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

Chui Yan Ma[1], Cheng Li[1], Xiaoya Zhou[2], Zhao Zhang[1], Hua Jiang[2], Hongsheng Liu[2], Can Liao[2], and Qizhou Lian[1]

[1]University of Hong Kong
[2]Guangzhou Medical University

April 05, 2024

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 Addison's disease. cALD is a life-threatening form that causes death in young children. 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-/- mouse models and ABCD1- rabbit models created in our lab, do not develop cALD phenotypes observed in human being. 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.

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

Chui Yan Ma[1], Cheng Li [1] , Xiaoya Zhou[3] , Zhao Zhang[1] , Hua Jiang[4], Hongsheng Liu[5] ,Can Liao[3] , Qizhou Lian[1,2,3]*

1. Department of Medicine; 2. HKUMed Laboratory of Cellular Therapeutics, the University of Hong Kong, Hong Kong; 3. Prenatal Diagnostic Centre and Cord Blood Bank; 4. Department of Haematology; 5. Department of radiology, Guangzhou Women and Children's Medical Centre, Guangzhou Medical University, Guangzhou, China.

***Address correspondence to:**

Qizhou Lian, MD, PhD (Email: qzlian@hku.hk)

Data Availability Statement

No data have been shared.

Word Count: 4138 words

Acknowledgements

We thank Sarah Aglionby for revising the manuscript. This research was in part supported by Natural Science Grant of China (No 31571407); Hong Kong Health and Medical Research Fund (HMRF) (No:06172956), and

Start-up Grant for Stem Cell and Regenerative Medicine (Guangzhou Women and Children's Medical Centre, Grant No:5001-4001010).

Conflict of Interest Statement

All authors declare that there is no conflict of interest.

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 Addison's disease. cALD is a life-threatening form that causes death in young children. 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* $^{-/-}$ mouse models and *ABCD1*$^{-}$ rabbit models created in our lab, do not develop cALD phenotypes observed in human being. 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.

Keywords

X-linked adrenoleukodystrophy, X-ALD, ABCD1, Very long chain fatty acids, VLCFA, Neurodegeneration, Demyelination, Myelopathy

Introduction

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.(Berger, Forss-Petter, & Eichler, 2014; Köhler, Curiel, & Vanderver, 2018; Kemp, Huffnagel, Linthorst, Wanders, & Engelen, 2016) 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,(Wiesinger, Kunze, Regelsberger, Forss-Petter, & Berger, 2013) 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.

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, 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 brain and spinal cord in X-ALD mice.(van de Beek et al., 2016) 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).(Liberato et al., 2019; Paik, Kim, Yoon, & Kim, 2001) Demyelination may be present in the brain of cALD patients, commonly in the corpus callosum.(Nowak, Löbel, Wölfl, Schlegel, & Warmuth-Metz, 2015;

Santosh Rai, Suresh, Bhat, Sekhar, & Chakraborti, 2013) 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.(Castellano et al., 2016) 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 neglectable.(de Beer, Engelen, & van Geel, 2014)

Myelopathy and adrenal insufficiency are usually defined as common early symptoms of X-ALD.(Huffnagel, Laheji, et al., 2019) Most cALD cases, especially those occur in adulthood, already have myelopathy with gait disorder before cerebral demyelination.(Engelen, Kemp, & Poll-The, 2014) 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.(Huffnagel, van Ballegoij, et al., 2019; van de Stadt et al., 2020) 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.(Lee, Ko, & Lee, 2020) 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,(Bergner et al., 2019; F. S. Eichler et al., 2008) and macrophages were found to have lost the plasticity in X-ALD patients.(Weinhofer et al., 2018) 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.(Musolino et al., 2015) 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.(Deon, Marchetti, Donida, Wajner, & Vargas, 2016; Galea et al., 2012) Impaired mitochondrial oxidative phosphorylation and mitochondrial depletion have been demonstrated in X-ALD mice and cells.(Baarine, Beeson, Singh, & Singh, 2015; Fourcade, Ferrer, & Pujol, 2015) One example marker is the significant increase of oxidized glutathione, a strong antioxidant, in lymphocytes.(Petrillo et al., 2013) Heat shock proteins, which would response with cellular stress, was also found to be upregulated in X-ALD astrocyte before demyelination process.(Görtz et al., 2018)

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,(Wiesinger, Eichler, & Berger, 2015) even a single mutation can lead to cALD, AMN, or Addison's disease only.(Ozdemir Kutbay, Ozbek, Sarer Yurekli, & Demirbilek, 2019) It has also been reported that even in genetically identical twins with similar environmental impacts, different forms of X-ALD may occur.(Di Rocco, Doria-Lamba, & Caruso, 2001; Korenke et al., 1996) 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" that described an *ABCD1* mutation that was not the sole cause of X-ALD.(Singh & Pujol, 2010) The complex nature of the disease makes fully understanding its pathophysiology more difficult.

Current Clinical Treatments and Limitations

The first clinical case of X-ALD was reported in the early 1990s.(Aubourg et al., 1990) 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.(Kühl et al., 2017; Rockenbach et al., 2012; van Geel et al., 2015) Nonetheless although transplantation is an effective therapy for cALD, there remains a risk of mortality if performed during late-stage disease.(Bladowska et al., 2015; Jia et al., 2019; Kühl et al., 2018) Serious fatal complications such as graft-verse-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.(Cartier et al., 2012; F. Eichler et al., 2017) The first reported case in 2009 involved two boys (7 and 7.5 years old) for whom no suitable bone marrow donor was available.(Cartier et al., 2009) 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.(Luis, 2020; Marcucci et al., 2018) Some modified viral vectors, such as Lenti-D lentivirus, were shown to reduce the risk of mutagenesis.(F. Eichler et al., 2017) More evaluations are required to further assess the safeness 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.(Sassa, Wakashima, Ohno, & Kihara, 2014) Administration of LO can help significantly decrease plasma VLCFA levels.(Ahmed et al., 2016; Moser, Moser, Hollandsworth, Brereton, & Raymond, 2007; Stradomska, Drabko, Moszczyńska, & Tylki-Szymańska, 2014) 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.(Deon et al., 2008; Majori et al., 2014)

Current Animal Models of X-ALD

The first X-ALD mouse model was created in 1997 in different laboratories by disrupting the ABCD1 gene.(Forss-Petter et al., 1997; Kobayashi, Shinnoh, Kondo, & Yamada, 1997; Lu et al., 1997) 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.(Kobayashi et al., 1997) 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.(Moser, Moser, Singh, & O'Neill, 1984) 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 to 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,(Pujol et al., 2002) 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.(Pujol et al., 2004) These double knockout mice have dysfunctional ABCD1 proteins, as well as its close homolog, ABCD2 proteins.(Kawaguchi & Morita, 2016; Morita & Imanaka, 2012) 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 to 2-fold in whole animal extracts.(Strachan et al., 2017) 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.(Coppa et al., 2020) Nonetheless further investigations with these two novel models of X-ALD are required. They may be a potential model for drug screening and 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.(Sivachenko et al., 2016) 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.(Gordon, Valdez, & Letsou, 2018) The results demonstrated that environmental stress can modulate the phenotype expressions in the same genetic background.

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),(Baarine, Khan, Singh, & Singh, 2015; Son et al., 2017; Yeon et al., 2019) 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.(Morita, Shinbo, Asahi, & Imanaka, 2012; Muneer et al., 2014) 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 [See Additional file 1].

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.(Gong et al., 2015) Nonetheless blood plasma level of VLCFAs were unchanged. This may be a limitation of the*ABCD1*$^{-}$ mouse model where there is no elevation in plasma VLCFAs compared with WT mice and hampers its reliability to evaluate the effect of the gene therapy. 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.(Gong et al., 2019) 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 thyromimetics, such as sobetirome and its prodrug in *ABCD1*$^{-}$ mice significantly reduced VLCFA level in various tissues,(Hartley, Kirkemo, Banerji, & Scanlan, 2017; Hartley et al., 2020) 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.(López-Erauskin et al., 2012; Marchetti et al., 2015) In the last few years, dimethyl fumarate (DMF) has been reported to successfully prevent oxidative damage to proteins and lipids,(Ranea-Robles et al., 2018) 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.(Morató et al., 2013) Pioglitazone works by regulating PPARγ/PGC-1α-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.(Fourcade et al., 2020; Sghaier et al., 2019)*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.(van de Beek et al., 2017) 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.(Launay et al., 2017)

Autophagy dysfunction has been shown to be related to some neurodegenerative diseases such as Alzheimer's and Parkinson's.(Nixon, 2013; Orr & Oddo, 2013; Sarkar, 2013) Autophagy was shown to provide protection to VLCFA enriched cells.(Doria et al., 2019) 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.(Launay et al., 2015)

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,(Casasnovas et al., 2019) which agreed with the pre-clinical trial in murine model.(López-Erauskin et al., 2011)

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.(Ciftciler et al., 2020) For patients who do not have suitable donors, haploidentical allogenetic HSCT, together with post-transplant cyclophosphamide treatment or infusion of umbilical cord blood, are currently proposed and under investigation.(Chen et al., 2019; Fernandes et al., 2018; Jiang et

al., 2015) 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.(Pierpont et al., 2017; Raymond et al., 2019)

There were reports that patients with advanced stage of cALD were treated by cord blood transplantation with reduced-intensity conditioning regimen.(Awaya et al., 2011; Niizuma et al., 2012) 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.(Zierfuss et al., 2020) 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.(Vogel et al., 2015) 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.(S. Lee et al., 2020) 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.(Barendsen et al., 2020; Wu et al., 2017) With careful considerations, such as ethical and feasibility, this trend may spread to more countries in the future.

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.(Banks, 2016) Drug distribution within the CNS is also another attribute that needed to be considered.(Warren, 2018)

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.(Maier et al., 2008) 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 created a novel*ABCD1*$^{-}$*/ABCD2*$^{-/-}$ microglial model that successfully showed biochemical changes similar to those in X-ALD cells, such as accumulation of VLCFAs and mitochondrial modifications.(Raas et al., 2019) 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.

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 taken into account 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 to 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.(Curiel et al., 2017) 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.

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 human. 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 (**Fig. 2**). Hopefully, the emerging new cell and animal models will provide potential means to investigate the disease pathophysiology and develop new therapies in the future.

References

Ahmed, M. A., Kartha, R. V., Brundage, R. C., Cloyd, J., Basu, C., Carlin, B. P., . . . Raymond, G. V. (2016). A model-based approach to assess the exposure-response relationship of Lorenzo's oil in adrenoleukodystrophy. *Br J Clin Pharmacol, 81* (6), 1058-1066. doi:10.1111/bcp.12897

Aubourg, P., Blanche, S., Jambaqué, I., Rocchiccioli, F., Kalifa, G., Naud-Saudreau, C., . . . et al. (1990). Reversal of early neurologic and neuroradiologic manifestations of X-linked adrenoleukodystrophy by bone marrow transplantation. *N Engl J Med, 322* (26), 1860-1866. doi:10.1056/nejm199006283222607

Awaya, T., Kato, T., Niwa, A., Hiramatsu, H., Umeda, K., Watanabe, K., . . . Adachi, S. (2011). Successful cord blood transplantation using a reduced-intensity conditioning regimen for advanced childhood-onset cerebral adrenoleukodystrophy. *Pediatr Transplant, 15* (6), E116-120. doi:10.1111/j.1399-3046.2009.01188.x

Baarine, M., Beeson, C., Singh, A., & Singh, I. (2015). ABCD1 deletion-induced mitochondrial dysfunction is corrected by SAHA: implication for adrenoleukodystrophy. *J Neurochem, 133* (3), 380-396. doi:10.1111/jnc.12992

Baarine, M., Khan, M., Singh, A., & Singh, I. (2015). Functional Characterization of IPSC-Derived Brain Cells as a Model for X-Linked Adrenoleukodystrophy. *PLoS One, 10* (11), e0143238.

doi:10.1371/journal.pone.0143238

Banks, W. A. (2016). From blood-brain barrier to blood-brain interface: new opportunities for CNS drug delivery. *Nat Rev Drug Discov, 15* (4), 275-292. doi:10.1038/nrd.2015.21

Barendsen, R. W., Dijkstra, I. M. E., Visser, W. F., Alders, M., Bliek, J., Boelen, A., . . . Kemp, S. (2020). Adrenoleukodystrophy Newborn Screening in the Netherlands (SCAN Study): The X-Factor. *Front Cell Dev Biol, 8* , 499. doi:10.3389/fcell.2020.00499

Berger, J., Forss-Petter, S., & Eichler, F. S. (2014). Pathophysiology of X-linked adrenoleukodystrophy. *Biochimie, 98* (100), 135-142. doi:10.1016/j.biochi.2013.11.023

Bergner, C. G., van der Meer, F., Winkler, A., Wrzos, C., Türkmen, M., Valizada, E., . . . Stadelmann, C. (2019). Microglia damage precedes major myelin breakdown in X-linked adrenoleukodystrophy and metachromatic leukodystrophy. *Glia, 67* (6), 1196-1209. doi:10.1002/glia.23598

Bladowska, J., Kulej, D., Biel, A., Zimny, A., Kałwak, K., Owoc-Lempach, J., . . . Sasiadek, M. J. (2015). The Role of MR Imaging in the Assessment of Clinical Outcomes in Children with X-Linked Adrenoleukodystrophy after Allogeneic Haematopoietic Stem Cell Transplantation. *Pol J Radiol, 80* , 181-190. doi:10.12659/pjr.893285

Cartier, N., Hacein-Bey-Abina, S., Bartholomae, C. C., Bougnères, P., Schmidt, M., Kalle, C. V., . . . Aubourg, P. (2012). Lentiviral hematopoietic cell gene therapy for X-linked adrenoleukodystrophy.*Methods Enzymol, 507* , 187-198. doi:10.1016/b978-0-12-386509-0.00010-7

Cartier, N., Hacein-Bey-Abina, S., Bartholomae, C. C., Veres, G., Schmidt, M., Kutschera, I., . . . Aubourg, P. (2009). Hematopoietic stem cell gene therapy with a lentiviral vector in X-linked adrenoleukodystrophy. *Science, 326* (5954), 818-823. doi:10.1126/science.1171242

Casasnovas, C., Ruiz, M., Schlüter, A., Naudí, A., Fourcade, S., Veciana, M., . . . Pujol, A. (2019). Biomarker Identification, Safety, and Efficacy of High-Dose Antioxidants for Adrenomyeloneuropathy: a Phase II Pilot Study. *Neurotherapeutics, 16* (4), 1167-1182. doi:10.1007/s13311-019-00735-2

Castellano, A., Papinutto, N., Cadioli, M., Brugnara, G., Iadanza, A., Scigliuolo, G., . . . Salsano, E. (2016). Quantitative MRI of the spinal cord and brain in adrenomyeloneuropathy: in vivo assessment of structural changes. *Brain, 139* (Pt 6), 1735-1746. doi:10.1093/brain/aww068

Chen, Y., Zhang, X. H., Xu, L. P., Liu, K. Y., Qin, J., Yang, Y. L., & Huang, X. J. (2019). [Haploidentical allogenetic hematopoietic stem cell transplantation for X-linked adrenoleukodystrophy]. *Beijing Da Xue Xue Bao Yi Xue Ban, 51* (3), 409-413. doi:10.19723/j.issn.1671-167X.2019.03.006

Ciftciler, R., Goker, H., Buyukasık, Y., Topcu, M., Gevher, N., & Demiroglu, H. (2020). The experience of allogeneic hematopoietic stem cell transplantation in a patient with X-linked adrenoleukodystrophy. *Transfus Apher Sci, 59* (1), 102583. doi:10.1016/j.transci.2019.06.019

Coppa, A., Guha, S., Fourcade, S., Parameswaran, J., Ruiz, M., Moser, A. B., . . . Pujol, A. (2020). The peroxisomal fatty acid transporter ABCD1/PMP-4 is required in the C. elegans hypodermis for axonal maintenance: A worm model for adrenoleukodystrophy. *Free Radic Biol Med, 152* , 797-809. doi:10.1016/j.freeradbiomed.2020.01.177

Curiel, J., Steinberg, S. J., Bright, S., Snowden, A., Moser, A. B., Eichler, F., . . . Vanderver, A. (2017). X-linked adrenoleukodystrophy in a chimpanzee due to an ABCD1 mutation reported in multiple unrelated humans. *Mol Genet Metab, 122* (3), 130-133. doi:10.1016/j.ymgme.2017.08.012

de Beer, M., Engelen, M., & van Geel, B. M. (2014). Frequent occurrence of cerebral demyelination in adrenomyeloneuropathy. *Neurology, 83* (24), 2227-2231. doi:10.1212/wnl.0000000000001074

Deon, M., Garcia, M. P., Sitta, A., Barschak, A. G., Coelho, D. M., Schimit, G. O., . . . Vargas, C. R. (2008). Hexacosanoic and docosanoic acids plasma levels in patients with cerebral childhood and asymptomatic X-

linked adrenoleukodystrophy: Lorenzo's oil effect. *Metab Brain Dis, 23* (1), 43-49. doi:10.1007/s11011-007-9079-9

Deon, M., Marchetti, D. P., Donida, B., Wajner, M., & Vargas, C. (2016). Oxidative Stress in Patients with X-Linked Adrenoleukodystrophy.*Cell Mol Neurobiol, 36* (4), 497-512. doi:10.1007/s10571-015-0234-2

Di Rocco, M., Doria-Lamba, L., & Caruso, U. (2001). Monozygotic twins with X-linked adrenoleukodystrophy and different phenotypes. *Ann Neurol, 50* (3), 424. doi:10.1002/ana.1220

Doria, M., Nury, T., Delmas, D., Moreau, T., Lizard, G., & Vejux, A. (2019). Protective function of autophagy during VLCFA-induced cytotoxicity in a neurodegenerative cell model. *Free Radic Biol Med, 137* , 46-58. doi:10.1016/j.freeradbiomed.2019.04.016

Eichler, F., Duncan, C., Musolino, P. L., Orchard, P. J., De Oliveira, S., Thrasher, A. J., . . . Williams, D. A. (2017). Hematopoietic Stem-Cell Gene Therapy for Cerebral Adrenoleukodystrophy. *N Engl J Med, 377* (17), 1630-1638. doi:10.1056/NEJMoa1700554

Eichler, F. S., Ren, J. Q., Cossoy, M., Rietsch, A. M., Nagpal, S., Moser, A. B., . . . Ransohoff, R. M. (2008). Is microglial apoptosis an early pathogenic change in cerebral X-linked adrenoleukodystrophy?*Ann Neurol, 63* (6), 729-742. doi:10.1002/ana.21391

Engelen, M., Kemp, S., & Poll-The, B. T. (2014). X-linked adrenoleukodystrophy: pathogenesis and treatment. *Curr Neurol Neurosci Rep, 14* (10), 486. doi:10.1007/s11910-014-0486-0

Fernandes, J. F., Bonfim, C., Kerbauy, F. R., Rodrigues, M., Esteves, I., Silva, N. H., . . . Hamerschlak, N. (2018). Haploidentical bone marrow transplantation with post transplant cyclophosphamide for patients with X-linked adrenoleukodystrophy: a suitable choice in an urgent situation. *Bone Marrow Transplant, 53* (4), 392-399. doi:10.1038/s41409-017-0015-2

Forss-Petter, S., Werner, H., Berger, J., Lassmann, H., Molzer, B., Schwab, M. H., . . . Nave, K. A. (1997). Targeted inactivation of the X-linked adrenoleukodystrophy gene in mice. *J Neurosci Res, 50* (5), 829-843. doi:10.1002/(sici)1097-4547(19971201)50:5<829::Aid-jnr19>3.0.Co;2-w

Fourcade, S., Ferrer, I., & Pujol, A. (2015). Oxidative stress, mitochondrial and proteostasis malfunction in adrenoleukodystrophy: A paradigm for axonal degeneration. *Free Radic Biol Med, 88* (Pt A), 18-29. doi:10.1016/j.freeradbiomed.2015.05.041

Fourcade, S., Goicoechea, L., Parameswaran, J., Schlüter, A., Launay, N., Ruiz, M., . . . Pujol, A. (2020). High-dose biotin restores redox balance, energy and lipid homeostasis, and axonal health in a model of adrenoleukodystrophy. *Brain Pathol, 30* (5), 945-963. doi:10.1111/bpa.12869

Görtz, A. L., Peferoen, L. A. N., Gerritsen, W. H., van Noort, J. M., Bugiani, M., & Amor, S. (2018). Heat shock protein expression in cerebral X-linked adrenoleukodystrophy reveals astrocyte stress prior to myelin loss. *Neuropathol Appl Neurobiol, 44* (4), 363-376. doi:10.1111/nan.12399

Galea, E., Launay, N., Portero-Otin, M., Ruiz, M., Pamplona, R., Aubourg, P., . . . Pujol, A. (2012). Oxidative stress underlying axonal degeneration in adrenoleukodystrophy: a paradigm for multifactorial neurodegenerative diseases? *Biochim Biophys Acta, 1822* (9), 1475-1488. doi:10.1016/j.bbadis.2012.02.005

Gong, Y., Berenson, A., Laheji, F., Gao, G., Wang, D., Ng, C., . . . Eichler, F. (2019). Intrathecal Adeno-Associated Viral Vector-Mediated Gene Delivery for Adrenomyeloneuropathy. *Hum Gene Ther, 30* (5), 544-555. doi:10.1089/hum.2018.079

Gong, Y., Mu, D., Prabhakar, S., Moser, A., Musolino, P., Ren, J., . . . Eichler, F. S. (2015). Adenoassociated virus serotype 9-mediated gene therapy for x-linked adrenoleukodystrophy. *Mol Ther, 23* (5), 824-834. doi:10.1038/mt.2015.6

Gordon, H. B., Valdez, L., & Letsou, A. (2018). Etiology and treatment of adrenoleukodystrophy: new insights from Drosophila. *Dis Model Mech, 11* (6). doi:10.1242/dmm.031286

Hartley, M. D., Kirkemo, L. L., Banerji, T., & Scanlan, T. S. (2017). A Thyroid Hormone-Based Strategy for Correcting the Biochemical Abnormality in X-Linked Adrenoleukodystrophy. *Endocrinology, 158* (5), 1328-1338. doi:10.1210/en.2016-1842

Hartley, M. D., Shokat, M. D., DeBell, M. J., Banerji, T., Kirkemo, L. L., & Scanlan, T. S. (2020). Pharmacological Complementation Remedies an Inborn Error of Lipid Metabolism. *Cell Chem Biol, 27* (5), 551-559.e554. doi:10.1016/j.chembiol.2020.02.008

Huffnagel, I. C., Laheji, F. K., Aziz-Bose, R., Tritos, N. A., Marino, R., Linthorst, G. E., . . . Eichler, F. (2019). The Natural History of Adrenal Insufficiency in X-Linked Adrenoleukodystrophy: An International Collaboration. *J Clin Endocrinol Metab, 104* (1), 118-126. doi:10.1210/jc.2018-01307

Huffnagel, I. C., van Ballegoij, W. J. C., van Geel, B. M., Vos, J., Kemp, S., & Engelen, M. (2019). Progression of myelopathy in males with adrenoleukodystrophy: towards clinical trial readiness. *Brain, 142* (2), 334-343. doi:10.1093/brain/awy299

Jia, M. R., Wu, W. Z., Li, C. M., Cai, X. H., Zhang, L., Yan, F., . . . Gu, M. H. (2019). Clinical characteristics and phenotype distribution in 10 Chinese patients with X-linked adrenoleukodystrophy. *Exp Ther Med, 18* (3), 1945-1952. doi:10.3892/etm.2019.7804

Jiang, H., Jiang, M. Y., Liu, S., Cai, Y. N., Liang, C. L., & Liu, L. (2015). Combination of a Haploidentical Stem Cell Transplant With Umbilical Cord Blood for Cerebral X-Linked Adrenoleukodystrophy.*Pediatr Neurol, 53* (2), 163-165.e161. doi:10.1016/j.pediatrneurol.2015.05.002

Köhler, W., Curiel, J., & Vanderver, A. (2018). Adulthood leukodystrophies. *Nat Rev Neurol, 14* (2), 94-105. doi:10.1038/nrneurol.2017.175

Kühl, J. S., Suarez, F., Gillett, G. T., Hemmati, P. G., Snowden, J. A., Stadler, M., . . . Arnold, R. (2017). Long-term outcomes of allogeneic haematopoietic stem cell transplantation for adult cerebral X-linked adrenoleukodystrophy. *Brain, 140* (4), 953-966. doi:10.1093/brain/awx016

Kühl, J. S., Kupper, J., Baqué, H., Ebell, W., Gärtner, J., Korenke, C., . . . Steinfeld, R. (2018). Potential Risks to Stable Long-term Outcome of Allogeneic Hematopoietic Stem Cell Transplantation for Children With Cerebral X-linked Adrenoleukodystrophy. *JAMA Netw Open, 1* (3), e180769. doi:10.1001/jamanetworkopen.2018.0769

Kawaguchi, K., & Morita, M. (2016). ABC Transporter Subfamily D: Distinct Differences in Behavior between ABCD1-3 and ABCD4 in Subcellular Localization, Function, and Human Disease. *Biomed Res Int, 2016* , 6786245. doi:10.1155/2016/6786245

Kemp, S., Huffnagel, I. C., Linthorst, G. E., Wanders, R. J., & Engelen, M. (2016). Adrenoleukodystrophy - neuroendocrine pathogenesis and redefinition of natural history. *Nat Rev Endocrinol, 12* (10), 606-615. doi:10.1038/nrendo.2016.90

Kobayashi, T., Shinnoh, N., Kondo, A., & Yamada, T. (1997). Adrenoleukodystrophy protein-deficient mice represent abnormality of very long chain fatty acid metabolism. *Biochem Biophys Res Commun, 232* (3), 631-636. doi:10.1006/bbrc.1997.6340

Korenke, G. C., Fuchs, S., Krasemann, E., Doerr, H. G., Wilichowski, E., Hunneman, D. H., & Hanefeld, F. (1996). Cerebral adrenoleukodystrophy (ALD) in only one of monozygotic twins with an identical ALD genotype.*Ann Neurol, 40* (2), 254-257. doi:10.1002/ana.410400221

López-Erauskin, J., Galino, J., Bianchi, P., Fourcade, S., Andreu, A. L., Ferrer, I., . . . Pujol, A. (2012). Oxidative stress modulates mitochondrial failure and cyclophilin D function in X-linked adrenoleukodystrophy. *Brain, 135* (Pt 12), 3584-3598. doi:10.1093/brain/aws292

López-Erauskin, J., Fourcade, S., Galino, J., Ruiz, M., Schlüter, A., Naudi, A., . . . Pujol, A. (2011). Antioxidants halt axonal degeneration in a mouse model of X-adrenoleukodystrophy. *Ann Neurol, 70* (1), 84-92.

doi:10.1002/ana.22363

Launay, N., Aguado, C., Fourcade, S., Ruiz, M., Grau, L., Riera, J., . . . Pujol, A. (2015). Autophagy induction halts axonal degeneration in a mouse model of X-adrenoleukodystrophy. *Acta Neuropathol, 129* (3), 399-415. doi:10.1007/s00401-014-1378-8

Launay, N., Ruiz, M., Grau, L., Ortega, F. J., Ilieva, E. V., Martínez, J. J., . . . Fourcade, S. (2017). Tauroursodeoxycholic bile acid arrests axonal degeneration by inhibiting the unfolded protein response in X-linked adrenoleukodystrophy. *Acta Neuropathol, 133* (2), 283-301. doi:10.1007/s00401-016-1655-9

Lee, H., Ko, J. M., & Lee, S. H. (2020). Generalized skin hyperpigmentation as the only manifestation of X-linked adrenoleucodystrophy. *Br J Dermatol, 182* (1), 239-240. doi:10.1111/bjd.18310

Lee, S., Clinard, K., Young, S. P., Rehder, C. W., Fan, Z., Calikoglu, A. S., . . . Taylor, J. L. (2020). Evaluation of X-Linked Adrenoleukodystrophy Newborn Screening in North Carolina. *JAMA Netw Open, 3* (1), e1920356. doi:10.1001/jamanetworkopen.2019.20356

Liberato, A. P., Mallack, E. J., Aziz-Bose, R., Hayden, D., Lauer, A., Caruso, P. A., . . . Eichler, F. S. (2019). MRI brain lesions in asymptomatic boys with X-linked adrenoleukodystrophy. *Neurology, 92* (15), e1698-e1708. doi:10.1212/wnl.0000000000007294

Lu, J. F., Lawler, A. M., Watkins, P. A., Powers, J. M., Moser, A. B., Moser, H. W., & Smith, K. D. (1997). A mouse model for X-linked adrenoleukodystrophy. *Proc Natl Acad Sci U S A, 94* (17), 9366-9371. doi:10.1073/pnas.94.17.9366

Luis, A. (2020). The Old and the New: Prospects for Non-Integrating Lentiviral Vector Technology. *Viruses, 12* (10). doi:10.3390/v12101103

Maier, E. M., Mayerhofer, P. U., Asheuer, M., Köhler, W., Rothe, M., Muntau, A. C., . . . Berger, J. (2008). X-linked adrenoleukodystrophy phenotype is independent of ABCD2 genotype. *Biochem Biophys Res Commun, 377* (1), 176-180. doi:10.1016/j.bbrc.2008.09.092

Majori, M., Scarascia, A., Anghinolfi, M., Pisi, R., Gnetti, L., & Casalini, A. G. (2014). Lipoid pneumonia as a complication of Lorenzo's oil therapy in a patient with adrenoleukodystrophy. *J Bronchology Interv Pulmonol, 21* (3), 271-273. doi:10.1097/lbr.0000000000000084

Marchetti, D. P., Donida, B., da Rosa, H. T., Manini, P. R., Moura, D. J., Saffi, J., . . . Vargas, C. R. (2015). Protective effect of antioxidants on DNA damage in leukocytes from X-linked adrenoleukodystrophy patients. *Int J Dev Neurosci, 43* , 8-15. doi:10.1016/j.ijdevneu.2015.03.004

Marcucci, K. T., Jadlowsky, J. K., Hwang, W. T., Suhoski-Davis, M., Gonzalez, V. E., Kulikovskaya, I., . . . June, C. H. (2018). Retroviral and Lentiviral Safety Analysis of Gene-Modified T Cell Products and Infused HIV and Oncology Patients. *Mol Ther, 26* (1), 269-279. doi:10.1016/j.ymthe.2017.10.012

Morató, L., Galino, J., Ruiz, M., Calingasan, N. Y., Starkov, A. A., Dumont, M., . . . Pujol, A. (2013). Pioglitazone halts axonal degeneration in a mouse model of X-linked adrenoleukodystrophy.*Brain, 136* (Pt 8), 2432-2443. doi:10.1093/brain/awt143

Morita, M., & Imanaka, T. (2012). Peroxisomal ABC transporters: structure, function and role in disease. *Biochim Biophys Acta, 1822* (9), 1387-1396. doi:10.1016/j.bbadis.2012.02.009

Morita, M., Shinbo, S., Asahi, A., & Imanaka, T. (2012). Very long chain fatty acid β-oxidation in astrocytes: contribution of the ABCD1-dependent and -independent pathways. *Biol Pharm Bull, 35* (11), 1972-1979. doi:10.1248/bpb.b12-00411

Moser, H. W., Moser, A. B., Hollandsworth, K., Brereton, N. H., & Raymond, G. V. (2007). "Lorenzo's oil" therapy for X-linked adrenoleukodystrophy: rationale and current assessment of efficacy.*J Mol Neurosci, 33* (1), 105-113. doi:10.1007/s12031-007-0041-4

Moser, H. W., Moser, A. E., Singh, I., & O'Neill, B. P. (1984). Adrenoleukodystrophy: survey of 303 cases: biochemistry, diagnosis, and therapy. *Ann Neurol, 16* (6), 628-641. doi:10.1002/ana.410160603

Muneer, Z., Wiesinger, C., Voigtländer, T., Werner, H. B., Berger, J., & Forss-Petter, S. (2014). Abcd2 is a strong modifier of the metabolic impairments in peritoneal macrophages of ABCD1-deficient mice.*PLoS One, 9* (9), e108655. doi:10.1371/journal.pone.0108655

Musolino, P. L., Gong, Y., Snyder, J. M., Jimenez, S., Lok, J., Lo, E. H., . . . Eichler, F. S. (2015). Brain endothelial dysfunction in cerebral adrenoleukodystrophy. *Brain, 138* (Pt 11), 3206-3220. doi:10.1093/brain/awv250

Niizuma, H., Uematsu, M., Sakamoto, O., Uchiyama, T., Horino, S., Onuma, M., . . . Tsuchiya, S. (2012). Successful cord blood transplantation with reduced-intensity conditioning for childhood cerebral X-linked adrenoleukodystrophy at advanced and early stages. *Pediatr Transplant, 16* (2), E63-70. doi:10.1111/j.1399-3046.2011.01539.x

Nixon, R. A. (2013). The role of autophagy in neurodegenerative disease.*Nat Med, 19* (8), 983-997. doi:10.1038/nm.3232

Nowak, J., Löbel, U., Wölfl, M., Schlegel, P. G., & Warmuth-Metz, M. (2015). MRI demyelination pattern and clinical course in a child with cerebral X-linked adrenoleukodystrophy (X-ALD). *Acta Radiol Open, 4* (4), 2047981615573655. doi:10.1177/2047981615573655

Orr, M. E., & Oddo, S. (2013). Autophagic/lysosomal dysfunction in Alzheimer's disease. *Alzheimers Res Ther, 5* (5), 53. doi:10.1186/alzrt217

Ozdemir Kutbay, N., Ozbek, M. N., Sarer Yurekli, B., & Demirbilek, H. (2019). A Distinct Clinical Phenotype in Two Siblings with X-linked Adrenoleukodystrophy. *Neuro Endocrinol Lett, 40* (1), 36-40.

Paik, M. J., Kim, K. R., Yoon, H. R., & Kim, H. J. (2001). Diagnostic patterns of very-long-chain fatty acids in plasma of patients with X-linked adrenoleukodystrophy. *J Chromatogr B Biomed Sci Appl, 760* (1), 149-157. doi:10.1016/s0378-4347(01)00263-8

Petrillo, S., Piemonte, F., Pastore, A., Tozzi, G., Aiello, C., Pujol, A., . . . Bertini, E. (2013). Glutathione imbalance in patients with X-linked adrenoleukodystrophy. *Mol Genet Metab, 109* (4), 366-370. doi:10.1016/j.ymgme.2013.05.009

Pierpont, E. I., Eisengart, J. B., Shanley, R., Nascene, D., Raymond, G. V., Shapiro, E. G., . . . Miller, W. P. (2017). Neurocognitive Trajectory of Boys Who Received a Hematopoietic Stem Cell Transplant at an Early Stage of Childhood Cerebral Adrenoleukodystrophy. *JAMA Neurol, 74* (6), 710-717. doi:10.1001/jamaneurol.2017.0013

Pujol, A., Ferrer, I., Camps, C., Metzger, E., Hindelang, C., Callizot, N., . . . Mandel, J. L. (2004). Functional overlap between ABCD1 (ALD) and ABCD2 (ALDR) transporters: a therapeutic target for X-adrenoleukodystrophy. *Hum Mol Genet, 13* (23), 2997-3006. doi:10.1093/hmg/ddh323

Pujol, A., Hindelang, C., Callizot, N., Bartsch, U., Schachner, M., & Mandel, J. L. (2002). Late onset neurological phenotype of the X-ALD gene inactivation in mice: a mouse model for adrenomyeloneuropathy.*Hum Mol Genet, 11* (5), 499-505. doi:10.1093/hmg/11.5.499

Raas, Q., Gondcaille, C., Hamon, Y., Leoni, V., Caccia, C., Ménétrier, F., . . . Savary, S. (2019). CRISPR/Cas9-mediated knockout of Abcd1 and Abcd2 genes in BV-2 cells: novel microglial models for X-linked Adrenoleukodystrophy. *Biochim Biophys Acta Mol Cell Biol Lipids, 1864* (5), 704-714. doi:10.1016/j.bbalip.2019.02.006

Ranea-Robles, P., Launay, N., Ruiz, M., Calingasan, N. Y., Dumont, M., Naudí, A., . . . Pujol, A. (2018). Aberrant regulation of the GSK-3β/NRF2 axis unveils a novel therapy for adrenoleukodystrophy.*EMBO Mol Med, 10* (8). doi:10.15252/emmm.201708604

Raymond, G. V., Aubourg, P., Paker, A., Escolar, M., Fischer, A., Blanche, S., . . . Lund, T. (2019). Survival and Functional Outcomes in Boys with Cerebral Adrenoleukodystrophy with and without Hematopoietic Stem Cell Transplantation. *Biol Blood Marrow Transplant, 25* (3), 538-548. doi:10.1016/j.bbmt.2018.09.036

Rockenbach, F. J., Deon, M., Marchese, D. P., Manfredini, V., Mescka, C., Ribas, G. S., . . . Vargas, C. R. (2012). The effect of bone marrow transplantation on oxidative stress in X-linked adrenoleukodystrophy. *Mol Genet Metab, 106* (2), 231-236. doi:10.1016/j.ymgme.2012.03.019

Santosh Rai, P. V., Suresh, B. V., Bhat, I. G., Sekhar, M., & Chakraborti, S. (2013). Childhood adrenoleukodystrophy - Classic and variant - Review of clinical manifestations and magnetic resonance imaging. *J Pediatr Neurosci, 8* (3), 192-197. doi:10.4103/1817-1745.123661

Sarkar, S. (2013). Regulation of autophagy by mTOR-dependent and mTOR-independent pathways: autophagy dysfunction in neurodegenerative diseases and therapeutic application of autophagy enhancers. *Biochem Soc Trans, 41* (5), 1103-1130. doi:10.1042/bst20130134

Sassa, T., Wakashima, T., Ohno, Y., & Kihara, A. (2014). Lorenzo's oil inhibits ELOVL1 and lowers the level of sphingomyelin with a saturated very long-chain fatty acid. *J Lipid Res, 55* (3), 524-530. doi:10.1194/jlr.M044586

Sghaier, R., Zarrouk, A., Nury, T., Badreddine, I., O'Brien, N., Mackrill, J. J., . . . Lizard, G. (2019). Biotin attenuation of oxidative stress, mitochondrial dysfunction, lipid metabolism alteration and 7β-hydroxycholesterol-induced cell death in 158N murine oligodendrocytes. *Free Radic Res, 53* (5), 535-561. doi:10.1080/10715762.2019.1612891

Singh, I., & Pujol, A. (2010). Pathomechanisms underlying X-adrenoleukodystrophy: a three-hit hypothesis. *Brain Pathol, 20* (4), 838-844. doi:10.1111/j.1750-3639.2010.00392.x

Sivachenko, A., Gordon, H. B., Kimball, S. S., Gavin, E. J., Bonkowsky, J. L., & Letsou, A. (2016). Neurodegeneration in a Drosophila model of adrenoleukodystrophy: the roles of the Bubblegum and Double bubble acyl-CoA synthetases. *Dis Model Mech, 9* (4), 377-387. doi:10.1242/dmm.022244

Son, D., Quan, Z., Kang, P. J., Park, G., Kang, H. C., & You, S. (2017). Generation of two induced pluripotent stem cell (iPSC) lines from X-linked adrenoleukodystrophy (X-ALD) patients with adrenomyeloneuropathy (AMN). *Stem Cell Res, 25* , 46-49. doi:10.1016/j.scr.2017.10.003

Strachan, L. R., Stevenson, T. J., Freshner, B., Keefe, M. D., Miranda Bowles, D., & Bonkowsky, J. L. (2017). A zebrafish model of X-linked adrenoleukodystrophy recapitulates key disease features and demonstrates a developmental requirement for abcd1 in oligodendrocyte patterning and myelination. *Hum Mol Genet, 26* (18), 3600-3614. doi:10.1093/hmg/ddx249

Stradomska, T. J., Drabko, K., Moszczyńska, E., & Tylki-Szymańska, A. (2014). Monitoring of very long-chain fatty acids levels in X-linked adrenoleukodystrophy, treated with haematopoietic stem cell transplantation and Lorenzo's Oil. *Folia Neuropathol, 52* (2), 159-163. doi:10.5114/fn.2014.43787

van de Beek, M. C., Dijkstra, I. M., van Lenthe, H., Ofman, R., Goldhaber-Pasillas, D., Schauer, N., . . . Kemp, S. (2016). C26:0-Carnitine Is a New Biomarker for X-Linked Adrenoleukodystrophy in Mice and Man. *PLoS One, 11* (4), e0154597. doi:10.1371/journal.pone.0154597

van de Beek, M. C., Ofman, R., Dijkstra, I., Wijburg, F., Engelen, M., Wanders, R., & Kemp, S. (2017). Lipid-induced endoplasmic reticulum stress in X-linked adrenoleukodystrophy. *Biochim Biophys Acta Mol Basis Dis, 1863* (9), 2255-2265. doi:10.1016/j.bbadis.2017.06.003

van de Stadt, S. I. W., van Ballegoij, W. J. C., Labounek, R., Huffnagel, I. C., Kemp, S., Nestrasil, I., & Engelen, M. (2020). Spinal cord atrophy as a measure of severity of myelopathy in adrenoleukodystrophy. *J Inherit Metab Dis, 43* (4), 852-860. doi:10.1002/jimd.12226

van Geel, B. M., Poll-The, B. T., Verrips, A., Boelens, J. J., Kemp, S., & Engelen, M. (2015). Hematopoietic cell transplantation does not prevent myelopathy in X-linked adrenoleukodystrophy: a retrospective study. *J Inherit Metab Dis, 38* (2), 359-361. doi:10.1007/s10545-014-9797-1

Vogel, B. H., Bradley, S. E., Adams, D. J., D'Aco, K., Erbe, R. W., Fong, C., . . . Caggana, M. (2015). Newborn screening for X-linked adrenoleukodystrophy in New York State: diagnostic protocol, surveillance protocol and treatment guidelines. *Mol Genet Metab, 114* (4), 599-603. doi:10.1016/j.ymgme.2015.02.002

Warren, K. E. (2018). Beyond the Blood:Brain Barrier: The Importance of Central Nervous System (CNS) Pharmacokinetics for the Treatment of CNS Tumors, Including Diffuse Intrinsic Pontine Glioma. *Front Oncol, 8* , 239. doi:10.3389/fonc.2018.00239

Weinhofer, I., Zierfuss, B., Hametner, S., Wagner, M., Popitsch, N., Machacek, C., . . . Berger, J. (2018). Impaired plasticity of macrophages in X-linked adrenoleukodystrophy. *Brain, 141* (8), 2329-2342. doi:10.1093/brain/awy127

Wiesinger, C., Eichler, F. S., & Berger, J. (2015). The genetic landscape of X-linked adrenoleukodystrophy: inheritance, mutations, modifier genes, and diagnosis. *Appl Clin Genet, 8* , 109-121. doi:10.2147/tacg.S49590

Wiesinger, C., Kunze, M., Regelsberger, G., Forss-Petter, S., & Berger, J. (2013). Impaired very long-chain acyl-CoA β-oxidation in human X-linked adrenoleukodystrophy fibroblasts is a direct consequence of ABCD1 transporter dysfunction. *J Biol Chem, 288* (26), 19269-19279. doi:10.1074/jbc.M112.445445

Wu, C., Iwamoto, T., Igarashi, J., Miyajima, T., Hossain, M. A., Yanagisawa, H., . . . Eto, Y. (2017). Application of a diagnostic methodology by quantification of 26:0 lysophosphatidylcholine in dried blood spots for Japanese newborn screening of X-linked adrenoleukodystrophy. *Mol Genet Metab Rep, 12* , 115-118. doi:10.1016/j.ymgmr.2017.06.004

Yeon, G. B., Son, D., Kang, P. J., Oh, S. M., Lim, H., Kang, H. C., . . . Kim, D. S. (2019). Generation of an induced pluripotent stem cell (iPSC) line from a 42-year-old adult cerebral type X-linked adrenoleukodystrophy (X-ALD) patient. *Stem Cell Res, 36* , 101425. doi:10.1016/j.scr.2019.101425

Zierfuss, B., Weinhofer, I., Kühl, J. S., Köhler, W., Bley, A., Zauner, K., . . . Berger, J. (2020). Vorinostat in the acute neuroinflammatory form of X-linked adrenoleukodystrophy. *Ann Clin Transl Neurol, 7* (5), 639-652. doi:10.1002/acn3.51015

Tables

		C26:0/C22:0 increase Compared to Wild Type	C26:0/C22:0 increase Compared to Wild T
	Onset Age	Blood Plasma/Serum	Brain/Spinal cord
Human cALD	<10	4˜5-fold	3˜14.5 fold
Human AMN	20-30		
ABCD1- Mice	20-month	0˜1-fold	4˜5 fold
Double KO Mice	12-month	3˜4-fold	6˜7-fold

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

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

Improve motor function in Double KO mice	Not tested	Not tested
Halt axonal degeneration in Double KO mice	Not tested	Not tested
Biochemical Changes in *ABCD1*- Mice	Reduced VLCFA levels in brain and spinal cord	Reduced VLCFA levels in vario

Improve motor function in Double KO mice	Not tested	Not tested
Mechanism/ Targeted pathway	Gene regulation	Upregulate ABCD2 expression
Treatment	AAV	Thyromimetics

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

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

Figure Titles and Legends

Fig. 1 , Characteristics of *ABCD1*- rabbit model. (a). Plasma C26:0/C22:0 ratio in *ABCD1*- mutated rabbits. All three rabbits have 4 to 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*- mutated rabbit brain. No abnormalities including demyelination in the interested regions, the corpus callosum (top) and periventricular white matter (bottom) were found in the brain.

Fig. 2 , Common and different phenotypes of ABCD1 deficiency between human being and mouse.