Lipotoxicity in adrenoleukodystrophy

Size matters!

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Chapter 1

Introduction
**Introduction**

**History**

In 1910, Haberfeld and Spieler reported a case of a previously healthy 6 year old boy that had developed hyperpigmentation of the skin and decreased visual acuity. Additionally his school performances deteriorated. In the months that followed, the young boy lost his ability to speak, became incontinent and eventually was unable to walk. When he was 7 years old, he was hospitalized and died 8 months later. Autopsy revealed that he had suffered from an extensive cerebral demyelination that was associated with a perivascular inflammatory response. In retrospect, this was the first reported case of X-linked adrenoleukodystrophy (ALD, MIM #300100). In the 1950’s, ALD was known as Schilder’s disease, after Paul Schilder, who had reported several cases in the previous years. By 1963, nine similar cases had been reported in literature and since all cases involved male patients, it was proposed that it was an X-linked recessive inheritable disease. The striking co-occurrence of a leukodystrophy with adrenocortical insufficiency led to the introduction of the name adrenoleukodystrophy in 1970. By the end of the 1970’s and during the 1980’s, the clinical description of ALD was improved. The research group led by Dr. Hugo Moser at the Kennedy Krieger Institute in Baltimore USA ensured that medical professionals became aware of the disease and that the clinical characterization of ALD became more specified.

A diagnostic test for ALD became available in 1981 when it was found that very long-chain fatty acid (VLCFA) levels are increased in fibroblasts, plasma, red blood cells and amniocytes of ALD patients. In the same year, the ALD locus was mapped to the terminal segment of the long arm of the X-chromosome, Xq28. In 1993, the ALD gene, nowadays referred to as the \textit{ABCD1} gene, was identified.

To date, ALD is no longer considered as a disease with various distinct phenotypes but as a disease with a clinical spectrum of symptoms. Furthermore, females are no longer merely referred to as carriers, but women with ALD, since >80% of the affected women develop signs and symptoms of ALD. Since 2014, New York State in the USA is screening for ALD as part of the newborn screening program and in 2015 ALD was added to the US Recommended Uniform Screening Panel for newborn screening. In the Netherlands, ALD was added to the newborn screening list in 2015, but screening has not yet been started. Inclusion of ALD into the Dutch newborn screening program is expected in 2018-2019.

Taken together, since the first reported ALD patient by Haberfeld and Spieler, major steps forward were made in diagnosing ALD and the understanding of the pathogenesis of the disease which is necessary for the development of a curative therapy.
Introduction

Clinical symptoms

ALD is a progressive neurometabolic disease with a birth incidence of 1:14,700 and characterized by a striking and unpredictable clinical spectrum (Fig. 1). All ALD patients are presymptomatic at birth. Most male patients develop adrenal insufficiency at some point during their life. In fact adrenal insufficiency is often the first manifestation of the disease. However, this adrenal insufficiency is often missed in the diagnosis. This is well illustrated in a study describing the findings in a cohort of 49 neurologically asymptomatic boys with ALD that were identified through extended family screening. Of these boys, 80% suffered from an unrecognized and hence untreated adrenal insufficiency. The youngest patient with undiagnosed adrenal insufficiency was only 5 months of age. An adrenal insufficiency can be treated effectively by corticosteroid replacement therapy. However, if left undiagnosed, patients may develop a life threatening adrenal crisis.

Figure 1. The clinical spectrum of ALD in men.

All ALD patients are born with a mutation in the ABCD1 gene and they are free of symptoms at birth. The first symptom, which is often adrenal insufficiency, can occur from the age of 5 months onwards. Virtually, all adult men develop a myelopathy. Male patients, boys and adults, are at risk to develop cerebral ALD. (Figure adapted from Kemp et al.)

A newborn male patient has a 35-40% risk to develop demyelinating lesions in the cerebral white matter (cerebral ALD) between 3 and 18 years of age but cerebral ALD can also occur during adulthood. The youngest patient ever reported with cerebral ALD was 2.8 years of age. The first clinical symptoms are generally cognitive deficits and behavioral problems which are reflected in declined school performances. These early clinical symptoms are not specific for ALD and are often attributed to other disorders such as attention deficit hyperactivity disorder (ADHD), which can lead to a delay in the diagnosis of ALD. If the disease progresses, overt neurological deficits become apparent including hearing and
visual impairment, cerebellar ataxia, spasticity of the limbs and sometimes even seizures. At this stage progression is extremely rapid. Affected patients can lose the ability to understand language and walk within a few months. Eventually, patients are bedridden, blind, unable to speak or respond, requiring full-time nursing care and feeding by nasogastric or gastrostomy tube. In general death occurs 2 to 4 years after onset of the initial symptoms. However, if patients are well cared for, they may remain in this apparent vegetative state for years. The only effective treatment available for cerebral ALD at this moment is an allogeneic hematopoietic stem cell transplantation (HSCT). It can arrest or even reverse cerebral demyelination in ALD provided that the transplant is performed in the earliest stage of the disease. For this, early diagnosis is of major importance. In the sections “diagnosis in boys and adult males” and “current therapy for ALD” this will be further addressed. Male patients who do not develop cerebral demyelination as a child, are still at risk as adults. The life-time risk for a male ALD patient to develop cerebral ALD is about 60%17. Virtually all males develop a progressive spinal cord disease (adrenomyeloneuropathy, AMN) as adults11. Most male patients develop AMN in the third decade of their life although it can also be much earlier (in the second decade) or later (up to the fifth decade)18. Patients may suffer from a neurological bladder dysfunction that can lead to full incontinence16, they may need assistance to walk and they can become wheelchair dependent over time19. As with many X-linked diseases, it was thought that females were only carriers of ALD and that only a minor proportion would develop symptoms. Recent studies, however, have demonstrated that around 80% of the female carriers develop symptoms of ALD12, 20. The age of onset is later in women than in men; around the fifth decade of life12. As in men with AMN, the disease is progressive. But, the disease progression appears to be slower in women compared to men12. Especially striking is the occurrence of faecal incontinence that was reported in 28% of the affected women12. This has not been reported for men with AMN. Although it is now clear that women develop symptoms of ALD, diagnosis can be difficult and the possibility of misdiagnosis remains. In the section ‘diagnosis in women’ and in chapter 4 of this thesis, this topic will be further addressed.

Diagnosis

Diagnosis in boys and adult males

If ALD is suspected, the diagnosis can be confirmed by biochemical and/or genetic laboratory tests. In >99.9% of boys and adult male ALD patients, the VLCFA levels in plasma are elevated21. However, two cases have been reported of male ALD patients with normal VLCFA concentrations in plasma22, 23. For that reason, additional biochemical tests in cultured skin fibroblasts were performed and these results showed clearly abnormal values23. Due to the defect in the ABCD1 protein, ALD patients have impaired peroxisomal beta-oxidation
of VLCFA and increased elongation activity. By using stable-isotope labelled docosanoic acid (D3-C22:0), it is possible to measure both the VLCFA beta-oxidation capacity and the fatty acid chain elongation activity in living cells (chapter 2 of this thesis). The combination of a decreased VLCFA beta-oxidation capacity and increased fatty acid elongation activity points towards several peroxisomal disorders, which includes ALD, Zellweger spectrum disorders, peroxisomal acyl-CoA oxidase 1 (ACOX1) deficiency, D-bifunctional protein (HSD1B4) deficiency, the “contiguous ABCD1 DXS1357E deletion syndrome” (CADDS) and acyl-CoA binding domain containing protein 5 (ACBD5) deficiency. Additional diagnostic test (e.g. measurement of other peroxisomal markers and mutation analysis) are necessary to confirm the diagnosis.

The adrenal insufficiency that can occur in male patients can be diagnosed by measuring the levels of adrenocorticotropic hormone (ACTH) and cortisol in plasma. High levels of ACTH and low levels of cortisol are markers of adrenal insufficiency. With the current diagnostic tests available it is not possible to verify whether the adrenal insufficiency is a consequence of ALD or caused by other factors, for example an autoimmune response. Of ALD patients, 80% develop adrenal insufficiency before the onset of neurological symptoms. Therefore, when a boy or man is diagnosed with primary adrenal insufficiency he should always be tested for ALD.

After ALD is diagnosed, young boys and male patients will need to undergo a MRI scan on a regular basis to identify signs of cerebral demyelination in the earliest stage possible.

**Diagnosis in females**

The diagnosis of ALD in females can be difficult in some cases. Of all women with ALD 15-20% have normal VLCFA levels in plasma and fibroblasts. For this reason, it is recommended to perform ABCD1 mutation analysis in women when ALD is suspected. However, when a woman with symptoms consistent with ALD has normal VLCFA levels in plasma and the mutation analysis reveals a variation in ABCD1 that has not been described as a pathogenic mutation, the diagnosis or the exclusion of ALD can be problematic. Especially if no male relatives are present. In 2016, Schackmann et al. published a method that provides a solution for this problem. In this method, fibroblasts from women with a variation of unknown significance (VUS) in ABCD1 are immortalized and clonal cell lines are selected. Since one cell expresses the normal allele and the other cell expresses the VUS-containing gene, both cell types can be tested biochemically. If the VUS-containing clone shows reduced levels of VLCFA beta-oxidation, this points towards a pathogenic mutation and confirms the diagnosis of ALD. However, since this method is highly labor-intensive and therefore costly, new biomarkers to diagnose women with ALD with a much higher sensitivity are warranted. To this end, we used the ALD 2.0 mouse model, which is an Abcd1 knockout model with enhanced ELOVL1 expression in oligodendrocytes, with the aim to
identify new biomarkers. In chapter 3 we show that C26:0-lysophosphatidylcholine (C26:0-lysoPC) and C26:0-carnitine are elevated in brain and spinal cord of the ALD 2.0 mouse. In addition, C26:0-lysoPC and C26:0-carnitine levels were elevated in dried blood spots of the ALD 2.0 mice, but also in dried blood spots of male ALD patients. From this study it was concluded that C26:0-carnitine could be used as a new biomarker for ALD. In the newborn screen, C26:0-lysoPC is currently used as the biomarker for ALD\textsuperscript{14, 34, 35}. In chapter 4 we measured C26:0-carnitine and C26:0-lysoPC in dried blood spots of 49 women with ALD to investigate if these biomarkers allow a more accurate diagnosis. Our results show that C26:0-carnitine is only elevated in 32/49 women with ALD. In contrast, C26:0-lysoPC was elevated in all ALD women compared to control levels. This study shows that, in comparison to the currently existing biomarker (plasma VLCFA), C26:0-lysoPC is a superior biomarker for the diagnosis of females suspected to have ALD.

**Newborn screening**

For young boys that often suffer from an undiagnosed adrenal insufficiency or who are at risk to develop cerebral ALD, an early diagnosis of ALD is of major importance. To detect ALD in the newborn screen, the levels C26:0-lysoPC are measured in dried blood spots\textsuperscript{14, 34}. In all ALD males and in all the 49 women in whom we measured C26:0-lysoPC in dried blood spots, the levels of C26:0-lysoPC were elevated (chapter 4). The quantification of C26:0-lysoPC requires a dedicated, separate method. The implementation of ALD into newborn screening programs would benefit if this dedicated and separate method is no longer needed. A group of diseases that is often screened for in newborn screening programs are the mitochondrial fatty acid disorders. The detection of mitochondrial fatty acid disorders involves the quantification of acylcarnitine levels in dried blood spots. As described in the previous section, we identified C26:0-carnitine as a potential new biomarker for ALD. To validate C26:0-carnitine as a biomarker for ALD in newborns, dried blood spots of 200 control newborns and 11 newborns with ALD were used to measure the C26:0-carnitine levels. The data showed that C26:0-carnitine was not increased in all newborns with ALD. The results were in line with data from our collaborators in this project. The New York State Newborn Screening Program analyzed approximately 270,000 dried blood spots from newborns for C26:0-carnitine. In addition, C26:0-carnitine was measured in 64 dried blood spots with increased levels of C26:0-lysoPC (these samples were from newborns diagnosed with ALD, Zellweger spectrum disorders or Aicardi Goutières Syndrome). Although the C26:0-carnitine levels in these increased C26:0-lysoPC samples were increased compared to control levels, there was still an overlap between the two groups. From these two studies was concluded that C26:0-carnitine is not a proper biomarker for ALD to use in the newborn screening as it would result in false negative results (chapter 4 of this thesis).
**Therapy for ALD**

**Current therapy**

To date, no fully curative therapy is available for ALD but there are treatment options for adrenal insufficiency and cerebral demyelination. Adrenal insufficiency can be treated via corticosteroid replacement therapy. The cerebral demyelination can be halted or even reversed by an allogeneic HSCT if this procedure is performed in the earliest stage of the demyelination with only minor white matter abnormalities visible on an MRI scan. After a successful HSCT the lesions will stabilize after 6 to 12 months. The exact mechanism behind the curative effect of a HSCT is not known, but it might involve the replacement of ABCD1-deficient microglia progenitor cells by bone marrow-derived microglia and this process lasts several months. A requirement for an allogeneic HSCT is a matching donor. In the near future, it might become possible to perform autologous HSCT with cells that are genetically corrected via gene therapy. Two patients have already been treated with gene therapy. At the time of the treatment the patients were 7 and 7.5 years of age. The patients received an intravenous injection with granulocyte colony-stimulating factor. Next, peripheral blood mononuclear cells (PBMCs) were taken from the patients and CD34+ cells were isolated. The cells were infected with a replicon-defective HIV-1-derived lentiviral factor which expressed normal control ABCD1 cDNA. After performance of replicon-competent lentiviral safety assays, the transduced CD34+ cells were reinfused into the patients. The MRI scan of the patients showed inflammatory demyelinating lesions before the treatment. Between 12 to 18 months post-transplant, no signs of inflammatory demyelinating lesions were visible on the MRI scans of the patients. In addition, the neurological outcome of both patients indicated that the procedure was successful. These results suggest that gene therapy may be as effective as conventional allogeneic HSCT. The main advantages of an autologous HSCT over an allogeneic HSCT is that there is no matching donor needed and the morbidity and mortality rate will decrease since allogeneic HSCT is associated with a 10% mortality rate mainly caused by graft-versus-host disease. Currently, the safety and tolerability of gene therapy for ALD is investigated in a phase 2/3 investigational trial (The Starbeam Study, sponsored by Bluebird bio). In December 2016, 25 boys diagnosed with cerebral ALD, but for whom a matched donor was not available, had been treated with autologous CD34+ hematopoietic stem cells that were transduced with Lenti-D lentiviral vector ex-vivo. After completing the first phase of the study (2018), the patients will enter a new study for an additional 13 years to evaluate long-term safety and efficacy.
Unsuccessful therapeutic strategies

Anti-inflammatory drugs
The cerebral demyelination in ALD is caused by a severe inflammatory response. In the past several attempts have been made to counteract or reverse this inflammation with anti-inflammatory drugs. Unfortunately, cerebral demyelination could not be halted or reversed by cyclophosphamide\(^{41,42}\), cytoxan\(^{41,42}\), interferon-beta\(^{43}\), cyclosporine\(^{7}\), or immunoglobulins\(^{44}\).

Lorenzo’s oil
In the 1980’s it was shown that C26:0 levels were normalized in fibroblast from ALD patients when these fibroblasts were cultured in the presence of oleic acid (C18:1) and erucic acid (C22:1)\(^{45}\). Based on these studies, Lorenzo’s oil was developed. Lorenzo’s oil is a 4:1 mixture of the triglyceride form of C18:1 and C22:1. When ALD patients received oral administration of Lorenzo’s oil, the plasma C26:0 levels normalized within one month\(^{46,47}\). However, there is no effect of Lorenzo’s oil on C26:0 levels in the central nervous system\(^{48}\). In addition, several open-label studies with Lorenzo’s oil could not show any improvement in the endocrine and neurological function or an arrest in disease progression in ALD patients\(^{49-51}\). In 2005, a follow-up study was published of 89 asymptomatic boys that had taken Lorenzo’s oil and were on a moderate fat restriction diet\(^{52}\). After a mean follow-up of 6.9 years, 24% of the patients developed MRI abnormalities. The authors of this study advised, based on these results, to provide Lorenzo’s oil treatment to asymptomatic boys with ALD. There are, however, certain limitations in this study. Since it was considered to be unethical to refuse a possible curative therapy to patients, no placebo controlled patient group was included in this study. The results of the follow-up were compared to historical data that had showed that the percentage of boys who develop cerebral ALD is 37%\(^{7}\). This percentage however might be an overestimation due to a selection bias in the direction of the severely affected patients. Another study from the Netherlands had indeed reported a lower percentage of 31% of boys who developed cerebral ALD\(^{18}\).

Statins
In 1998, it was reported that treatment with lovastatin resulted in normalization of plasma VLCFA in ALD patients\(^{53}\). Statins, including lovastatin, is a group of drugs that lowers low-density lipoprotein (LDL) cholesterol. In another study simvastatin was provided to six children with ALD but no significant decrease in plasma VLCFA concentrations was found\(^{44}\). In ALD mice, no significant decrease on VLCFA levels in brain, heart and liver was found after treatment with simvastatin. In 2010, a randomized, double-blind, cross-over trial comparing lovastatin to placebo was published that showed that there was no normalization of plasma C26:0 in ALD patients after treatment with lovastatin\(^{55}\). Furthermore, the levels of C26:0 in
erythrocytes and lymphocytes did not change after treatment with lovastatin\textsuperscript{56}. The reason that Singh \textit{et al}\textsuperscript{53} found a decrease in plasma VLCFA levels is probably caused by the decrease in the levels of LDL cholesterol. VLCFA are basically water-insoluble and have a very low binding affinity to albumin. The majority of VLCFA in plasma are transported as cholesterol-esters associated to lipoprotein particles such as LDL. Taken together, from this study it was concluded that lovastatin should not be used as a therapy to lower VLCFA in ALD patients.

\textbf{Genetics and biochemistry}

All ALD patients have mutations in the \textit{ABCD1} gene, that is located on the X-chromosome. The majority of the patients inherit a mutated gene via one of the parents. However, 4\% of the patients are affected by a mutation that has occurred in the germ line, a \textit{de novo} mutation\textsuperscript{57}. To date, >750 unique mutations have been identified and registered in the ALD database (www.x-ald.nl). There is no correlation between the severity of the disease and the type of mutation. Cases have been reported of monozygotic twins where one sibling suffered from cerebral ALD and the other did not\textsuperscript{58-60}.

The \textit{ABCD1} gene encodes the peroxisomal transmembrane protein (ABCD1 protein), also referred to as ALDP, a member of the ATP-binding cassette (ABC) transporters family\textsuperscript{10, 61}. The ABCD1 protein has the structure of an ABC half-transporter and transports VLCFA as CoA esters into the peroxisome\textsuperscript{62, 63} where these are degraded via beta-oxidation\textsuperscript{24}. Due to the defective ABCD1 protein in ALD, the VLCFA are unable to enter the peroxisome and subsequently they accumulate in plasma and the cells of all tissues including white matter of the brain, spinal cord and adrenal cortex\textsuperscript{64, 65}. Only a small portion of the accumulated VLCFA are of dietary origin. The majority is the result of endogenous elongation of long-chain fatty acids by the ELOVL enzymes\textsuperscript{66, 67}. The main enzyme responsible for this is the protein ‘elongation of very long-chain fatty acids 1’ (ELOVL1)\textsuperscript{68}. The enhanced elongation activity is not the result of increased levels ELOVL1 protein but the result of increased VLCFA CoA esters due to the primary defect in peroxisomal VLCFA beta-oxidation\textsuperscript{25, 68}.

In addition to the ABCD1 protein, the peroxisomal membrane contains two additional ABC half-transporters: the ABCD2 protein (ALDRP)\textsuperscript{69, 70} and the ABCD3 protein (PMP70)\textsuperscript{71, 72}. ABCD1 and ABCD2 are highly homologous and have some overlap in the substrate specificity for saturated and mono-unsaturated VLCFA-CoA esters\textsuperscript{73-75}. The ABCD3 protein transports more hydrophilic fatty acids such as long-chain unsaturated fatty acids and long-chain dicarboxylic acids\textsuperscript{76}. In fibroblasts from ALD patients, the residual beta-oxidation activity is 15-20\% compared to the activity in control fibroblasts\textsuperscript{27, 78}. Since the ABCD2 expression in human fibroblasts is virtually absent, this residual beta-oxidation activity is most likely due to the VLCFA import into the peroxisome via ABCD3\textsuperscript{61}. In line with this, over-expression of either ABCD2 or ABCD3 in ALD deficient fibroblasts corrects the biochemical deficiency\textsuperscript{73} and over-expression of ABCD2 in ALD knockout mice corrects VLCFA levels\textsuperscript{79}. 
Fatty acid toxicity

All ALD patients accumulate VLCFA. It is hypothesized that the accumulated VLCFA cause adrenal insufficiency and myelopathy but that an external trigger and/or variations in modifier genes are necessary for the initiation of cerebral demyelination\(^1\). This hypothesis is supported by the finding that young boys who received a hematopoietic stem cell transplantation to treat the cerebral demyelination still have increased VLCFA levels and can develop myelopathy as adults\(^7\). If VLCFA by themselves are so toxic that increased levels cause adrenal insufficiency and myelopathy, than the question arises “what is the mechanism underlying VLCFA toxicity”? It is of major importance to clarify the underlying mechanism of VLCFA toxicity, because there is no therapy available to treat myelopathy. All male patients, and >80% of the female patients, will develop myelopathy during their lives. Many studies have been done to gain insight into the toxicity of VLCFA. In human ALD fibroblasts that were exposed to supraphysiological levels of VLCFA oxidative lesions, reactive oxygen species, decreased mitochondrial membrane potential and reduced glutathione levels were found\(^8\). Lopez-Erauskin et al\(^8\) showed that high levels of C26:0 added to the tissue culture medium of ALD fibroblasts cause oxidation of mitochondrial DNA which impairs the oxidative phosphorylation resulting in the production of mitochondrial reactive oxygen species. In addition, oxidative lesion markers were also demonstrated in the spinal cord of Abcd1 deficient mice\(^8\). Oligodendrocytes and astrocytes that were isolated from rat brain and cultured in the presence of excess amounts of C22:0, C24:0 or C26:0 showed increased levels of cell death within one day\(^8\). In glia cells and neurons depolarization of mitochondria induced by VLCFA was found in situ\(^8\). Furthermore, Hein et al\(^8\) reported that the myelin producing oligodendrocytes were affected by VLCFA to the largest degree which is interesting since demyelination is one of the main problems in ALD. In addition, astrocytes from Abcd1 deficient mice are more sensitive to VLCFA than astrocytes from wild type mice\(^8\). Mitochondria in ALD brain show increased levels of reactive oxygen species and depolarization\(^8\). Much evidence that oxidative stress and mitochondrial dysfunction play a role in the pathogenesis of ALD has been obtained by using cell cultures and mouse models. However, evidence of increased oxidative stress and mitochondrial dysfunction has been obtained in ALD patients as well. In plasma from ALD patients levels of free radicals were increased and the anti-oxidant defense capacity was reduced\(^8\). In leukocytes from ALD patients, increased levels of DNA damage were reported which is associated with increased levels of free radicals\(^8\). From all these data, it can be concluded that oxidative stress and mitochondrial dysfunction has a role in the pathogenesis of ALD. However, the exact mechanism by which VLCFA activate these responses has not been resolved at the cellular level.

In 2013, Volmer et al\(^8\) showed that high levels of palmitic acid (C16:0, 500 µM) resulted in the direct activation of the endoplasmic reticulum (ER) transmembrane proteins PERK (protein
kinase RNA-like endoplasmic reticulum kinase) and IRE1 (inositol-requiring enzyme 1α) (Fig. 2). These transmembrane proteins can activate an ER stress response that is associated with the accumulation of misfolded or unfolded proteins in the ER. The mis- or unfolded proteins are detected via the luminal parts of PERK, IRE1α and ATF6 (activating transcription factor 6) and as a result the unfolded protein response is activated. Volmer et al.86 showed that high levels of fatty acids also activate an ER stress response but that this is initiated via direct activation of the transmembrane domains of IRE1α and PERK instead of the luminal stress-sensing domain. Based on these findings we decided to investigate whether VLCFA can induce an ER stress response (chapter 5 of this thesis). The data showed that only VLCFA but not long-chain fatty acids induce an ER stress response in fibroblasts from ALD patients. In control fibroblasts, no ER stress response was induced neither by VLCFA nor by long-chain fatty acids87. In 2008, Eichler et al.88 injected C16:0-lysoPC and C24:0-lysoPC into the cortex of wildtype mice and showed that only C24:0-lysoPC caused microglial activation and apoptosis. These results indicate as well that fatty acid toxicity is chain length dependent. Besides this, we showed that exposure of ALD fibroblasts to VLCFA results in increased levels of cell death87. Further support that ER stress indeed has a role in the pathogenesis of ALD was demonstrated by the finding that the PERK/IRE1α/ATF4 pathway in the spinal cord of Abcd1 knockout mice and in brain samples of ALD patients is activated89.

**Figure 2. ER stress pathway.**

Upon ER stress, the ER transmembrane protein PERK and IRE1 form a dimer that becomes auto-phosphorylated. Downstream of PERK, eIF2α is phosphorylated and downstream of IRE1, 26 nucleotides are removed from XBP1unspliced (XBP1u) mRNA, causing a frame-shift which results in the transcription factor XBP1spliced (XBP1s) that activated its target EDEM1. Severe or long-lasting ER stress results in increased levels of GADD34 and pro-apoptotic factor CHOP.
Therapeutic targets

Degradation of VLCFA via omega-oxidation

VLCFA can undergo ω-oxidation resulting in the generation of very long-chain dicarboxylic acids. Dicarboxylic acids can enter the peroxisome via the ABCD3 protein. The beta-oxidation of long-chain dicarboxylic acids is normal in ALD patients. The transporter for very long-chain dicarboxylic acids has not yet been identified, but it is likely that this function is carried out by ABCD3. Stimulation of the ω-oxidation pathway is an interesting target since it provides an escape route for the deficient beta-oxidation in ALD patients. CYP4F2 and CYP4F3B are key enzymes in the omega-oxidation of VLCFA. In 2016, it was reported that a polymorphism in CYP4F2 (rs2108622 C.1297G>A causing a substitution of valine for methionine at position 433) increases the risk to develop cerebral ALD. This polymorphism does not reduce the enzymatic activity of CYP4F2 but causes a major decrease in the amount of protein and thereby a reduction in the ω-oxidation (rescue pathway) capacity. Since decreased levels of ω-oxidation are associated with an increased risk of developing cerebral ALD, patients might benefit from increased activity of the ω-oxidation pathway which results in an increased capacity of the rescue pathway.

Inhibition of ELOVL1

Since ELOVL1 is the main enzyme responsible for the elongation of long-chain fatty acids to VLCFA, the inhibition of ELOVL1 will directly decrease the synthesis of newly formed VLCFA. This makes the ELOVL1 protein and ELOVL1 inhibitors interesting targets for a therapeutic strategy for ALD. Bezafibrate was described as a compound that reduces VLCFA levels in fibroblasts from ALD patients via the direct inhibition of ELOVL1. Unfortunately, a clinical trial with bezafibrate showed that the C26:0 levels were not reduced in plasma and lymphocytes nor was C26:0-lysoPC reduced in dried blood spots. These results were most likely caused by a too low biological availability of bezafibrate. However, inhibition of ELOVL1 is still a highly interesting therapeutic target.

Activation of ABCD2

In 1998, it was shown that the beta-oxidation activity in cells from ALD patients and Abcd1 knockout mice can be increased by treatment with 4-phenylbuterate (4PBA). 4PBA resulted in increased levels of ABCD2 protein and peroxisome proliferation. In Abcd1 knockout mice that were fed a diet supplemented with 4PBA, the levels of C24:0 were completely normalized and the levels of C26:0 were lowered by 80% after six weeks in brain. In the adrenal glands a 90% normalization of both C24:0 and C26:0 was reported. Furthermore, additional inactivation of Abcd2 in Abcd1 knockout mice (the Abcd1/Abcd2 double knockout mouse model), resulted in higher VLCFA levels when compared to the Abcd1 knockout.
mouse model. This observation further supported that there is an overlapping substrate specificity for ABCD1 and ABCD2. In line with this, overexpression of Abcd2 in an Abcd1 knockout mouse resulted in normalization of VLCFA in spinal cord, sciatic nerve and adrenal gland. Various attempts have been made to activate ABCD2. Fenofibrate and the thyroid hormone 3,5,3'-iodothyronine (T₃) were able to increase Abcd2 mRNA expression in rat liver after dietary supplementation. Fenofibrate did not cause an increase of Abcd2 mRNA in the brain, probably due to the inability of fenofibrate to cross the blood-brain-barrier. After the supplementation of T₃ to rats, no increased mRNA levels of Abcd2 were found in the brain. However, the incubation of oligodendrocyte-differentiated CG4 cells with T₃ resulted in increased mRNA levels of Abcd2 and Abcd3. This suggests that it is possible to increase ABCD2 and ABCD3 activity in an oligodendrocyte cell type, a cell type that is a target for any therapy for ALD. Furthermore it was shown that the thyroid hormone receptor agonist sobetirome increased Abcd2 mRNA levels in brain and liver of wild type mice. Adult Abcd1 knockout mice were treated for 11-12 weeks with sobetirome after which the levels of C26:0-lysoPC in serum and brain and the C26:0/C22:0 ratio in adrenal glands, testes and brain were decreased by approximately 20%. ABCD2 expression can also be regulated via intracellular cholesterol levels. In human fibroblasts and monocytes, ABCD2 expression was increased upon sterol depletion.

It has been shown that in cultured cells ABCD2 expression can be induced by retinoic acid. To investigate if retinoic acid can induce ABCD2 expression in monocytes, THP-1, a monocyte/macrophage-like suspension cell line, was cultured in the presence of retinoic acid and it was found that ABCD2 expression levels were increased. Retinoic acid is used to treat patients that suffer from severe acne. Weber et al. isolated monocytes and lymphocytes from acne patients that were treated with retinoic acid and from healthy controls. There was no difference in the ABCD2 expression levels between healthy controls and acne patients that were given retinoic acid. From this study it was concluded that retinoic acids are probably not able to increase ABCD2 to a high enough level and are not a realistic therapeutic option for ALD.

Redirection of fatty acid synthesis via SCD1 activation

In chapter 6 of this thesis we show that mono-unsaturated VLCFA are less toxic than saturated VLCFA in ALD fibroblasts. We hypothesized that it could be beneficial for ALD patients if the fatty acid synthesis pathway could be diverted from the saturated towards the mono-unsaturated fatty acids (Fig. 3). This is possible via the activation of the main enzyme responsible for the desaturation ‘stearoyl-CoA-desaturase 1’ (SCD1). SCD1 generates a cis-double bond at the omega-9 position of C16:0 and C18:0 resulting in C16:1 and C18:1. These mono-unsaturated are then further elongated to generate mono-unsaturated VLCFA (C24:1 and C26:1).
Figure 3. Fatty acid synthesis pathway.
Activation of SCD1 causes a shift from the saturated towards the mono-unsaturated fatty acids. C16:1 and C18:1 are further elongated to C24:1 and C26:1.

The LXR agonist TO901317 is a compound that has been described in literature to increase SCD1 activity\textsuperscript{106}. Chapter 6 shows that exposure of ALD fibroblasts to different LXR agonists results in increased levels and activity of SCD1, increased synthesis of mono-unsaturated very long-chain fatty acids and decreased levels of saturated very long-chain fatty acids. In addition, it is demonstrated that these compounds are able to reduce endogenous C26:0 levels in ALD fibroblasts to the levels measured in control fibroblasts. To study the effect of LXR agonists \textit{in vivo}, we are currently investigating if TO901317 affects VLCFA levels in Abcd1 knockout mice with our collaborators at the Medical University of Vienna Dr. Sonja Forss-Petter and Prof. Johannes Berger.

Outline of this thesis
During my Ph.D. project I have worked on several ALD-related subjects. The first part of this thesis is focused on the improvement of the diagnosis for ALD. First, in chapter 2, a method is presented that can be used to measure peroxisomal beta-oxidation and de novo synthesis of C26:0 in living cells. This method has been added to the diagnostic array of tests of our laboratory. Chapter 3 describes the development of the “ALD 2.0” mouse model and the identification of C26:0-carnitine as a new biomarker for ALD in mice and men. In chapter 4 the comparison of the sensitivity of C26:0-carnitine and C26:0-lysoPC for the diagnosis of ALD newborns and women with ALD is presented. The results show that C26:0-carnitine is not a sensitive enough biomarker for the diagnosis of ALD in newborns or women with
ALD. However, C26:0-lysoPC is a superior marker in comparison with C26:0 plasma levels to diagnose women with ALD. Levels of C26:0-lysoPC were increased in all women, even the women with normal C26:0 plasma concentrations. In chapter 5 the role of VLCFA in the pathophysiology of ALD was investigated. It is shown that saturated VLCFA induce an ER stress response and lipid-induced cell death in ALD cells which provides new insight in the pathophysiology of the disease. In chapter 6 it is hypothesized that upregulation of SCD1 would redirect fatty acid synthesis towards the much less toxic mono-unsaturated fatty acids. In addition, it is demonstrated that SCD1 activation results in a complete normalization of endogenous C26:0 levels in ALD. This new approach might be a first step towards a curative therapy for ALD aimed at the correction of the biochemical deficiency.
References

68. Offman, R. et al. The role of ELOVL1 in very long-chain fatty acid homeostasis and X-linked adrenoleukodystrophy. EMBO Mol Med 2, 90-7 (2010).