a confirmed pathogenic variant in *ABCD1* may not develop ccALD and yet similar to (ii) would be exposed to serial sedated MRI and some may even possibly undergo HSCT (if nonspecific findings on brain imaging are interpreted as ccALD lesions), a therapy modality that is not shown to be beneficial to the AMN or the Addison only presentation.

The molecular evidence of glial oxidative damage due to VLCFA accumulation and increased apoptosis, along with the pro-inflammatory cellular response, provides clues but not insights into the pathophysiology of either ccALD or AMN. To bridge the gap, vertebrate models for X-ALD can provide both cellular and tissue-level evidence for processes leading to dying-back axonopathy and myelin destabilization, while invertebrate models can assist in uncovering unexplored pathological mechanisms in neuroglia (Coutinho-Budd & Freeman, 2013). Both vertebrate and invertebrate animal models were constructed and provided invaluable information about X-ALD-related oxidative stress and mitochondrial involvement. However, X-ALD's unique phenotypic heterogeneity complicates the creation of a unifying disease model in animals and limits our comprehension of several hallmark aspects of the disease, in particular demyelination and immune invasion.

2 | MOUSE AND ZEBRAFISH MODELS OF X-ALD

2.1 | Mouse (*Mus musculus*)

Mice have genes orthologous to human *ABCD1* as well as *ABCD2*: Murine *Abcd1* and *Abcd2* orthologs bear 92% and 94% identity to the human proteins, respectively, with 0% gaps (Altschul, 1993). *Abcd1*-deficient mice show a reduced β-oxidation of VLCFAs that lead to a significant rise in levels of saturated VLCFAs in the brain, adrenal gland, heart, liver, and kidney (Forss-Petter et al., 1997; Kobayashi et al., 1997; Lu et al., 1997). Electron microscopy of adrenocortical cells from 6-month-old *Abcd1* null mice show cleft-like cytoplasmic and lysosomal lipid inclusion bodies, reminiscent of but not identical to human adrenal pathology in which ballooning and trilamellar lipid leaflets are observed. However, the lysosomal lipid cleft-like inclusions have been found in generalized peroxisomal disorders and in macrophages of ALD and AMN patients (Forss-Petter et al., 1997). At 12–13 months, electron-dense condensation of mitochondrial cristae with mitochondrial dissolution was observed (McGuinness et al., 2003).

CNS/PNS pathology	Pathophysiological lessons
• Axonal degeneration of sciatic nerve and spinal cord long tracts • Myelin thickening in sciatic nerve • Slow nerve conduction velocity with normal amplitude • Axonal damage reversed with antioxidant supplementation • No CNS immune system invasion • No CNS demyelination	• AMN-like late onset, progressive neurodegenerative phenotype with peripheral nerve axonal damage and hypermyelination. Axonal damage precedes myelin abnormalities • Oxidative stress as a driving force for axonal damage • Abcd2 overexpression reverts PNS neurodegeneration • No CNS involvement • No increase in neuroglial apoptosis
• Reduction in myelin component proteolipid protein 1a • Reduction in myelinated axons • 30% reduction of oligodendrocyte progenitor cells	• Model shows increase in C26:0, competitive disadvantage, and reduction in oligodendrocyte progenitor cells, reversible with human ABCD1 rescue • Competitive disadvantage • Increase in brain apoptosis, however not of oligodendrocytes

CNS/PNS pathology	Pathophysiological lessons
• Oligodendrocyte-produced myelin basic protein was unaltered	• Modest increase in VLCFA, life span reduction and decrease in oligodendrocytes
• Axonal damage of GABAergic motor neurons • Similar axonal damage was seen by knockdown of mitochondrial complex I subunit	• Model shows increase C26:0, axonal damage, and susceptibility to ROS • Axonal damage similar to mitochondrial dysfunction, ameliorated by antioxidants • Accumulation of lipid droplets • Decrease in life span when exposed to complex I, II, and IV inhibitors • Axonal damage was not rescued by the overexpression of WT Pmp-4 • No similar redox imbalance with peroxisomal thiolase deficiency
• Vacuoles and disrupted pigment cells between ommatidia • Lipid droplet formation	• Model shows neuronal (retinal) degeneration • No information regarding VLCFA accumulation, flight quality, or life span • Neuron-specific and not glia-specific knockdown of dABCD caused retinal degeneration
• Similar to Abcd1 deficiency	• Similar to Abcd1 deficiency
• Loss of photoreceptors • Vacuoles and disrupted pigment cells between ommatidia • Suppression by medium chain rich food	• Neuronal (retinal) degeneration seen also with disruption of the “upstream” long-chain fatty acid activation pathway • Model shows C26:1 elevation, reduced life span and decreased flight ability • Reversed VLCFA elevation upon supplementation with glyceryl trioleate oil • Similar retinal neurodegeneration observed with knocking down dABCD and elongase (dELOVL), both independently responsible for activated fatty acid products • No exacerbation of symptoms with VLCFA-rich food • Medium-chain rich food did not revert reduced life span or decreased flight quality

Abcd1 null mouse models in general resemble the AMN phenotype with myelin alterations and axonal degeneration in the sciatic nerve and long tracts of the spinal cord (Pujol et al., 2002). At 16 months of age, *Abcd1* hemizygous null mice show prominent thickening of myelin in the sciatic nerve and demonstrate motor disability by 20 months. This late-onset neurologic phenotype in *Abcd1* hemizygous null mice also leads to significant loss of motor coordination (Pujol et al., 2002, 2004; Singh & Pujol, 2010). Nerve conduction studies show slow velocity but preserved amplitude, suggesting a demyelination process (Pujol et al., 2002). The clinical symptoms of X-ALD are not evident in these mice up to 4 months; abnormal neurological and locomotor phenotype become visible at 16 and 20 months old, respectively. Axonopathy was demonstrated by ubiquitinated amyloid precursor protein (APP) and synaptophysin, which are found along axonal swellings (Pujol et al., 2002). *Abcd1:Abcd2* double null mice present an earlier onset of symptoms with more aggravated motor coordination defects, axonal degeneration, and myelin alterations (Pujol

et al., 2004). Moreover, the same authors also report a rescue of conduction velocity and axonal swelling by the overexpression of *Abcd2* cDNA in *Abcd1* null mice. Both the *Abcd1* null and the *Abcd1:Abcd2* double null mice do not exhibit cerebral demyelination nor cerebral inflammatory changes (see Table 1).

The mouse model therefore serves as the primary animal model for X-ALD axonopathy and its relation with oxidative stress and damage (Fourcade et al., 2014, 2015; López-Erauskin et al., 2011, 2013). Interestingly, oxidative damage was observed as early as 3.5 months in the spinal cord of mice (malondialdehyde-lysine) followed by its near-normalization and elevation of aminoadipic semialdehyde and Nε-carboxyethyl-lysine at 12 months, the latter two also normalize at 18 months, all before the development of symptoms (Fourcade et al., 2008). In contrast, no oxidative damage was seen at 3 months in cerebellar Purkinje cells (where murine *Abcd1* is known to be expressed) (Powers et al., 2005). The same authors also found no significant adrenal fibrosis. The spinal, cerebellar, and adrenal findings are in contrast to the human adrenal cortex and brain tissues that show extensive oxidative stress, particularly of lipid peroxidation (Powers et al., 2005). Nonetheless, X-ALD mice model has enabled several investigatory approaches for the amelioration of AMN-related symptoms: Oral 4-phenylbutyrate is shown to function as a peroxisomal proliferator, restores VLCFA levels in the brain and adrenal tissue of *Abcd1* null mice, and also partially rescues mitochondrial abnormality in the adrenal cortical cells (Kemp et al., 1998; McGuinness et al., 2003); Dimethyl fumarate, an activator for the antioxidant regulator nuclear factor erythroid 2-like 2, resulted in in vitro improved oxidative damage profile and increased mitochondrial DNA levels, demonstrating its role in improving the mitochondrial stress that is associated with X-ALD, and also halts axonal degeneration in *Abcd1*/*Abcd2* null mice (Ranea-Robles et al., 2018). The injection of insulin-like growth factor-1 (IGF1) and neurotrophin-3 (NT-3) into the cisterna magna resulted in improved motor coordination and myelination in the spinal cord, demonstrating IGF1 and NT-3 role as myelin inducers (Mastroeni et al., 2009). In search for disease biomarkers, VLCFAs incorporated at the sn-1 position of phosphatidylcholine and phosphatidylethanolamine were found to be enriched in *Abcd1* null mice, similar to a profile seen in fibroblasts from Zellweger spectrum disorder patients (Hama et al., 2018). More recently, the induction of experimental autoimmune encephalitis (EAE, achieved by injecting a truncated MOG peptide with bacterial adjuvant) in such mice resulted in decrease of both serum C20:3-LPC and C20:4-LPC in subjects in which EAE resulted in paralysis compared with null mice unaffected by the EAE or control (Kettwig et al., 2021). Translating to humans, a twofold decrease in both species was observed only in ccALD patients prior to MRI changes but not in those with positive Loes score at the time of analysis, complicating the interpretation of these findings as X-ALD-related neuroinflammation biomarkers. Taken together X-ALD mice model provides a valuable system to study the disease pathology and therapeutic options for the AMN phenotype.

2.2 | Zebrafish (*Danio rerio*)

The zebrafish ortholog, *Abcd1*, has 68% identity (1% gap), and is expressed in the interrenal gland, the analog of the adrenal gland in zebrafish, the CNS and spinal cord, and oligodendrocytes. A null allele construct showed a 1.9-fold increase in C26:0, decrease

in proteolipid protein 1a (PLP1a, a major component of myelin anchoring) with a reduction in myelin sheath content, and decrease in oligodendrocyte precursor cells in the spinal cord (Strachan et al., 2017). Contrary to mice, the loss of oligodendrocytes and myelin in zebrafish is promising for ccALD phenotype modeling; however, only a modest increase in VLCFAs and no change in cortisol expression was observed (Strachan et al., 2017). Additionally, the adaptive immune system, a key element in the etiopathogenesis of ccALD, matures in zebrafish only 14 days postfertilization (Rutherford & Hamilton, 2019), while the above findings were seen 3–8 days postfertilization. This may suggest a nonimmunologic role for Abcd1 in oligodendrocyte maintenance.

3 | INVERTEBRATE MODELS OF *ABCD1* AND B-OXIDATION FUNCTION

3.1 | Nematodes (*Caenorhabditis elegans*)

More recently, a model of X-ALD in *C. elegans* had been characterized (Coppa et al., 2020). The ortholog of *ABCD1* in *C. elegans* is *pmp-4* (57% identity, 2% gap), and its loss leads to axonal degeneration and locomotor disability (Coppa et al., 2020). These *pmp-4* mutants showed VLCFA accumulation and impaired mitochondrial redox homeostasis, and the mitochondria-targeted antioxidant MitoQ prevented axonal degeneration and locomotor disability. Importantly, the overexpression of PMP-4 solely in the hypodermis, a myelin-like tissue, rescued the axonal and locomotion abnormalities, suggesting that the PMP-4 is required for the nematode nervous system, especially in myelin-like tissues (Coppa et al., 2020).

3.2 | Fly (*Drosophila melanogaster*)

The fruit fly *D. melanogaster* is particularly well suited for these studies because of the availability of tools for sophisticated genetic manipulation, its short generation time, and the fact that about 75% of human genes have Drosophila homologs (Wangler et al., 2017). Indeed, research on Drosophila in collaboration with other human geneticists has provided diagnoses in many rare human genetic diseases (Ansar et al., 2019; Chung, Mao, et al., 2020; Marcogliese et al., 2018). In Drosophila, the study of two neurodegenerative mutants has parallels to X-ALD disease pathogenesis, namely Drosophila's bubblegum (*bgm*) and double-bubble (*dbb*) as both are involved in VLCFA biology as acyl-CoA synthases (human ortholog: ACSBG2—acyl-CoA synthetase bubblegum family member 2) (Wang et al., 2017). *Bgm* homozygous null mutants exhibit impaired vision associated with optic lobe defects with many vacuoles and loss of photoreceptors and pigment cells (glial cells supporting the photoreceptors) (Min & Benzer, 1999). Importantly, these defects were mostly suppressed by dietary treatment with glyceryl trioleate oil, one of the components of "Lorenzo's oil" by blocking the accumulation of excess VLCFAs (Min & Benzer, 1999). Recent studies reported that the double knockout of both *bgm* and *dbb* exhibit much-enhanced neurodegeneration in fly eyes with severe loss of lipid-and membrane-rich pigment photoreceptors and surrounding glia, suggesting a redundant function between these two proteins (Gordon et al., 2018). However, LCFA/VLCFA supplementation did not exacerbate the phenotypes in *bgm* and *dbb* mutants, arguing that the product loss is causative of neurodegeneration caused by loss of *bgm* or *dbb* (Sivachenko et al., 2016). Of note, the Bgm and dbb have been labeled in some studies as useful X-ALD fly models and while

they provide insight into VLCFA metabolism, it is important to note that they are not the homologs of *ABCD1* in humans. Fly has *dABCD* as a sole homolog for human *ABCD1*. dABCD shares 53% identity and 71% similarity to human ABCD1 (Wang et al., 2017). The ubiquitous expression of *dABCD RNAi* caused defects in pigment glia and progressive photoreceptor loss that are very similar to the phenotypes observed in *bgm* and *dbb* double mutants (Sivachenko et al., 2016). These defects were suppressed by high medium-chain fatty acids food (7% coconut oil) post-eclosion. The fly model for X-ALD has thus far focused on acyl-CoA synthetase loss of function with less data available on the effect of dABCD loss of function on neurons and particularly on glial cells.

ACOX1, the second enzyme in the peroxisomal β-oxidation, which desaturates imported esterified VLCFAs into 2-trans-enoyl acyl-CoA, is highly conserved in fruit flies. We recently discovered that dACOX1 (the fly homolog of ACOX1) is mostly expressed and required in glia and that *dACOX1* loss leads to developmental delay and pupal death (Chung, Wangler, et al., 2020). Flies that escape death exhibit a severely reduced life span, impaired synaptic transmission, and pronounced glial and axonal loss. Bezafibrate that reduces VLCFA synthesis by inhibiting ELOVL1, a VLCFA synthase, was beneficial to the glial loss observed in *dACOX1* mutant flies. We further identified three pediatric patients with heterozygous mutations in *ACOX1* who all displayed progressive myeloneuropathy and sensorineural hearing loss. Although they are from unrelated families, these patients shared the same de novo mutation in *ACOX1* (N237S). Unlike patients with *ACOX1* deficiency, an increase in VLCFA was not observed in *ACOX1 p.N237S* patients, suggesting that a different molecular mechanism may underlie neurological problems. Interestingly, the p.N237S mutation causes increased levels of ACOX1 and ROS in insulating glia in flies and Schwann cells in mice. Similarly, *ACOX1 p.N237S* patients exhibit a severe loss of Schwann cells and motor and sensory neurons. Treatment with an antioxidant (NAC) suppresses the loss of glia in flies, and primary Schwann mouse cells and NAC have been beneficial to the patients with p.N237S mutation demonstrating that gain- and loss-of-function mutations in the peroxisomal *ACOX1* gene cause neurodegeneration via distinct molecular pathways in glia (Chung, Wangler, et al., 2020).

Taken together, these data suggest a shared metabolic disorder of several altered VLCFA processing pathways resulting in a neurodegenerative disease in the fly, a disorder that in other models had not been explored (Gordon et al., 2018). This broader view of X-ALD might offer diagnostic opportunities for undiagnosed leukodystrophy patients and may provide more therapeutic targets (Gordon et al., 2014, 2018).

4 | CONCLUSION

The various animal models contributed, separately, distinct pieces of information for the large puzzle of X-ALD pathophysiology, in which much is yet to be discovered. Together, they recapitulate features of the AMN disease phenotype with VLCFA accumulation, most notably C26:0 (C26:1 in fly), reduced locomotion, normal to a mildly reduced lifespan, and neuronal degeneration (see Table 1). While the mice model shows specific axonal pathology as in men, other models provide additional cellular and molecular insights into the pathophysiology. Zebrafish model highlight the loss of oligodendrocyte, a phenomenon seen

in demyelinating lesions in ccALD (Feigenbaum et al., 2000). In nematodes, the increased ROS were shown to be of mitochondrial origin and amendable to antioxidant, supporting the notion of VLCFA mitotoxicity and impaired ROS repair (Singh & Pujol, 2010). Lipid droplets (LDs), a sign of oxidative stress (Jarc & Petan, 2019; Nguyen & Olzmann, 2017), were seen in both nematodes and flies. In flies, VLCFA acyl-CoA synthases deficiency showed LD accumulation in glial cells, highlighting mitochondrial dysfunction and neurodegeneration (Liu et al., 2015). The latter was also observed another β-oxidation defect, ACOX1 deficiency, showing shared pathological features of neurodegeneration (a feature not seen in nematodes, nonetheless). Taken together, animal models demonstrate the role of oxidative stress, mitochondrial dysfunction, and mitochondrial ROS production pointing to a possible ROS stress-driven sequence of events from VLCFA accumulation to oligodendrocyte loss and its effect on axonal survival, as seen in AMN. The models do not, however, provide molecular or cellular clues on how this sequence lead to demyelination, blood–brain barrier disruption, and macrophage and lymphocytes presence in the perivascular spaces within the CNS tissue, all of which are significant pathological findings of ccALD. Moreover, none of the models show adrenal fibrosis, nor oxidative damage in brain or adrenal gland (Powers et al., 2005), leaving room for more research into the effect of mitochondrial dysfunction and oxidative damage in target tissues, and what is the trigger for immune activation seen in ccALD. This shortcoming might suggest that the cerebral phenotype is unique to primates (Curiel et al., 2017) or might imply a peroxisomal biogenesis defect resulting from stochastic or environmental factors. Improved modeling of the pathogenesis of X-ALD could address yet another fundamental unanswered question of X-ALD: what is the precise role of the accumulation of VLCFA, in light of the (a) prevention of neuronopathic phenotype of ccALD with HSCT despite unaltered elevation in VLCFA level, and (b) irreversibility of the disease progression even when a VLCFA-restricted diet is provided despite leading to substantial reduction in their plasma levels (Moser et al., 2007).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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DATA AVAILABILITY STATEMENT

Data sharing not applicable to this article as no data sets were generated or analyzed during the current study.

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Significance

X-linked adrenoleukodystrophy is a severe disorder of the myelin sheath, adrenal gland, and/or axons. It is caused by mutations in *ABCD1* gene encoding a peroxisomal very long-chain fatty acid importer, and characterized by plasma elevation of these fatty acids. Remarkably, the same mutations in *ABCD1* can result in distinct phenotypes of either rapidly progressive demyelinating disease, a slowly progressive axonopathy, or adrenal insufficiency. Animal models successfully recapitulated the disease's biochemical signature and the axonopathic phenotype, while demonstrating the central role played by glial dysfunction early in the disease's pathogenesis, and opening the door for possible therapies targeting the glia.

TABLE 1

Phenotype, biochemical findings, pathophysiological insights, and limitations of each animal model of X-ALD

Species	Gene k/o (% identity to ABCD1)	Phenotype / Pathology		
		C26:0 elevation	**Locomotion (test)**	**Life span**
Mouse (hemizygotes)	Abcd1 (92%)	• Adrenal: × 2–8 • Sciatic nerve and spinal cord: × 6 • CNS: × 2–5	• Decreased coordination (rotarod) • Decreased exploration (open field)	Unaltered
Zebrafish	abcd1 (68%)	× 1.4–1.9 (whole animal extracts)	Reduced (evoked) swimming distance	20%–30% reduction in competitive survival to 30 days. Unaltered life span when reared separately
Caenorhabditis elegans	Pmp-4 (57%)	25% increase	• 10% reduction in lateral swimming motion • Increased lethality when exposed to oxidative stress	Unaltered
Drosophila	dABCD (53%)	Not reported	Not reported	Unaltered
Selected additional phenotypes models with deficiency in genes related to ABCD1				
Mouse	Abcd1 and Abcd2 double k/o	• Adrenal: × 16 • Sciatic nerve and spinal cord: × 6	• Locomotion defects begins at 12 months instead of 20 months • Reversibility of symptoms with antioxidant treatment	Unaltered
Drosophila	bgm and dbb (VLCFA acyl-CoA synthases)	• ×2 increase for bgm (× 1.5 for C26:1)	• 50% reduction (beam breaks assay)	• bgm: 0%–30% reduction • dbb: 15% reduction