



Review

Management of adrenoleukodystrophy: From pre-clinical studies to the development of new therapies

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ABSTRACT

X-linked adrenoleukodystrophy (X-ALD) is an inherited neurodegenerative disorder associated with mutations of the *ABCD1* gene that encodes a peroxisomal transmembrane protein. It results in accumulation of very long chain fatty acids in tissues and body fluid. Along with other factors such as epigenetic and environmental involvement, *ABCD1* mutation-provoked disorders can present different phenotypes including cerebral adrenoleukodystrophy (cALD), adrenomyeloneuropathy (AMN), and peripheral neuropathy. cALD is the most severe form that causes death in young childhood. Bone marrow transplantation and hematopoietic stem cell gene therapy are only effective when performed at an early stage of onsets in cALD. Nonetheless, current research and development of novel therapies are hampered by a lack of in-depth understanding disease pathophysiology and a lack of reliable cALD models. The *Abcd1* and *Abcd1/Abcd2* knock-out mouse models as well as the deficiency of *Abcd1* rabbit models created in our lab, do not develop cALD phenotypes observed in human beings. In this review, we summarize the clinical and biochemical features of X-ALD, the progress of pre-clinical and clinical studies. Challenges and perspectives for future X-ALD studies are also discussed.

1. Background

X-linked adrenoleukodystrophy (X-ALD) is a neurodegenerative disease that mostly affects males. There are three main forms with varying degrees of severity: cerebral adrenoleukodystrophy (cALD), Adrenomyeloneuropathy (AMN), and Addison's disease [1–3]. The gene involved in X-ALD pathology, *ABCD1*, encodes adenosine triphosphate (ATP)-binding cassette subfamily D member 1 (ABCD1) protein, a peroxisomal transmembrane protein. Defective ABCD1 protein fails to transport fatty acyl-CoAs, including very long chain fatty acid (VLCFA)-CoAs into peroxisomes from the cytoplasm for β -oxidation with consequent accumulation of VLCFAs in the cells [4], leading to neuron

damages. To date, the underlying pathology of X-ALD is poorly understood. A lack of reliable cALD animal models is a hurdle of X-ALD studies. In this review, we will summarize current knowledge of X-ALD, as well as pre-clinical and clinical studies. We will also introduce a novel *Abcd1* rabbit model created by our lab and discuss potential development of future X-ALD investigation.

2. Clinical and biochemical features of X-ALD

The diagnostic markers of X-ALD include an increase in VLCFAs in blood plasma, especially lignoceric acid (C24:0) and hexacosanoic acid (C26:0) in tissues and body fluid. Since the amount of shorter fatty acid,

Abbreviations: AAV, adeno-associated virus; ABCD1, encodes adenosine triphosphate-binding cassette subfamily D member 1; AMN, Adrenomyeloneuropathy; BMT, bone marrow transplant; cALD, cerebral adrenoleukodystrophy; CNS, central nervous system; DMF, dimethyl fumarate; ER, endoplasmic reticulum; GVHD, graft-verse-host disease; HSCT, hematopoietic stem cell transplantation; iPSCs, induced pluripotent stem cells; LO, Lorenzo's oil; MRI, magnetic resonance imaging; X-ALD, X-linked adrenoleukodystrophy; VLCFA, very long chain fatty acid.

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such as docosanoic acid (C22:0) or even shorter (carbon chain length <22), is little affected by dysfunctional ABCD1 proteins. C26:0/C22:0 is particularly an important index of X-ALD diagnosis. However, this index does not necessarily reflect disease severity. Recently, levels of 1-hexacosanoyl-2-hydroxy-*sn*-glycero-3-phosphocholine (C26:0 LysoPC) and C26:0-carnitine in dry blood spot are proposed to be more sensitive biomarkers that correlate with VLCFA levels in the brain and spinal cord in X-ALD mice [5]. More work is needed to investigate the relationship between the levels of these two markers and disease progression.

Cerebral demyelination is a hallmark of cALD and can be diagnosed on magnetic resonance imaging (MRI) [6,7]. Demyelination may be present in the brain of cALD patients, commonly in the corpus callosum [8,9]. Such lesions can lead to motor impairment, memory loss and seizures. Patients often have rapid spread of demyelination in the brain with death occurring within a few years.

In AMN, no such damage is evident in the brain although axonopathy is common in the spinal cord and peripheral nerves [10]. Compared with cALD, symptoms of AMN are less severe. Patients usually experience weak muscle control and poor control of urination. Patients will experience difficulty with activities of daily living but will not necessarily progress to a vegetative state or succumb to the disease on disease onset. However, the chance of developing cerebral demyelination after AMN onset is not negligible [11].

Defective *ABCD1* in females rarely develops cALD, but presents symptoms like AMN. Since symptoms in X-ALD females are not severe, and the disease progression is relatively slow, they are considered to be mild in the past. However, more studies have been demonstrated that more than 80% of X-ALD females develop spinal involvement at older ages [12,13]. Until now the mechanisms that affect symptom progression are still unclear.

Myelopathy and adrenal insufficiency are usually defined as common early symptoms of X-ALD [14]. Most cALD cases, especially those occur in adulthood, already have myelopathy with gait disorder before cerebral demyelination [15]. This comes to a thought that monitoring myelopathy level during early phase of the disease can somehow estimate the time or the probability of cALD onset. Recently, there are studies developing method to quantify progression of myelopathy in X-ALD [16,17]. These studies aim to provide new markers in detecting disease progression in future clinical studies.

For adrenal insufficiency, elevation of plasma adrenocorticotropic hormone and hyperpigmentation are the characteristics. Rarely, it was reported that generalized skin hyperpigmentation could be the only sign of X-ALD. In that case, a boy that had hyperpigmentation at gums, elbows and knees was diagnosed with X-ALD subsequently, although he appeared to be healthy when this manifestation was observed [18]. These reports have raised the awareness that patients who have signs of adrenal insufficiency, especially in young boys, should also be screened for X-ALD, in order to prevent delayed therapeutic treatments. Early diagnosis is also important for their siblings to have early DNA screening for X-ALD.

The main damaged site in cALD is the central nervous system (CNS). Microglia and macrophages play an important role in the progress of demyelination in cALD. Aberrant activation of microglia is considered to be involved in myelin degradation process [19,20], and macrophages were found to have lost the plasticity in X-ALD patients [21]. cALD macrophages lost the ability to switch to anti-inflammatory activation state to phagocytose destructive myelin, which may lead to progressive myelin damage. The blood-brain barrier permeability was also found to be altered at the demyelinating edge of the brain [22]. These findings provide new insights for the development of novel therapeutic targets.

On cellular level, oxidative stress is proven to be another hallmark of X-ALD [23,24]. Impaired mitochondrial oxidative phosphorylation and mitochondrial depletion have been demonstrated in X-ALD mice and cells [25,26]. One example marker is the significant increase of oxidized glutathione, a strong antioxidant, in lymphocytes [27]. Heat shock proteins, which would response with cellular stress, were also found to

be upregulated in X-ALD astrocytes before demyelination process [28].

X-ALD is a complex disease affecting different cell types and involving different pathological pathways. The level of *ABCD1* gene mutation is not necessarily correlated with the severity of the disease [29]. In clinical practice, it is not rare to observe that the same mutation of *ABCD1* in different individuals can lead to different phenotypes of cALD, AMN or Addison's disease only [30]. It has also been reported that even in genetically identical twins with similar environmental impacts, different forms of X-ALD may occur [31,32]. All these prove that genetic background is not the sole determinant of the phenotypic heterogeneity of X-ALD. There may be some underlying pathology that has not been clearly identified. In 2010, Singh and Pujol suggested a "Three-Hit Hypothesis" which described that an *ABCD1* mutation-provoked elevation of VLCFAs was not the sole cause of cALD phenotypes [33]. He proposed that elevated VLCFAs-induced oxidative stress is the first hit to develop AMN, neuroinflammation triggered by oxidative stress is the second hit, and peroxisomal dysfunction subsequently caused by neuroinflammation is the third hit, resulting in demyelination and neurodegeneration finally. The complex nature of the disease makes fully understanding its pathophysiology more difficult.

3. Current clinical treatments and limitations

The first clinical case of X-ALD was reported in the early 1990s [34]. A 7.5-year-old boy diagnosed with cALD received a bone marrow transplant (BMT). The therapy was successful and favorable results persisted for at least eight years. BMT and allogeneic hematopoietic stem cell transplantation (HSCT) were shown to have successfully reduced levels of VLCFAs as well as oxidative stress and, more importantly, to have improved the patients' performance in later trials performed during the early stage of cALD [35–37]. Nonetheless although transplantation is an effective therapy for cALD, there remains a risk of mortality if performed during late-stage of the disease [38–40]. Serious fatal complications such as graft-versus-host disease (GVHD) and graft failure can occur. Transplantation involves stressful surgery that may not be advisable during the middle and advanced stages of the disease. The availability of an HLA-matched donor is another barrier to transplantation. Patients may need to wait a long time for a suitable donor by which time the disease has advanced beyond early stage.

Another clinical trial with lentiviral hematopoietic stem cell (HSC) gene therapy [41,42]. The first reported case in 2009 involved two boys (7 and 7.5 years old) for whom no suitable bone marrow donor was available [43]. Both patients enjoyed favorable therapeutic effects for up to 30 months. The advantage of HSC gene therapy over BMT is that the patient's own stem cells are used, obviating the need to wait for a suitable donor and avoiding the risk of GVHD. This approach is currently in phase II/ III of clinical research. Nonetheless safety is a main concern of lentiviral HSC gene therapy. Due to the mechanism of lentiviral infection, there is a risk of developing insertional mutagenesis following gene therapy, which can trigger tumor formation [44,45]. Some modified viral vectors, such as Lenti-D lentivirus, were shown to reduce the risk of mutagenesis [42]. More evaluations are required to further assess the safety in clinical trials.

Dietary therapy is another approach to lower VLCFA level in patients. Lorenzo's oil (LO), which is a mixture of glyceryl trioleate oil and glyceryl trierucate oil in a ratio of 4:1, has been found to reduce accumulation of VLCFAs by inhibiting endogenous synthesis [46]. Administration of LO can help significantly decrease plasma VLCFA levels [47–49]. It is a comparably safe treatment, but its limitation is that while it may be able to reduce VLCFA levels during the early stage of X-ALD and slow disease progression, it has not been proven effective for cALD. The effect of arresting disease progression, especially in patients who already have neurological involvement, is still questionable [50,51].

4. Current animal models of X-ALD

The first X-ALD mouse model was created in 1997 in different laboratories by disrupting the *ABCD1* gene [52–54]. All the three *Abcd1* mouse models showed some biochemical changes and late onset resembling AMN. VLCFA level was increased in various tissues compared with wild type (WT) mice. Accumulation of VLCFAs was evident in the brain with an approximate 5-fold increase in C26:0/C22:0 ratio in the model compared with WT mice. In a human sample, the ratio in a X-ALD brain demonstrated a 3- to 15- fold increase compared with normal samples, depending on the level of demyelination in different brain regions [54]. These results were exciting because it showed that the animal model was moderately successful. Nonetheless surprisingly, the *Abcd1* mouse model showed no significant changes in blood plasma C26:0/C22:0 level. In contrast, in human patients, accumulated VLCFA levels can be easily measured in blood plasma, with an average 5-fold increase [55]. This may imply that the accumulation of VLCFAs in mice is not as severe as that in humans.

No *Abcd1* mice showed any behavioral changes at a young age. On the contrary, in humans, the most severe form of X-ALD, cALD, often has an onset in childhood, certainly below the age of 10 years. AMN often has an onset at ages 20–30 years. In *Abcd1* mice, some neurological phenotypes in the spinal cord started to appear at 16-months of age, and clinical presentations such as decreased muscle strength were evident in 20-month-old mice [56], equating to over 50 years of age in humans. No demyelination was found in the brain. *Abcd1* mice can be said to mimic AMN according to its onset age and the site of neuron degeneration.

Scientists have also endeavored to knockout both *ABCD1* and *ABCD2* genes in mice [57]. These double knockout mice have dysfunctional ABCD1 proteins, as well as its close homolog, ABCD2 proteins [58,59]. It was expected that this genetic modification would lead to more severe symptoms, similar to the characteristics of cALD. Excitingly, compared with single knockout *Abcd1* mice, the double knockout mouse model exhibited earlier disease onset at 12-months of age. In addition, the phenotypes, such as enhanced VLCFA level in spinal cord and level of oxidative damage, were more obvious. Nonetheless disappointingly, and similar to *Abcd1* mice, no demyelination was found in the brain. Both models developed AMN-like pathology (Table 1).

In 2017, a new zebrafish model of X-ALD was developed. Absence of ABCD1 proteins in zebrafish results in an elevated level of C26:0 by 1.5–2-fold in whole animal extracts [60]. Altered myelin development was found in this new model. Excitingly, this model displays impaired motor function very early, within a week of life. In 2020, the deletion of the *pmp-4* gene in worm, which is an orthologue of *ABCD1*, also demonstrated some AMN phenotypes, such as a 1.25-fold increase in C26:0 accumulated in lysophosphatidylcholine from whole body extract, axonal dysregulation, and impaired locomotion [61]. Nonetheless further investigations with these two novel models of X-ALD are required. They may be a potential model for drug screening and

Table 1
Summary of phenotypes in X-ALD mouse models and human patients.

	Onset Age	C26:0/C22:0 increase Compared to Wild Type		Main site of neural damages
		Blood Plasma/ Serum	Brain/ Spinal cord	
Human cALD	< 10	4–5-fold	3–14.5 fold	Brain
Human AMN	20–30			Spinal cord/ Peripheral nerves
<i>Abcd1</i> Mice	20-month	0–1-fold	4–5 fold	Spinal Cord
Double KO Mice	12-month	3–4-fold	6–7-fold	Spinal Cord

Abbreviations: AMN, Adrenomyeloneuropathy; cALD, adrenoleukodystrophy; KO, knockout

molecular study of X-ALD but are not appropriate for assessing effect of therapy at this stage.

Apart from *ABCD1* and *ABCD2*, studies suggested that double mutation of *bubblegum* (*bgm*) and *double bubble* (*dbb*) that cause acyl-CoA synthetases (ACS) dysfunction in *Drosophila* showed some X-ALD phenotypes, such as accumulation of VLCFAs and loss of neurons and neuron supporting cells in the brain [62]. ACS is an enzyme that activates free fatty acids to become the substrates for ABCD1 transporter. It is particularly exciting that this model showed neuron impairment in the brain, which is potentially important for studying the relationship between evaluated VLCFA levels and neuron damages in the brain. More interestingly, due to the fact that X-ALD is not genotype-phenotype correlated, scientists used this fly model to study the gene-environment interactions by exposing the flies in different light patterns [63]. The results demonstrated that environmental stress can modulate the phenotype expressions in the same genetic background.

5. Pre-clinical studies of X-ALD for new therapeutic development

To understand the pathogenesis of X-ALD, reliable cell and animal models are essential to find out the causes of the disease. Apart from patient fibroblasts and patient-derived cell models such as a patient's induced pluripotent stem cells (iPSCs) [64–66], researchers started to rely on *Abcd1* and *Abcd1/Abcd2* mouse models and their cells to study pathologies and develop promising therapeutic strategies for X-ALD [67, 68]. To date, different underlying pathological pathways have been proposed, and corresponding drug treatments and therapies investigated using X-ALD models. The *Abcd1* mouse model is often used to assess biochemical signs, but the *Abcd1/Abcd2* mouse model is commonly used to determine a therapeutic effect by studying the clinical signs. We summarize some examples of in vivo therapeutic studies that have targeted different pathways over the past few years (Table 2).

Gene therapy is a direct approach for treating X-ALD. Studies showed that overexpression of the *ABCD1* gene using adeno-associated virus (AAV) serotype 9 in an *Abcd1* mouse model is promising. In these studies, level of VLCFAs in *Abcd1* mice in multiple tissues was lowered after injecting AAV intravenously [69]. Nonetheless blood plasma level of VLCFAs were unchanged. This may be a limitation of the *Abcd1* mouse model where the *Abcd1* knockout mouse only develops a mild phenotype that resembles the myelopathy of ALD at around 20 months of age. More recently an *Abcd1* knockout mouse with a Cre-inducible *ELOVL1* transgene, the key enzyme in VLCFAs synthesis, was established. These transgenic mice displayed highly elevated levels of VLCFAs in the central nervous tissues, which could serve as a new animal model to better understand impacts of VLCFAs on cerebral phenotypes [5]. Studies also showed that injecting AAV intrathecally into *Abcd1* mice can lower 20% of VLCFAs in spinal cord, which is the main lesion site of AMN [70]. It is believed that this injection method will be more effective in the treatment for AMN.

Reducing VLCFA level is a simple concept for halting symptom development. Overexpression of ABCD2 has been an approach to compensate the function loss of ABCD1. In 2017 and 2020, studies showed that upregulation of ABCD2 by thyroid hormone agonists, such as sobetirome and its prodrug in *Abcd1* mice significantly reduced VLCFA level in various tissues [71,72], up to 50% in peripheral tissues and 15–20% in the brain. In their studies, the *Abcd1* mouse model did not show serious impairment of motor functions, so it was difficult to access its effectiveness in curing X-ALD. Nonetheless it is a promising approach that should be further studied in reliable X-ALD disease models.

Oxidative stress was suggested as a key factor of neurodegenerative and metabolic diseases inducing X-ALD. Drugs that target antioxidant pathways are thought to be effective in arresting X-ALD disease progression [73,74]. In the last few years, dimethyl fumarate (DMF) has been reported to successfully prevent oxidative damage to proteins and

Table 2
Examples of in vivo therapeutic trials using X-ALD mouse models.

Treatment	Mechanism/ Targeted pathway	Biochemical Changes in <i>Abcd1</i> Mice	Halt axonal degeneration in Double KO mice	Improve motor function in Double KO mice
AAV	Gene regulation	Reduced VLCFA levels in brain and spinal cord	Not tested	Not tested
Thyromimetics	Upregulate ABCD2 expression	Reduced VLCFA levels in various tissues and the CNS	Not tested	Not tested
Pioglitazone	Mitochondria biogenesis	Normalize oxidative stress, Prevent mitochondria depletion	Yes	Yes
High-dose biotin	Regulate energy metabolism	Restore redox homeostasis, stimulate mitochondrial biogenesis	Yes	Yes
DMF	Antioxidant pathway	Reduce oxidative damages, Prevent mitochondria depletion	Yes	Yes
TUDCA	ER stress	Normalize ER stress	Yes	Yes
Temsirolimus	Autophagy dysfunction	Restore autophagy, prevent accumulation of oxidized proteins	Yes	Yes

Abbreviations: AAV, adeno-associated virus; CNS, central nervous system; DMF, dimethyl fumarate; ER, endoplasmic reticulum; KO, knockout, TUDCA, tauroursodeoxycholate,

lipids [75], and more importantly, arrest axonal degeneration and restore normal motor activities in *Abcd1/Abcd2* mice.

Novel therapeutic strategies have also tried to target mitochondria related pathways to cure X-ALD. For example, a common drug for type 2 diabetes, Pioglitazone, was found to be a potential drug for X-ALD [76]. Pioglitazone works by regulating PPAR γ /PGC-1 α -dependent-pathways and thereby stimulating mitochondrial biogenesis in *ABCD1* deficient cells. The mouse models showed therapeutic effects upon Pioglitazone treatment.

Researchers continue to search for methods to normalize redox balance in X-ALD mouse models. Most recently, high-dose biotin has been reported to help stimulate mitochondrial biogenesis and restore redox balance [77,78]. *Abcd1/Abcd2* mice clinical presentations were improved upon such treatment. These successful trials support the concept that restoring mitochondrial functions are one of the key approaches in X-ALD treatment.

Apart from mitochondria, the endoplasmic reticulum (ER) has been proven to be another important organelle that is associated with VLCFA-induced oxidative stress [79]. Recent findings suggest that administration of a bile acid tauroursodeoxycholate (TUDCA), which is an ER stress inhibitor, can halt axonal degeneration and locomotor impairment in *Abcd1/Abcd2* mice [80].

Autophagy dysfunction has been shown to be related to some neurodegenerative diseases such as Alzheimer's and Parkinson's [81–83]. Autophagy was shown to provide protection to VLCFA enriched cells [84]. Researchers have tried to target this mechanism to cure X-ALD. One example of attempts to restore autophagy in X-ALD

mice is the use of Temsirolimus, an mTOR inhibitor. Results showed an ability to stop disease progression in double knockout mice models [85].

6. Novel clinical trials and development

The *Abcd1* and *Abcd1/Abcd2* murine models pave the way to new therapeutic development. From the pre-clinical studies, we learn that oxidative stress plays an important role in X-ALD pathogenesis. Recently, a phase II study of AMN suggested that administration of a combination of antioxidants, α -tocopherol, N-acetylcysteine and α -lipoic acid to patients improved their motor performance [86], which agreed with the pre-clinical trial in murine model [87].

To date, BMT, HSCT and HSC gene therapies remain the only effective treatments for cALD. A recent successful case of HSCT reported in 2020 was of a 31-year-old man diagnosed with X-ALD at the early stage of the disease [88]. For patients who do not have suitable donors, haploidentical allogeneic HSCT, together with post-transplant cyclophosphamide treatment or infusion of umbilical cord blood, are currently proposed and under investigation [89–91]. Transplantation is undoubtedly beneficial, but a post-transplantation follow-up study showed that worsening in neurocognitive function can still occur in cALD survivors who have undergone HSCT at early stage. This observation is more likely to happen in younger boys, and patients with more advanced disease prior the time of transplantation [92,93].

There were reports that patients with advanced stage of cALD were treated by cord blood transplantation with reduced-intensity conditioning regimen [94,95]. These trials not only demonstrated promising treatments for advanced stage of the disease with favorable effects in the follow-up period, but also provide an alternative therapy for patients who lack suitable donor for HSCT. More large-scale and robust studies should be carried out to further assess its efficacy.

On the other hand, there was a new therapeutic approach for patients who were in advanced stage of cALD in 2020. Disappointingly it was not successful. This study included three cALD patients who received Vorinostat orally, a drug that could stimulate ABCD2 expression. In this study, the drug failed to stop disease progression and all patients developed thrombocytopenia [96]. Until now, treatments for late stage of cALD is still lacking.

In view of this, screening of X-ALD in babies is thought to be beneficial for timely treatment. In 2013, New York implemented newborn screening (NBS) to screen for newborn X-ALD patients, using dry blood spots as the testing samples [97]. In 2016, X-ALD was added to the federal Recommended Uniform Screening Panel (RUSP), more states began NBS for X-ALD. An NBS pilot study was carried out in North Carolina and reported in 2020 [98]. With the experience of NBS in the US, other countries such as Japan and Netherland also started to develop reliable protocols for screening X-ALD newborns [99,100]. With careful considerations, such as ethical and feasibility, this trend may spread to more countries in the future.

7. Challenges in the development of new therapies for X-ALD

Given that most current treatments are effective only at the very early stage of X-ALD, they are clearly not ideal for such a rapidly progressing disease. For cALD, the brain is the major affected site. This makes treatment more difficult because of the inability of drugs to penetrate the blood-brain barrier [101]. Drug distribution within the CNS is also another attribute that needed to be considered [102].

Study of X-ALD is currently hampered by the lack of a suitable animal disease model. As mentioned before, the *Abcd1* mouse model shows only symptoms that mimic AMN. *Abcd1/Abcd2* double knockout mice show earlier onset and more severe biochemical alterations and are thought to be a better model for AMN studies. Nonetheless it is not perfect. There is no direct evidence that *ABCD2* genotyping is associated with X-ALD phenotypes [103]. The absence of ABCD2 may cause alterations to other pathways that may cause phenotypes that are irrelevant to X-ALD.

Apart from animal models, scientists recently knocked out both *ABCD1* and *ABCD2* in microglia that successfully showed biochemical changes similar to those in X-ALD cells, such as accumulation of VLCFAs and mitochondrial modifications [104]. This would be a useful cell line to further investigate the pathology of X-ALD. Nevertheless, the accuracy of mimicking tissue cells in vivo is limited by 2D cell culture models.

Current studies tend to focus on the upstream pathology pathways, such as *ABCD1* gene defect, VLCFA accumulation, mitochondria depletion and oxidative stress. All these biochemical characteristics can be studied in both X-ALD cells and animal models. The downstream pathologies that lead to cerebral demyelination in cALD, the life-threatening form of X-ALD, remain unknown. Although favorable therapeutic effects can be seen in AMN mouse models, we still cannot estimate the effectiveness in curing cALD. A reliable animal model of cALD is vital to study the underlying pathology and to develop novel therapies.

8. Perspectives

The difficulties in creating an ideal animal model for a complex disease such as X-ALD are understandable. Many factors apart from genetic involvement need to be considered when efforts to create a cALD animal model. It is possible that living environment, lifestyle and eating patterns should also be considered when developing new animal models of X-ALD. Much work remains to be done. The failure of *Abcd1* and *Abcd1/Abcd2* mouse models to develop cALD might imply some compensation mechanisms in response to the absence of *ABCD1* proteins in rodents that differ to human beings.

In this regard, our lab tried to use rabbits (*Oryctolagus cuniculus*) to create X-ALD model by knocking out *ABCD1* using CRISPR/Cas9 technologies. Three male rabbits with mutations in *ABCD1* gene exon 1, namely Rabbit 1, 2 and 3, were successfully created. All of them have 4–4.5-fold increase of C26:0/C22:0 in blood plasma, which is more similar to human X-ALD compared with both *Abcd1* and *Abcd1/Abcd2*

mouse models. Nonetheless, no rabbits showed cALD phenotypes in the 2-year observational period. No behavioral and locomotion disability were observed, and body weights were steady. No abnormalities were found in brain MRI (Fig. 1). More examination is needed to evaluate the pathophysiological changes in this novel model.

Recently there was a case reporting a chimpanzee with an *ABCD1* mutation developed cALD [105]. This fact gives hope that chimpanzee could be a suitable model for cALD. Despite the increasing ethical concerns, few numbers of offspring in long term and high cost, creating an X-ALD model using *ABCD1*-absent non-human primates may still be a direction for better disease studies.

Before the establishment of reliable X-ALD, particularly cALD animal models, 3D cell culture models may play a contributive role in the disease studies. Human PSC-derived brain organoid may serve as a disease model that mimics in vivo environment, offering an opportunity to better understand cell-to-cell interactions or metabolic profile in a diseased brain, and to develop new therapeutic interventions in near future.

9. Conclusions

There is currently no effective treatment for severe cALD that remains a rapidly progressing fatal disease. The full pathophysiology of X-ALD is poorly understood. The mechanism that drives *ABCD1* mutation into different types of X-ALD remains a mystery. The absence of a reliable cALD animal model is the biggest hurdle to cALD pathological study and therapeutic development. Researchers have relied on *Abcd1* and *Abcd1/Abcd2* mouse models in X-ALD studies but these fail to simulate cALD phenotypes in humans. Although there are a number of promising therapeutic strategies being tested in mouse models, these novel interventions can only be applied to AMN phenotypes. There remains a lack of investigations for cALD. Hopefully, the emerging new cell and animal models will provide potential means to investigate the disease pathophysiology and develop new therapies in the future.

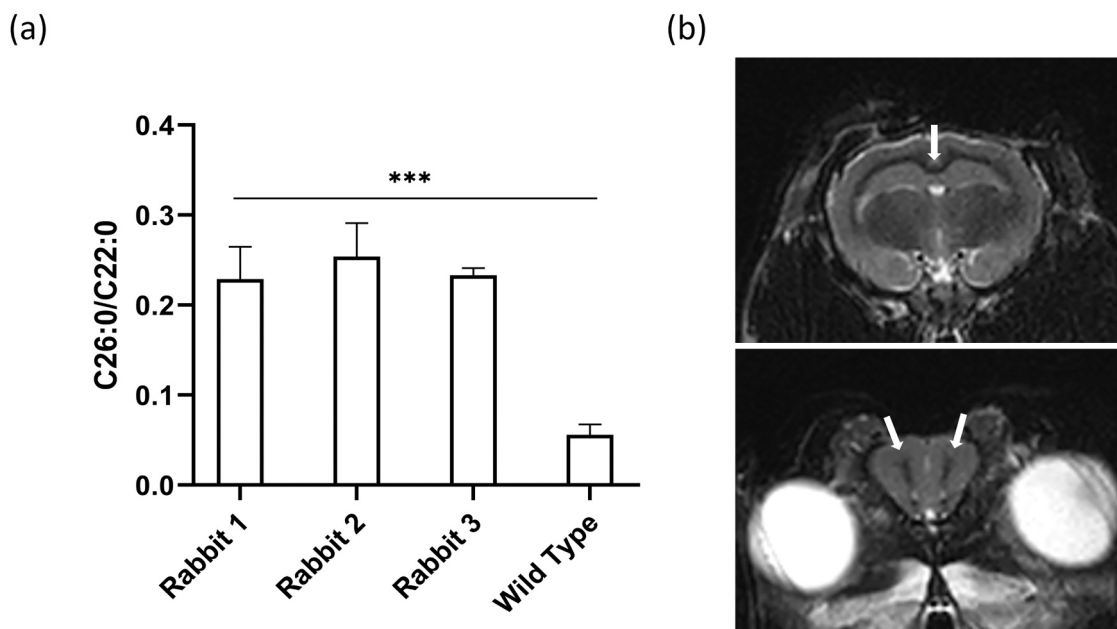


Fig. 1. Characteristics of *Abcd1* rabbit model. (a). Plasma C26:0/C22:0 ratio in wild type and *Abcd1* rabbits. Gas chromatography - mass spectrometry (GC - MS) was used to detect VLCFAs in rabbits' blood plasma. VLCFAs were derivatized in silane solution and methylated before subjected to GC-MS detection. Qualitative analysis of derived products was conducted based on the total ions detected in chromatogram and mass spectra. The VLCFA levels in plasma samples, including dodecanoic, tetraanoic and hexadecanoic acids, were calculated according to the selective ion chromatogram. All three rabbits have 4–4.5-fold increase compared to a wild type rabbit with the same sex and age. (Data are presented as mean ± SD. ***, $p = 0.0001$ vs. WT, significant difference was analyzed by one-way ANOVA followed by Dunnett's test). (b). Representative T2-weighted MRI scans for a 21-month-old *Abcd1* rabbit brain. No abnormalities including demyelination in the interested regions, the corpus callosum (top) and periventricular white matter (bottom) were found in the brain.

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Authors' contributions

Ma CY, Li C, Zhou XY and Lian Q wrote and revised manuscript with assistance by Zhang Z, Liao C, Jiang H, Liu HS, Chen HH, Tse HF and Liao C. Lian Q conceptualized and supervised the whole project and finalized the manuscript.

CRediT authorship contribution statement

Ma Chui Yan: Writing – Original Draft, Writing – Review & Editing, **Li Cheng:** Writing - Review & Editing, **Zhou Xiaoya:** Writing – Review & Editing, **Zhang Zhao:** Resources, **Jiang Hua:** Resources, **Liu Hongsheng:** Resources, **Chen Huanhuan Joyce:** Resources, **Tse Hung Fat:** Resources, **Liao Can:** Resources, **Lian Qizhou:** Conceptualization, Supervision, Writing- Review & Editing.

Conflict of interest statement

The authors declare that there is no conflict of interest

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Declarations

All animal experiments have followed the guidelines from Committee review of animal experiments in Guangzhou Medical University.

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