Biochemical and cell biological aspects of X-linked adrenoleukodystrophy

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Biochemical and cell biological aspects of X-linked adrenoleukodystrophy

M.J.A. Schackmann
BIOCHEMICAL AND CELL BIOLOGICAL ASPECTS OF X-LINKED ADRENOLEUKODYSTROPHY

Martinus Johannes Adrianus Schackmann
Biochemical and cell biological aspects of X-linked adrenoleukodystrophy

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Coverdesign: Ron Schackmann & Stefanie de Poot
BIOCHEMICAL AND CELL BIOLOGICAL ASPECTS OF X-LINKED ADRENOLEUKODYSTROPHY

ACADEMISCH PROEFSCHRIFT

ter verkrijging van de graad van doctor aan de Universiteit van Amsterdam op gezag van de Rector Magnificus prof. dr. ir. K.I.J. Maex
ten overstaan van een door het College voor Promoties ingestelde commissie,
in het openbaar te verdedigen in de Agnietenkapel op dinsdag 23 mei 2017, te 10:00 uur
door

Martinus Johannes Adrianus Schackmann

geboren te Amsterdam
Promotiecommissie:

Promotor: Prof. Dr. R.J.A. Wanders Universiteit van Amsterdam
Copromotor: Dr. S. Kemp Universiteit van Amsterdam

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Chapter 1

General Introduction
Introduction

X-linked adrenoleukodystrophy: clinical aspects

History

X-linked adrenoleukodystrophy (X-ALD) was first reported by Haberfeld & Spieler in 1910 [1] and Siemeling & Creutzfeldt in 1923 [2]. They described boys between 6 and 8 years of age who had developed disturbances in eye movement followed by deterioration in school performance and a progressive inability to walk. In addition, bronzing of the skin was noted. Upon postmortem autopsy of the brain Schilder observed a severe loss of myelin [3]. The discovery by Schilder prompted the name Schilder’s disease [3]. It was not until 1970 that the term adrenoleukodystrophy was introduced by Blaw thereby linking the leukodystrophy in patients with adrenocortical insufficiency [4].

The most striking biochemical marker was described in 1976 when it was found that lipid inclusions in adrenal cells derived from autopsy material from X-ALD patients [5] contained cholesterol esters with an excess of very long-chain fatty acids (VLCFA) from C24:0 up to C30:0 with C26:0 being the most prominent. Later Moser and coworkers discovered increased VLCFA levels in plasma of X-ALD patients too [6]. It was not until 1984 that VLCFA oxidation was found to take place in peroxisomes [7], which explains why X-ALD is classified as a peroxisomal disease.

In 1981 X-ALD was genetically mapped to the Xq28 chromosomal band [8]. In 1992 the X-ALD gene that codes for a protein referred to as ALDP was identified. This protein was found to share homology with members of the ATP-binding cassette (ABC) transporter family. ALDP is classified to subfamily D being member 1 (ABCD1) [9]. Experiments using immunofluorescence and immunoelectron microscopy were used to demonstrate that ALDP associates with the peroxisomal membrane [10].

In 1997, three independent laboratories reported the generation of Abcd1 knockout mouse model. Although the Abcd1 knockout mouse showed increased VLCFA levels, no neurological abnormalities were detected in these mice [11–13]. Importantly, in 2002 it was reported that mice older than 15 months develop adrenomyeloneuropathy (AMN) like symptoms [14], indicating that the mouse models could still be of use in the study of X-ALD.

Phenotypes in male patients

The phenotypic presentation of X-ALD is very heterogeneous. The two most frequent phenotypes are childhood cerebral ALD (CCALD) and adrenomyeloneuropathy (AMN). In a minority of patients there is only adrenocortical insufficiency (Addison-only) [15]. These patients, however, are at high risk to develop either CCALD or AMN.

Cerebral ALD

The most severe form of X-ALD is CCALD. The onset of CCALD is between three and ten years of age and is characterized by a rapidly progressive cerebral demyelination [16]. In CCALD patients initial development is normal until symptoms including decreasing school performance, behavioral disturbances, impaired vision and hearing and poor coordination start to present [17]. Symptoms that present as the disease progresses include seizures and dementia. After the initial manifestation of neurological symptoms progression is rapid leaving the patient in a vegetative state within approximately two years [17,18]. This fast progression is caused by an inflammatory demyelinating disease of the brain [19].
**Adrenomyeloneuropathy**

The most frequent phenotype of X-ALD is AMN, which typically presents in males in their twenties presenting with stiffness in the legs and generalized weakness. The symptoms are slowly progressive eventually leading to the need for a cane or wheelchair. Over time about 50% of male patients show neurological involvement [16]. A subset of AMN patients may develop the cerebral form of X-ALD later in life which mimics the childhood cerebral form [20]. Symptoms strongly resemble CCALD, but the initial progression of symptoms usually is slower [21].

**Addison-only**

Addison’s disease or adrenal insufficiency can present with fatigue and weakness. It can cause persistent vomiting, anorexia, hypoglycemia, poor weight gain in a child, or unexplained weight loss in an adult, malaise, fatigue, muscular weakness, unexplained isotonic or hyponatremic dehydration, hyperkalemia, hypotension, hypoglycemia and especially generalized hyperpigmentation [22]. Addison’s disease can have various causes one of them being X-ALD. In fact, it has been suggested that if the onset of Addison-only is before the age of 7.5 there is a high probability that X-ALD is the underlying cause [23]. With age patients with bona fide mutations in ABCD1 but with Addison-only can develop neurological symptoms. In most patients this will present as AMN when they are adults however, as the age of onset varies greatly this can also occur during childhood. It is important that males with idiopathic Addison disease or with progressive paraparesis are screened for X-ALD as therapies can be offered and delay of treatment can lead to severe morbidity and even death. Furthermore, it has important implications for genetic counseling [15].

**Phenotypes in female patients**

It has already been known for some time that female carriers can also present with symptoms which is in marked contrast to most X-linked diseases [24–26]. Early on female carriers of X-ALD were diagnosed with AMN-like symptoms such as abnormal gait, extensor plantar responses, hypertonia, and urinary symptoms [27]. More recent studies have shown that eventually up to 80% of female X-ALD carriers develop neurological symptoms [28,29]. These reports also demonstrate that development of neurological symptoms increases with age. On rare occasions there have been reports of female patients developing the cerebral form of X-ALD [30–32].

Screening of female carriers for X-ALD can be difficult because of X chromosome inactivation. X-inactivation is generally random, with both X chromosomes having an equal probability of inactivation. X-inactivation ensures equivalence in expression of X-linked genes in females and males [33]. For female carriers of X-ALD this means that their cells will have a mosaic expression of the X-linked gene involved and thus the protein. This implies that some cells will have normal expression of ALDP whereas in other cells the mutant form of ALDP is expressed. To explain the different phenotypes in X-ALD carriers skewed inactivation of the X-ALD gene has been proposed. However no correlation could be found [28,34]. If the female patient is the first in the family to be diagnosed they are often misdiagnosed as having multiple sclerosis [35,36]. Furthermore, even if a genetic variant in ABCD1 is found in a female patient the pathogenicity of the mutation can only be established if the variant has been described earlier as pathogenic, or if there is an affected male relative with the same mutation [37]. Correct diagnosis is of importance for female
patients as it will help in the identification of present and future health issues and allows for correct genetic counseling. When genetic studies identify a novel genetic variant and there is no known male relative with X-ALD the pathogenicity of the variant cannot be established. In that case additional laboratory studies are needed (see chapter 4) [38].

**X-linked adrenoleukodystrophy: biochemistry**

**VLCFA**

A major breakthrough in X-ALD research was the finding by Igarashi et al. [5] who discovered the presence of abnormal cholesterol esters in adrenal cortex and cerebral white matter of X-ALD patients. The cholesterol esters of X-ALD patients contained VLCFA (C24:0 up to C30:0) while in controls these cholesterol esters were virtually absent. Increased levels of VLCFA are also present in cultured skin fibroblasts [39] and in plasma of patients [6]. The effects of increased levels of VLCFA on the properties of lipid bilayers were studied using artificial membranes. These studies revealed that the microviscosity is increased, making the membrane more rigid [40]. The change in microviscosity of the membrane was found to coincide with a decrease in the adrenocorticotropic hormone (ACTH) receptor response [41]. Stimulation of this receptor normally leads to production of cortisol which functions to: 1) increase blood sugar levels through enhanced gluconeogenesis; 2) suppress the immune system and; 3) to stimulate metabolism [42].

**Peroxisomes**

Peroxisomes are subcellular organelles which were first described morphologically upon electron microscopy analysis of mouse kidney cells (see Nagotu et al. 2012 for review). In 1973 peroxisomes were first linked to a human disease when it was found that kidney and liver tissues from Zellweger syndrome patients were devoid of peroxisomes [44]. Since then many additional peroxisomal diseases have been identified. Inborn errors of peroxisome deficiency can be classified into two categories: 1) peroxisome biogenesis disorders (PBD) (see [45–47] for review) and 2) single peroxisomal enzyme deficiencies (see Wanders 2014 for review). Peroxisomes have important functions in a number of metabolic pathways including: 1) the biosynthesis of ether phospholipids which are phospholipids characterized by an ether bond at the sn-1 position of the glycerol backbone; 2) the α- and β-oxidation of fatty acids; 3) the detoxification of glyoxylate [49,50] and other functions.

**Peroxisomal β-oxidation**

The main oxidative pathway in the peroxisome is the β-oxidation pathway which is not only involved in the metabolism of VLCFA but also in the degradation of dihydroxycholestanolic acid (DHCA), trihydroxycholestanolic acid (THCA) and pristanic acid [48]. However, before oxidation can commence different acyl-CoA synthetases (ACS) need to activate the free fatty acid to the corresponding thioester. Although multiple acyl-CoA synthetases have been identified assignment of a specific ACS to a specific pathway has not yet been achieved [51]. The first step involved in peroxisomal β-oxidation is catalyzed by different flavoproteins known as acyl-CoA oxidases (ACOX). In humans, two acyl-CoA-oxidases have been identified (ACOX1 and ACOX2). Of these two ACOX1 is specific for the CoA-ester of VLCFA and prostaglandins whereas ACOX2 is specifically involved in the β-oxidation of
other acyl-CoAs including pristanoyl-CoA, and di- and trihydroxycholestanoyl-CoA [52]. The second and third steps in peroxisomal β-oxidation are catalyzed by D-bifunctional protein (DBP), also called multifunctional enzyme 2 (MFE2/MFP2) which has a D-3-hydroxy-acyl-CoA ester as intermediate (see [50] for review). The final step involves a thiolase of which the human peroxisome contains two, including: 1) the straight-chain 3-oxoacyl-CoA thiolase (ACAA1) that is specific for the 3-ketoacyl-CoA-esters of VLCFA, and 2) sterol carrier protein X (SCPx) that reacts with intermediates formed from other β-oxidation substrates [49,53] for review). Many disorders are caused by a defect in one of the peroxisomal β-oxidation enzymes. However, in patients with X-ALD the increase in VLCFA is caused by a defect in VLFA transport across the peroxisomal membrane.

Transport of VLCFA

In mammalian peroxisomes three ATP-binding cassette (ABC) half-transporters have been identified and classified into subfamily D. These are adrenoleukodystrophy protein (ABCD1/ALDP), ALDP-related protein (ABCD2/ALDR) and the 70-kDa peroxisomal membrane protein (ABCD3/PMP70) [9,54,55]. The ABCD half-transporters have a transmembrane domain (TMD) with six segments and a nucleotide binding (NBD) domain containing the Walker A, Walker B and ATP-binding cassette signature sequence (see [50,56,57] for review). The TMD allows passage through the membrane while the NBD binds the ATP that is hydrolyzed to facilitate the transport [58]. To be functional transporters, two half-transporters need to form either homo- or heterodimers. Using a yeast two-hybrid system and co-immunoprecipitation experiments it was shown that ALDP, ALDRP and PMP70 can form both homo- and heterodimers [59]. However, experiments with mouse liver proteins have shown mainly homodimeric interactions for PMP70 and ALDP [60]. Furthermore, FRET experiments in intact cells have shown higher values for ALDP homodimerization than for PMP70 homodimerization or ALDP/PMP70 heterodimerization [61], which suggests that ALDP and PMP70 are mainly present as homodimers in mammalian peroxisomal membranes [62]. Studies using Saccharomyces cerevisiae as a model system have shown that human ALDP forms a functional homodimer and that ALDP most likely transports acyl-CoA esters [63]. Substrates for ALDP are saturated and monounsaturated VLCFA-CoA esters were found [65]. ABCD2 has a high sequence similarity to ABCD1 [66] and it has been shown that there is functional overlap between the two half-ABC-transporters in terms of their substrate specificity [67–69]. Despite the overlapping substrate specificity, Abcd1 and Abcd2 knockout mice have different phenotypes, indicating that ALDP and ALDR do perform different roles in lipid metabolism [70]. Abcd2 knockout mice have reduced levels of the poly-unsaturated fatty acid docosahexaenoic acid (C22:6ω3) in primary neurons [71]. It was also found that expression of ABCD2 in Saccharomyces cerevisiae increases β-oxidation of C24:6ω3 [69]. Overexpression of ABCD3 has been shown to stimulate C16:0 β-oxidation activity in CHO cells [72]. Studies in Saccharomyces cerevisiae have shown that ABCD3 (PMP70) is involved in the oxidation of dicarboxylic acids (DCA) and (2R)-methyl fatty acids [64]. Recently a patient with an ABCD3 deficiency was described [73]. The patient had accumulated peroxisomal C27-bile acid intermediates in plasma, severe liver disease and hepatosplenomegaly. This paper also shows that in Abcd3 -/- mice, there was a bile acid biosynthesis defect.
**VLCFA elongation**

While some VLCFA are taken up through the diet, most are synthesized endogenously from LCFA, via a process called chain elongation [74] which takes place at the membrane of the endoplasmic reticulum. Chain elongation involves four consequential reactions catalyzed by different enzymes [75]. The initial reaction is catalyzed by a transferase that allows the condensation of an acyl-CoA ester with malonyl-CoA to produce 3-ketoacyl-CoA. This transferase is part of a family of elongase proteins known as the elongase of very long-chain (ELOVL) fatty acid-like proteins. The second reaction reduces the 3-ketoacyl-CoA to 3-hydroxyacyl-CoA using NADPH as a cofactor. This reduction is executed by a ketoacyl-CoA reductase. Then the 3-hydroxyacyl-CoA dehydratase (HACD) enzyme dehydrates 3-hydroxyacyl-CoA to trans-2,3-enoyl-CoA. In the final reaction the enzyme trans-2,3-enoyl-CoA reductase (TECR) uses NADPH to reduce trans-2,3-enoyl-CoA to an acyl-CoA. The final result is an acyl-CoA ester that is extended by 2 carbon atoms, which can subsequently be elongated further via additional elongation cycles or be used in other metabolic processes. Seven different ELOVL proteins (ELOVL1 – ELOVL7) have been identified in mammals [76,77] which differ in substrate specificity and expression pattern in various cell types [76,77]. ELOVL1 has been identified as the elongase responsible for the elongation of C22:0 to C24:0 and C26:0 [65]. ELOVL1 is ubiquitously expressed in all tissues including the adrenal gland, brain and testis [78], which are the tissues primarily affected in X-ALD. Furthermore ELOVL1 has been linked directly to sphingolipid synthesis [78].

**VLCFA in lipid biosynthesis**

Fatty acids are incorporated in membranes and are needed for the biosynthesis of distinct lipid species such as phospholipids, sphingolipids and glycerolipids. Elongation of fatty acids contributes to the homeostasis of FA together with other factors. Disruption of this homeostasis leads to shifts in fatty acids content in lipids and membranes, which may in turn affect physiological systems [79]. In patients with X-ALD VLCFA β-oxidation is decreased giving rise to higher VLC-acyl-CoA levels that subsequently result in an increase in elongation, thus disrupting fatty acids homeostasis [65,80]. Desorption of free fatty acids from phospholipid membranes decreases greatly with longer fatty acids, from C14:0 to C26:0, and the incorporation of C26:0 in model membranes disrupts membrane structure [81]. Studies with erythrocytes from patients with X-ALD have shown increased levels of C25:0, C26:0 and C27:0 fatty acids in membranes and a subsequent increase in membrane microviscosity [40]. Another study showed that a shift from C24:0 to C16:0 in sphingolipid composition was associated with an increased susceptibility to apoptosis in HeLa cells [82]. Although the shift is opposite to what is found in X-ALD patients it does illustrate the importance of lipid homeostasis and that disruption can have major consequences.

**Fatty acid ω-oxidation**

Long-chain dicarboxylic acids are exclusively β-oxidized in peroxisomes and β-oxidation of long-chain dicarboxylic acids is not affected in fibroblasts from X-ALD patients [83]. It has been shown that fatty acids can be converted to dicarboxylic acids by ω-oxidation [84]. The human cytochrome P450 enzymes, CYP4F2 and CYP4F3B were shown to hydroxylate C26:0 to its dicarboxylic acid [84]. However,
the ω-oxidation of fatty acids is a minor oxidation pathway that accounts for approximately 5–10% of total fatty acid oxidation in the liver [85]. This implies that the pathway would need to be induced in order to be useful in the treatment of X-ALD. The expression of CYP4 genes is under the control of multiple transcription factors including the peroxisome proliferator activated receptor α (PPARα) and can be induced by fasting, ethanol or different fibrates including clofibric acid, at least in rodents [86,87].

X-linked adrenoleukodystrophy: therapy

Hormone replacement therapy

X-ALD patients with Addison’s disease, which presents in the majority of male patients, should receive adrenal hormone therapy. Although important for adrenocortical insufficiency, which can be lethal if untreated, it does not affect neurological progression [88].

Hematopoietic stem cell transplantation

At this time there is only one effective treatment for cerebral ALD and that is hematopoietic stem cell transplantation (HSCT) often referred to as bone marrow transplantation.

In 2004 a study in which data had been gathered from 94 boys with cerebral ALD who had a HSCT between 1982 and 1999, revealed that the 5- and 8-year survival were both 56% [89]. A 2011 study in which long-term disease stabilization and survival of 60 boys receiving HSCT from 2000 to 2009 was analyzed showed an estimated 5-year survival of 91% when boys without clinical cerebral ALD were treated [90]. In this study survival decreased to 66% for boys with neurologic dysfunction. Survival of the patients was greatly dependent on the severity of the neurological impairment at the start of HSCT, as determined by the Loes score [91]. In general when treated in the early stages of the disease HSCT halts [92] or may even reverse [93] the effects of X-ALD. However HSCT is not without risk as 8-10% of the recipients experienced severe acute graft-versus-host disease in some cases leading to death [89,90]. HSCT is dependent on having a human leukocyte antigen (HLA) matching donor. However, the chance of finding a HLA matching donor is small even among siblings [94]. Matching can be improved by combining pre-implantation genetic diagnosis (PGD) with HLA [95]. If a matching sibling is born stem cells from umbilical cord blood can be used for HSCT. This has been done for multiple disorders including X-ALD [96] and the outcome of pre-implantation HLA matching has been reviewed [97]. In these cases no serious complications were observed among recipients. Therefore preimplantation HLA matching is a viable treatment option for couples with an affected sibling.

Hematopoietic stem cell gene therapy

The main drawbacks of HSCT are the need for a bone marrow donor and the risk of graft-versus-host disease. Hematopoietic stem cell gene therapy removes not only the need to find a suitable donor but also the risk of graft-versus-host disease as the patient’s own cells are used for the treatment. Broadly speaking peripheral blood mononuclear cells are taken from the patient. From this blood CD34+ cells are selected and then infected with a lentiviral vector expressing normal ABCD1 cDNA.
These cells are then vigorously checked and conditioned before they are re-infused. Follow up studies strongly suggest that hematopoietic stem cells were transduced in the patients. In two patients the progressive cerebral demyelination stopped. Furthermore the effects were comparable to those seen with allogeneic HCT [98].

**Lorenzo’s oil**

The finding that most VLCFA are the result of endogenous synthesis [74], through elongation of medium and long-chain fatty acids taken up through the diet, was the reason for using a dietary therapy in an attempt to treat X-ALD. The most widely used dietary therapeutic approach to treat X-ALD is Lorenzo’s oil (LO), which is a mixture of glyceryl trioleate and glyceryl trierucate (4:1). Treatment lowers VLCFA in patient fibroblasts [99] and plasma in a matter of weeks [99]. However, treatment does not reverse or stop neurological progression in symptomatic patients [100,101]. In a follow-up study of 89 asymptomatic patients treated with LO Moser and coworkers have recommended, that LO therapy should be offered to neurologically asymptomatic male patients [15]. However, this recommendation is based on “strongly suggestive, albeit not fully definitive, evidence of a preventive effect combined with our awareness of the severe prognosis of the untreated patients” [15]. A recent study on the mechanism of LO revealed that LO inhibits ELOVL1 and lowers the levels of VLCFA containing sphingomyelin [102].

**Drug therapies**

Lovastatin, an inhibitor of 3-hydroxy-3-methyl-glutaryl-coenzyme A (HMG-CoA) reductase was claimed to normalize VLCFA in cultured skin fibroblasts by stimulating lignoceric acid (C24:0) oxidation [103]. However, studies in X-ALD mice [104] and a randomized, double-blind, placebo-controlled, crossover trial [105] did not show a specific decrease in VLCFA levels. Bezafibrate was reported to lowers VLCFA in X-ALD fibroblasts by inhibiting ELOVL1 (Chapter 2) [106]. However, in a proof-of-principal clinical trial bezafibrate was unable to lower VLCFA levels in plasma or lymphocytes in X-ALD patients [107]. Most likely this can be attributed to the low bezafibrate levels which were reached in patients.

Other drug therapies have focused on increasing ABCD2 gene expression (see [108] for review) although none of these have been proven useful in the treatment of X-ALD patients. This has been done in a PPAR dependent manner with fenofibrate, indirectly increasing expression of ALDRP via sterol regulatory-binding protein 2 [109,110]. Increasing ABCD2 gene expression can also be achieved in a PPAR independent manner with compounds like thyroid hormone (T3) [111] and 4-phenylbutyrate (4PBA) [67]. The treatment of X-ALD fibroblasts with T3 corrected the VLCFA. However the effect is limited to certain cell types [111]. Treatment with 4PBA increased expression of the ABCD2 gene in fibroblasts from X-ALD patients and Abcd1 knockout mice and lowered brain VLCFA in the brain of Abcd1 knockout mice [67]. The effects of 4PBA on the ABCD2 gene are probably mediated via histone deacetylase 1 [112].
Scope of this thesis

X-linked adrenoleukodystrophy is a devastating peroxisomal disorder with only limited options for treatment as described above. Recent findings however have pointed towards fatty acid elongation as a possible target for therapeutic intervention of X-ALD [65]. Chapter 2 describes how bezafibrate reduces VLCFA levels in X-ALD fibroblasts by inhibiting fatty acid chain elongation [106]. Based on these results, an open-label pilot study was performed to evaluate the effect of bezafibrate on VLCFA accumulation in blood cells of AMN patients. Unfortunately, bezafibrate failed to lower VLCFA levels in blood cells of X-ALD patients. Most likely this is attributable to its inability to reach adequate drug levels in vivo [107]. In chapter 3 the kinetic characteristics of ELOVL1 and further investigation of the effect of fibrates on fatty acid chain elongation are described. This revealed that bezafibrate had the strongest effect in intact cells while the CoA-ester of gemfibrozil was the strongest inhibitor of VLCFA elongation activity in vitro. The CoA esters of bezafibrate and gemfibrozil inhibit chain elongation by specifically inhibiting the first step of the elongation system, catalyzed by ELOVL1.

Correct diagnosis of patients is a major focus of the Laboratory Genetic Metabolic Diseases. When a female patient with AMN-like symptoms, but with normal plasma VLCFA levels and a novel genetic variant in the ABCD1 gene presented, the question came up whether this patient was truly heterozygous for X-ALD or not. In the absence of any other family members, carrying the same genetic variant, the need for a new diagnostic method came up which would be able to demonstrate whether this novel DNA change is disease causing or merely a polymorphism. In chapter 4 a method is reported to resolve the pathogenicity of novel ABCD1 variants of unknown significance. Correct diagnosis is of importance to all X-ALD patients as it will help in the identification of present and future health issues and allows for the correct counseling on the reproductive risk of the disease. Chapter 5 is focused on the intracellular metabolism of a range of fatty acids and the role of peroxisomes therein. To this end the differences in de novo fatty acid metabolism between control, ABCD1 deficient, ABCD3 deficient and PBD cell lines were studied. Furthermore, the effects of mitochondrial deficiencies on peroxisomal metabolism were investigated. Finally, studies were done to resolve how PBD and ABCD1 deficiency change the fatty acid composition of different lipid species. In chapter 6 a summary is given of the main findings of this thesis work followed by a discussion of the results obtained plus future perspectives.
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Chapter 2

Bezafibrate lowers very long-chain fatty acids in X-linked adrenoleukodystrophy fibroblasts by inhibiting fatty acid elongation

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Published in:
Journal of Inherited Metabolic Disease, Volume 35, Issue 6, November 2012, Pages 1137–1145
Abstract

X-linked adrenoleukodystrophy (X-ALD) is caused by mutations in the ABCD1 gene encoding ALDP, an ATP-binding-cassette (ABC) transporter located in the peroxisomal membrane. ALDP deficiency results in impaired peroxisomal β-oxidation and the subsequent accumulation of very long-chain fatty acids (VLCFA; > C22:0) in plasma and tissues. VLCFA are primarily derived from endogenous synthesis by ELOVL1. Therefore inhibiting this enzyme might constitute a feasible therapeutic approach. In this paper we demonstrate that bezafibrate, a PPAR pan agonist used for the treatment of patients with hyperlipidaemia reduces VLCFA levels in X-ALD fibroblasts. Surprisingly, the VLCFA-lowering effect was independent of PPAR activation and not caused by the increase in either mitochondrial or peroxisomal fatty acid β-oxidation capacity. In fact, our results show that bezafibrate reduces VLCFA synthesis by decreasing the synthesis of C26:0 through a direct inhibition of fatty acid elongation activity. Taken together, our data indicate bezafibrate as a potential pharmacotherapeutic treatment for X-ALD. A clinical trial is currently ongoing to evaluate the effect in patients with X-ALD.

Take-home message: Pharmacological inhibition of ELOVL1 with bezafibrate lowers very long-chain fatty acids in X-linked adrenoleukodystrophy fibroblasts and might constitute a new approach to treatment.
Introduction

X-linked adrenoleukodystrophy (X-ALD: OMIM 300100) is an inherited metabolic disorder characterized by impaired peroxisomal β-oxidation of very long-chain fatty acids (VLCFA; ≥C22) and accumulation of VLCFA (mainly ≥C26:0) in plasma and tissues of patients [1]. It is caused by mutations in the ABCD1 gene (http://www.x-ald.nl), encoding a peroxisomal transmembrane protein named ALD protein (ALDP: OMIM 300371) [2]. Clinically, X-ALD is characterized by a striking and unpredictable variation in phenotypic expression, ranging from the rapidly progressive childhood cerebral form (CCALD) to the more slowly progressive adult form adrenomyeloneuropathy (AMN) and variants without neurological involvement (“Addison-only” phenotype) [1].

Experimental pharmacotherapy in X-ALD was aimed at normalizing VLCFA β-oxidation and VLCFA levels. Over the years several compounds have been investigated, such as Lorenzo’s oil [3,4], 4-phenylbutyrate [5], and lovastatin [6,7]. These treatments were shown to be either unpractical or ineffective in clinical trials and therefore other drugs are needed.

Fenofibrate (a PPAR-alpha agonist) was shown to induce expression of ALDR (Abcd2) in the liver of Abcd1−/− mice [8]. ALDRP is a functional homolog of ALDP [5]. However, fenofibrate has no effect on Abcd2 expression in the brain of Abcd1−/− mice, possibly because it is a substrate for the Mdr1 transporter at the blood brain barrier and therefore does not penetrate into the brain very effectively [9]. For this reason, we investigated the effect of several other drugs known to activate PPAR on VLCFA metabolism in cultured skin fibroblasts from patients with X-ALD. The results described in this paper show that bezafibrate (BF), but not fenofibrate, clofibrate or other PPAR agonists, could reduce VLCFA in cultured fibroblasts from patients with X-ALD.

The VLCFA which accumulate in X-ALD, are partly absorbed from the diet [10], but mostly result from endogenous synthesis through elongation of long-chain fatty acids [11]. Recently, we identified ELOVL1 as the key enzyme responsible for the synthesis of VLCFA and demonstrated that knock-down of ELOVL1 resulted in lower VLCFA synthesis and reduced levels of VLCFA in cultured X-ALD fibroblasts [12]. Hence, inhibiting fatty acid elongation (for example by inhibition of ELOVL1) by pharmacological means could be a potential treatment for X-ALD. Here, we show that BF lowers VLCFA in X-ALD fibroblasts by direct inhibition of fatty acid elongation.
Materials and Methods

Chemicals

Deuterium-labeled palmitate-16,16,16-D3 acid (D3C16:0) was purchased from CDN isotopes (Pointe-Claire, Canada). A 12.5 mM stock solution in dimethyl sulfoxide (DMSO) was prepared. BF, fenofibrate and clofibrate, WY14643, GW501516, and rosiglitazone were purchased from Sigma-Aldrich (St. Louis, MO, USA). Stock solutions in DMSO were made of 400 mM (BF and clofibrate), 50 mM (fenofibrate), 10 mM (WY14643 and rosiglitazone), 500 M (GW501516). MK-886 was purchased from Cayman Chemical (Ann Arbor, MI, USA) and a stock solution of 50 mM in DMSO was used. Prior to usage the stock solutions were vortex mixed and diluted in HAMF10 tissue culture medium to the final concentration. All chemicals used were of analytical grade.

Cell lines and cell culture

Primary human skin fibroblasts were obtained from X-ALD patients through the Neurology Outpatient Clinic of the Academic Medical Center. From each patient written informed consent was obtained. X-ALD diagnosis was confirmed by VLCFA and ABCD1 mutation analysis. Control fibroblasts were from male anonymous volunteers. Cells from patients with a peroxisomal biogenesis disorder were obtained from the laboratory cell bank. Cells were grown in HAMF10 supplemented with 10% fetal calf serum, 2.5 mM HEPES, 100 U/ml penicillin, 100 U/ml streptomycin and 2 mM glutamine. Cells were used between passage numbers 6 and 20. Culture media were refreshed every 5 days.

Fatty acid synthesis

Synthesis of D3-VLCFA in intact cells was measured using D3-C16:0. Assays were performed in triplicate. Cells were seeded at 40% confluency in T75 flasks. The next day, medium was replaced by fresh medium supplemented with D3-C16:0 (dissolved in DMSO) at a final concentration of 50 µM. After 72 hr, cells were harvested and VLCFA analyzed as described [13].

Measurement of fatty acid beta-oxidation

Mitochondrial β-oxidation activity of intact fibroblasts was measured by quantifying the production of 3H2O from [9,10-3H(N)] oleic acid as described previously [14]. Peroxisomal β-oxidation activity of intact fibroblasts was measured using [1-14C]-26:0 as described previously [15]. All measurements were performed in triplicate for each cell line.

Immunofluorescence and counting of peroxisomes

Fibroblasts where grown on glass microscopy slides in 6-well plates with or without 400 µM BF. Cells were fixed with paraformaldehyde and permeabilized with Triton X-100. Peroxisomes were visualized by catalase immunofluorescence microscopy as described previously [16]. To count peroxisomes, we made images of immunofluorescence-stained cells after focusing on the cell nucleus, and determined the peroxisome number per cell with the aid of a colony counter. For each cell line and condition, 10 cells were counted at random.
Quantitative RT-PCR analysis

ELOVL1, ELOVL4, ELOVL6, ACOX1, ABCD3 and CPT1a mRNA levels in control and X-ALD fibroblasts growing in log phase were determined as described [17], with primer sets presented in Table S1 (Supporting Information Table S1).

Purification of mouse liver microsomes

Microsomes were isolated from livers from wild type and transgenic ELOVL1 over-expressing mice (Kemp et al manuscript in preparation) by differential centrifugation as described by Baudhuin et al [18], with minor modifications. Livers were washed with ice-cold homogenization buffer containing 250 mM sucrose, 2 mM EDTA, 2 mM DTT and 5 mM MOPS (pH 7.4), minced and homogenized using a potter tissue grinder with Teflon pestle with 5 strokes at 500 rpm. A post-nuclear supernatant was produced by centrifugation at 600 g for 10 min. The supernatant was centrifuged at 22,500 g for 10 min and the pellet was discarded. Next, the supernatant was centrifuged for 1 h at 100,000 g to obtain a microsomal fraction. The pellet was resuspended in homogenization buffer containing 10 mg/mL methyl-β-cyclodextrin and sonicated for 4 times 5 seconds at 8 W with a 1 minute interval. The microsomal membranes were collected by centrifugation at 100,000 g for 1 h. Finally, the microsomes were resuspended in homogenization buffer and stored at -80 °C until further use. All steps were carried out at 4 °C. Protein concentrations were determined using BCA as described [19].

Fatty acid elongation assay

The fatty acid elongation assay was carried out using a method adapted from Nagi et al (Nagi et al 1989). The reaction mixture contained 50 mM potassium phosphate buffer (pH 6.5), 10 mg/mL α-cyclodextrin, 1 mM NADPH, 5 µM rotenone, 60 µM [2-14C] malonyl-CoA (6.5 dpm/pmol) (American Radiolabeled Chemicals, St Louis, MO) and 20 µM C16:0-CoA or 20 µM C22:0-CoA (Avanti Polar Lipids, Alabaster, AL), in a total volume of 200 µL. The reaction was started by adding 100 µg protein of the microsomal fraction and allowed to proceed for 30 min at 37°C. NADPH dependency was tested by performing the reaction without NADPH. Reactions were carried out with or without BF or BF-CoA (100 – 400 µM). The reaction was stopped by adding 200 µL 5 M KOH in 10% methanol, saponified at 65°C for 1 h and acidified by adding 200 µL 5 N HCl and 200 µL 96% ethanol. Fatty acids were extracted 3 times with 1 mL hexane and the hexane phases were collected in a scintillation vial to which 10 mL scintillation cocktail (Ultima-Gold, Perkin Elmer) was added and radioactivity counted.

Synthesis of bezafibroyl-CoA (BF-CoA)

BF-CoA was synthesized by a method adapted from Rasmussen et al [20]. Dichloromethane (DCM) and tetrahydrofuran (THF) (Merck) were dried with molecular sieve deperox (Fluka). Triethylamine and ethylchloroformate (Merck) were diluted to 1 M with dry DCM. The reaction contained 36 µmol BF dissolved in 1.4 mL DCM/THF (5:2) and 40 µL 1 M triethylamine. The reaction mixture was incubated at room temperature for 10 min under constant stirring and under an atmosphere of nitrogen. After 10 min, 40 µL 1 M ethylchloroformate was added and the incubation was continued for 45 min. After the incubation, the mixture was dried under nitrogen and dissolved in 0.5 mL tert-butanol. Next, 40 µmol CoA trilithium
salt (Sigma-Aldrich) dissolved in 0.5 mL 0.4 M potassium bicarbonate was added and the sample was incubated for 30 min at room temperature. The reaction was stopped by adding 100 μL 0.1 N HCl. BF-CoA was purified using a C18 solid phase extraction column (JTBaker). The column was eluted by a gradient of acetonitrile and 40 mM ammonium acetate, starting with 10% acetonitrile and 90% 40 mM ammonium acetate increasing to 50% acetonitrile and 50% 40 mM ammonium acetate. Acetonitrile was evaporated and purity was checked by HPLC. BF-CoA was quantified using 5,5’-dithiobis-(2-nitrobenzoic) acid (DTNB) (Sigma-Aldrich). The method used was an adaptation of that of Ellman [21]. BF-CoA was diluted in 20 μM MES buffer pH 6.0, an equal volume of 2 M NaOH was added and the sample was incubated for 30 min at 50°C. The reaction was neutralized with 2 M HCl. Absorbance at 412 nm was measured and the concentration was determined using a calibration curve of CoA trilithium salt (Sigma-Aldrich).
Results

Effect of BF on endogenous VLCFA levels and de novo C26:0 synthesis in fibroblasts from patients with X-ALD

We tested the effect of several drugs from the fibrate class, but found that only BF reduces C26:0 levels, both fenofibrate and clofibrate being ineffective (Fig. 1A). BF is a PPAR pan-agonist activating all three PPARs. To determine whether the effect of BF on C26:0 levels is mediated by activation of either PPARα, PPARβ/δ or PPARγ, X-ALD fibroblasts were incubated with the PPARα ligand WY14643, PPARβ/δ ligand GW501516, or the PPARγ ligand rosiglitazone either alone or in all possible combinations. Only treatment with BF reduced C26:0 levels by about 30% after 7 days (Fig. 1B).

To examine if longer incubations with BF would result in a further decrease of C26:0 levels, we cultured X-ALD fibroblasts for up to 21 days. This resulted in a small additional decrease of C26:0 of 10% after 14 days and 15% after 21 days, respectively (Fig. 1C). No signs of cytotoxicity or impaired growth as determined by the MTS cell proliferation assay (CellTiter 96® Aqueous One Solution Cell Proliferation Assay) were observed (data not shown). Previously, we validated the use of stable-isotope labeled fatty acids to study VLCFA de novo synthesis in whole cells and demonstrated that the synthesis of D3-C26:0 from D3-C16:0 is elevated in X-ALD fibroblasts [12]. Earlier work has shown that BF is a potent inhibitor of long-chain fatty acid elongation, while clofibrate is not [22,23].

Therefore, we tested the effect of BF, clofibrate and fenofibrate on C26:0 de novo synthesis. Of all fibrates tested, only BF affected the de novo D3-C26:0 synthesis (Fig 2A). BF reduced D3-C26:0 synthesis in a concentration-dependent manner. At 400 µM BF, the synthesis of D3-C26:0 was reduced by 75% compared with untreated X-ALD fibroblasts (Fig. 2B). At this concentration, there was no difference in the amount of newly synthesized D3-C26:0 between X-ALD and control cells. We performed further experiments with BF, and not the other fibrates or PPAR agonists, to identify by which mechanism BF reduces C26:0 levels in fibroblasts from patients with X-ALD.
Figure 1. Only BF, and not other drugs from the fibrate class, reduces C26:0 in X-ALD fibroblasts. (A) C26:0 levels in 3 X-ALD cell lines cultured for 7 days without drugs (black bar), in the presence of BF (grey bar), fenofibrate (white bar), or clofibrate (hatched bar). (B) C26:0 levels in 3 X-ALD cell lines cultured for 7 days without drugs (black bar), in the presence of BF (grey bar), agonists of PPAR-alpha (WY 14643), PPAR-alpha (GW 501516) and PPAR-alpha (rosiglitazon), or with different combinations of PPAR agonists (white bars). (C) C26:0 levels in 8 control (white bar) and 8 X-ALD cell lines cultured without (black bar) or with 400 µM BF (grey bars) for up to 3 weeks. Fatty acid levels are in nmol/mg protein. Data are mean ± SD. * = p < 0.05, ** = P < 0.01, *** = P < 0.001 by ANOVA followed by Dunnett’s multiple comparison test compared with untreated X-ALD cells.
Figure 2. BF, but not other fibrates, inhibits D3-C26:0 synthesis. (A) D3-C26:0 synthesis from D3-C16:0 in 5 X-ALD cell lines cultured for 3 days without drugs (black bar) and in the presence of BF (grey bar), fenofibrate (FF, white bar), or clofibrate (CF, hatched bar). (B) D3-C26:0 analysis from D3-C16:0 in 4 control (white bar) and 4 X-ALD cell lines cultured for 3 days without (black bar) or with increasing concentrations of BF (grey bars). Fatty acid levels are in nmol/mg protein. Data are mean ± SD. ** = P < 0.01, *** = P < 0.001 by ANOVA followed by Dunnett’s multiple comparison test compared with untreated X-ALD cells.

The effect of BF on C26:0 levels is independent of induction of mitochondrial or peroxisomal beta-oxidation

BF and PPAR ligands in general are known to induce mitochondrial and peroxisomal β-oxidation [24] [25] [26] [27]. The effect of BF treatment on the rate of mitochondrial and peroxisomal fatty acid β-oxidation was determined in X-ALD fibroblasts. Exposure of X-ALD fibroblasts to BF caused a 50% increase in C16:0 β-oxidation (Fig 3A), and a 35% increase in C26:0 β-oxidation (Fig. 3B). Since it is known that fibrates induce peroxisome proliferation in rodents [26], we determined the amount of peroxisomes in X-ALD fibroblasts incubated for 10 days with BF. We did not find any evidence for an effect of BF treatment on peroxisome abundance (Fig.
BF reduced C26:0 levels in cultured X-ALD fibroblasts, while other drugs of the fibrate class or other synthetic PPAR agonists could not. This strongly suggested that the effect of BF on VLCFA levels is not dependent solely on induction of mitochondrial or peroxisomal beta-oxidation, since the other compounds should have been effective as well then [28,29]. BF treatment resulted in a 35% increase in the peroxisomal C26:0 beta-oxidation capacity (Fig 3B). To test if the induction of peroxisomal beta-oxidation by BF caused the reduction in C26:0 levels, we incubated fibroblasts from patients with a peroxisomal biogenesis disorder (PEX1, PEX6 and PEX26) with BF and measured the effect on D3-C26:0 de novo synthesis. The synthesis of D3-C26:0 from D3-C16:0 was reduced roughly 50% in peroxisome-deficient fibroblasts incubated with BF (Fig 4). This clearly indicated that the effect of BF on D3-C26:0 synthesis and C26:0 levels is only partially mediated by an induction of the peroxisomal beta-oxidation capacity.

![Figure 3](image)

**Figure 3. Effect of BF on mitochondrial and peroxisomal beta-oxidation and peroxisome number.** Measurement of (A) C16:0 beta-oxidation and (B) C26:0 beta-oxidation activity in 5 control cell lines (white bar) and 5 X-ALD cell lines cultured without (black bar) or with 400 µM BF (grey bar) for 7 days. Activities are in pmol/mg/hour. Data are mean ± SD. * = P < 0.05 and *** = P < 0.001 by ANOVA followed by Dunnett’s multiple comparison test compared with untreated X-ALD cells. (C) Peroxisome number in 10 X-ALD cell lines cultured without (white bar) or with 400 µM BF (grey bar) for 3 days. Mean and quartiles are indicated, the error bars represent the range.

To investigate whether the induction of mitochondrial beta-oxidation by BF is responsible for the reduction in C26:0, we used the PPARα inhibitor MK-886 [30]. The induction of mitochondrial beta-oxidation by BF in X-ALD fibroblasts could be reversed with 50 µM MK-886, suggesting that PPARα activation is completely blocked at this
concentration (Fig 5A). Next, we measured D3-C26:0 de novo synthesis in X-ALD fibroblasts incubated with BF in the presence of MK-886. Addition of MK-886 did not affect the reduction of D3-C26:0 synthesis (Fig 5B). Combined, these data are highly suggestive that the reduction of C26:0 levels by BF is PPAR independent and can not be explained by the increase in mitochondrial β-oxidation capacity and only partially by the increase in peroxisomal β-oxidation capacity. This suggests that BF inhibits the formation of D3-C26:0.

Figure 4. C26:0 reduction by BF is not mediated by increased peroxisomal β-oxidation. Analysis of de novo D3-C26:0 synthesis in 3 X-ALD (black bar) cell lines and 3 cell lines from patients with a peroxisomal biogenesis disorder (PBD, white bar). Cells were incubated with 50 µM D3-C16:0 for 3 days without or with 400 µM BF (cross-hatched bars). Fatty acid levels are in nmol/mg protein. Data are mean ± SD. *** = p < 0.001 by student’s unpaired t-test.
Figure 5. C26:0 reduction by BF is not mediated by increased mitochondrial β-oxidation. (A) C18:1 β-oxidation in 3 X-ALD cell lines cultured without (black bar), or for 48 hours with 400 µM BF (grey bar), or with BF and increasing concentrations of MK-886 (white bars). Activities are in pmol/mg/hour. Data are mean ± SD. (B) De novo D3-C26:0 synthesis in 4 X-ALD cell lines incubated with 50 µM D3-C16:0 without (black bar) or with 400 µM BF (grey bar), 50 µM MK-886 (white bar), or 400 µM BF and 50 µM MK-886 (cross-hatched bar). Fatty acid levels are in nmol/mg protein. Data are mean ± SD. ** = p < 0.01; *** = p < 0.001 by ANOVA followed by Dunnett’s multiple comparison test compared with untreated X-ALD cells.

BF directly inhibits fatty acid elongation

The amount of D3-C26:0 present is the net result of the elongation of D3-C16:0 to D3-C26:0 by ELOVL6 and ELOVL1, respectively [12] and the degradation of D3-C26:0 by peroxisomal β-oxidation. The inhibitory effect of BF on the formation of D3-C26:0 could either be indirect by affecting gene expression or direct by inhibition of key enzymes involved in VLCFA de novo synthesis [12]. To investigate the effect of BF on gene expression levels of several key enzymes involved in VLCFA synthesis, peroxisomal β-oxidation and mitochondrial β-oxidation we performed quantitative RT-PCR. As shown in Fig 6, there is only a small reduction in the mRNA levels of ELOVL6, which is not statistically significant. There was no increase in the expression of ACOX1. This is in line with previously published data [28] and our own data (Fig 3C) showing that there is no peroxisome proliferation, at least in cultured
human cells, treated with fibrates, in contrast to rodents [28]. BF did not induce the expression of ABCD3. Induction of this gene was detected in mice treated with fenofibrate, and considered to be the mechanism by which fibrates might be useful in correcting the metabolic defect in X-ALD [8,9]. Our data show that induction of ABCD3 did not occur in human X-ALD fibroblasts upon exposure to BF. In line with previous data [31], BF treatment resulted in increased expression of CPT1a.

The previous experiments suggested that the effect of BF on C26:0 levels and C26:0 synthesis could be mediated by a direct inhibiting effect on fatty acid elongation. VLCFA are synthesized by the concerted action of ELOVL6 and ELOVL1 [12]. ELOVL6 elongates C16:0 to C22:0 and ELOVL1 elongates C22:0 to C26:0. We measured the effect of free BF and BF esterified to coenzyme CoA (BF-CoA) on C16:0-CoA and C22:0-CoA elongation. In the elongation assay BF had no effect. However, BF-CoA inhibited the chain elongation activity of both C16:0 and C22:0 in a concentration dependent manner (Fig. 7). Both fenofibrate and clofibrate did not inhibit C22:0-CoA elongation (Fig. 7B). These data demonstrate that BF-CoA lowers C26:0 levels by direct inhibition of fatty acid chain elongation.

**Figure 6. Effect of BF on gene expression of genes involved in fatty acid metabolism.** Expression levels of genes involved in fatty acid metabolism in 3 X-ALD cell lines cultured without (black bars) or with 400 µM BF (grey bars) for 48 hours were analyzed by quantitative PCR. Data are mean ± SD. * = p < 0.05 by student’s unpaired t-test.
Figure 7. BF inhibits fatty acid elongation. (A) Fatty acid elongation activity of C16:0-CoA (a substrate of ELOVL6) in the presence of an increasing concentration of BF (○) and BF-CoA (●). (B) Fatty acid elongation activity of C22:0-CoA (a substrate of ELOVL1) in the presence of an increasing concentration of BF (○) and BF-CoA (●) or with an increasing concentration of fenofibrate (FF, □) or clofibrate (CF, △). Fatty acid elongation activity was measured using 20 µM C16:0-CoA or C22:0-CoA as substrate with concentrations of the inhibitor up to 400 µM. Error bars represent the standard deviation.
Discussion

Synthetic PPAR alpha ligands, like fibrates, are potentially interesting compounds to investigate as therapeutic agents in X-ALD because they are known to activate mitochondrial and peroxisomal fatty acid β-oxidation [32]. They might therefore reduce VLCFA accumulation by increasing VLCFA degradation. Indeed, Brown and colleagues demonstrated that treatment of two CCALD patients with clofibrate resulted in a reduction in VLCFA [33]. However, this reduction was not sustained. More recent experiments showed that fenofibrate induced expression of both ALDRP (ABCD2) and PMP70 (ABCD3) in the liver of Abcd1-deficient mice, but not in brain [9]. In Mdr1-/- knockout mice induction of ALDRP and PMP70 in brain did occur, suggesting that fenofibrate is indeed cleared from the brain by Mdr1 [9]. These studies, however, did not report the effect on VLCFA levels in tissues. In this paper, we studied the effect of several classical fibrates and other synthetic PPAR ligands in a cell model for X-ALD, and show that BF but not the other fibrates reduced endogenous C26:0 levels. This C26:0 reducing effect could not be mimicked by other PPAR-ligands which means that the effect is PPAR independent. BF has been demonstrated to induce mitochondrial beta-oxidation [24,31]. Blocking the induction of mitochondrial beta-oxidation with MK-886 did not prevent the reduction of D3-C26:0 de novo synthesis in X-ALD fibroblast by BF. We also showed that the peroxisomal C26:0 β-oxidation capacity in X-ALD skin fibroblasts increased with 35% upon treatment with BF. However this does not seem to be the only mechanism of reduction of VLCFA in fibroblasts incubated with BF, because in cells from patients with a peroxisome biogenesis disorder in which peroxisomal β-oxidation is completely deficient, BF lowered D3-C26:0 levels as well. This strongly suggested that BF might reduce C26:0 levels primarily by inhibiting C26:0 synthesis. To test this we measured de novo synthesis of D3-C26:0 from D3-C16:0 in X-ALD fibroblasts. BF indeed decreased de novo synthesis of D3-C26:0 in a concentration-dependent manner. At a concentration of 400 µM BF, D3-C26:0 levels in X-ALD fibroblasts were at the level of control fibroblasts. The rate limiting enzymes involved in synthesis of C26:0 from C16:0 are ELOVL6 (elongation of C16:0 to C22:0) and ELOVL1 (elongation of C22:0 to C26:0) [12,34]. By qPCR we showed that expression levels of these enzymes are not affected in fibroblasts incubated with BF suggesting a direct inhibition of VLCFA synthesis. Previously using rat liver microsomes other investigators showed that BF inhibits palmitoyl-CoA (C16:0-CoA) elongation in an in vitro assay [22,35]. We used purified microsomes from wild type and ELOVL1 over-expressing mice to test the effect of BF on elongation of both long-chain fatty acids (LCFA) and VLCFA. Our results demonstrate that BF-CoA, but not free BF, is a potent inhibitor of both LCFA and VLCFA elongation. It should be noted, however, that these results do not allow us to demonstrate at which level BF inhibits VLCFA synthesis. Fatty acid elongation requires four sequential reaction steps: (i) condensation between the fatty acyl-CoA and malonyl-CoA to form 3-ketoacyl-CoA; (ii) reduction using NADPH to form 3-hydroxyacyl-CoA; (iii) dehydration to trans-2-enoyl-CoA; and (iv) reduction to fully elongated fatty acyl-CoA. The initial condensation reaction is catalyzed by the enzyme referred to as “elongation of very long-chain fatty acids” (ELOVL) and is considered to be rate limiting (Cinti et al 1992). While seven elongases have been identified in mammals (designated ELOVL1-ELOVL7)(Jakobsson et al 2006), only a single enzyme has been identified yet for the subsequent reaction step (Jakobsson et al 2006). Identification of the specific
enzyme(s) affected by BF is not a trivial thing and requires detailed analysis of all enzymes involved, including: 3-ketoacyl-CoA reductase (HSD17B12), 3-hydroxyacyl dehydratase (HACD3) and the trans-2,3-enoyl-CoA reductase (TECR). This will be the subject of future studies.

**Concluding remarks**

The work described in the paper shows that inhibition of VLCFA synthesis by pharmacological means could be a feasible treatment option for X-ALD. BF is a good candidate for this approach. BF lowers the levels of C26:0 by a direct inhibition of the synthesis. Mouse studies to evaluate the in vivo effect of BF treatment on VLCFA in X-ALD mice would be interesting; however, the effect of fibrates is quite different in rodents and humans. Watanabe et al demonstrated that rats and mice are unusable as a model system (for primates) to the study the effect of BF [36]. BF has a proven safety profile for (long-term) use in humans. With a daily dose of 200 mg of BF peak plasma levels of 50 µM can be reached, with a maximum daily dose of 800 mg of BF therapeutic levels might be reached in plasma [37]. A small scale proof of principle clinical trial is currently ongoing to evaluate the effect in X-ALD patients.

**Acknowledgements**

We thank Femke Stet, Petra Mooyer, and Henk van Lenthe for expert technical assistance. This research was supported by the Netherlands Organization for Scientific Research (VIDI-grant number 91786328), the European Union Framework Programme 7 (grant number LeukoTreat 241622). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.
References


Chapter 3

Enzymatic characterization of ELOVL1, a key enzyme in very-long-chain fatty acid synthesis

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Published in:
Biochimica et Biophysica Acta (BBA) - Molecular and Cell Biology of Lipids, Volume 1851, Issue 2, February 2015, Pages 231–237
Abstract

X-linked adrenoleukodystrophy (X-ALD) is a neurometabolic disease that is caused by mutations in the ABCD1 gene. ABCD1 protein deficiency impairs peroxisomal very long-chain fatty acid (VLCFA) degradation resulting in increased cytosolic VLCFA-CoA levels, which are further elongated by the VLCFA-specific elongase, ELOVL1. In adulthood, X-ALD most commonly manifests as a gradually progressive myelopathy (adrenomyeloneuropathy; AMN) without any curative or disease modifying treatments. We recently showed that bezafibrate reduces VLCFA accumulation in X-ALD fibroblasts by inhibiting ELOVL1. Although, in a clinical trial, bezafibrate was unable to lower VLCFA levels in plasma or lymphocytes in X-ALD patients, inhibition of ELOVL1 remains an attractive therapeutic option. In this study, we investigated the kinetic characteristics of ELOVL1 using X-ALD fibroblasts and microsomal fractions from ELOVL1 over-expressing HEK293 cell lines and analyzed the inhibition kinetics of a series of fibrates. Our data show that the CoA esters of bezafibrate and gemfibrozil reduce chain elongation by specifically inhibiting ELOVL1. These fibrates can therefore serve as lead compounds for the development of more potent and more specific inhibitors for ELOVL1.
**Introduction**

X-linked adrenoleukodystrophy (X-ALD) is a progressive neurodegenerative disease caused by mutations in the ABCD1 gene [1] and is characterized by the accumulation of very long-chain fatty acids (VLCFA) in plasma and tissues [2]. Clinically, the disease ranges from the rapidly progressive cerebral form of X-ALD (cerebral-ALD), to the more slowly progressive adult form adrenomyeloneuropathy (AMN) and primary adrenocortical insufficiency [3]. In addition, over 80% of women with X-ALD develop AMN [4]. At present, treatment options are very limited. For early stage cerebral-ALD, bone-marrow transplantation is curative [5]. However, for AMN, which represents 85% of X-ALD cases (males and females combined), no disease modifying therapy is available [6].

The ABCD1 gene encodes a protein (ALDP) that transports very-long-chain fatty acids (VLCFA; ≥C22:0) into peroxisomes [7,8]. ALDP deficiency has two major consequences including: 1) the impaired oxidation of VLCFA in peroxisomes [9,10], and 2) raised cytosolic VLCFacyl-CoA levels which serve as substrates for further elongation to even longer fatty acids by ELOVL1, the human VLCFA-specific elongase [11,12].

In males, unambiguous diagnosis of X-ALD can be achieved by demonstration of elevated VLCFA levels in plasma [2]. The strongest increase in VLCFA has been reported in complex lipids in the central nervous system, in particular in myelin which is predominantly (>70%) composed of lipids [13,14]. While only a small part of VLCFA is taken up from the diet, the majority of VLCFA are synthesized endogenously from long-chain fatty acids, mainly C16:0, via chain elongation [15]. Fatty acid elongation consists of four enzymatic reactions (condensation, reduction, dehydration and reduction) [16]. The first step involves the malonyl-CoA driven formation of a 3-ketoacyl-CoA catalyzed by one of seven elongases (ELOVL1-7). In the second reaction, the 3-ketoacyl-CoA is reduced to 3-hydroxyacyl-CoA. This reaction requires NADPH and is carried out by a single enzyme named, 3-ketoacyl-CoA reductase, encoded by HSD17B12. In the third step, 3-hydroxyacyl-CoA is dehydrated to trans-2,3-enoyl-CoA by 3-hydroxyacyl-CoA dehydratase (HACD). Finally, trans-2,3-enoyl-CoA is reduced to a fatty acyl-CoA by trans-2,3-enoyl-CoA reductase (TECR), which also requires NADPH. The result is a fatty acyl-CoA ester that is extended with 2 carbon atoms, which can be elongated further during subsequent elongation cycles.

Previously, we identified ELOVL1 as the elongase responsible for the elongation of C22:0 to C24:0 and C26:0 [12] and demonstrated that knockdown of ELOVL1 lowers C26:0 synthesis and C26:0 levels in X-ALD fibroblasts [12], which provided proof-of-concept for ELOVL1 inhibition as a therapeutic option for X-ALD. Importantly, ELOVL1 is ubiquitously expressed in all tissues including the adrenal gland, brain and testis [17], the tissues which are primarily affected in X-ALD [3].

In search for pharmacological compounds able to reduce VLCFA levels in X-ALD fibroblasts, bezafibrate was identified as a VLCFA-lowering compound [18]. Surprisingly, the VLCFA lowering effect of bezafibrate, a well-known PPAR-ligand, was found to be independent of PPAR activation. This is in line with the observation that clofibrate and fenofibrate were unable to reduce VLCFA levels despite the fact that they are also potent PPAR ligands. Subsequently we found that bezafibrate reduces VLCFA synthesis by decreasing the synthesis of C26:0 through direct inhibition of fatty acid elongation. Additionally, we demonstrated that the CoA ester
of bezafibrate and not bezafibrate itself inhibits elongation. Based on these results, an open-label pilot study was performed to evaluate the effect of bezafibrate on VLCFA accumulation in blood cells of AMN patients. Unfortunately, bezafibrate failed to lower VLCFA levels in blood cells of the patients. Most likely this was attributable to its inability to reach adequate drug levels in vivo \[19\].

Since inhibition of chain elongation is an interesting target for future therapy of X-ALD, and ELOVL1 is the only enzyme that catalyses the initial step in VLCFA formation, we decided to investigate the kinetic characteristics of ELOVL1. We also studied the effects of bezafibrate, fenofibrate, clofibrate, ciprofibrate and gemfibrozil on the elongation of LCFAs to VLCFAs. The most potent elongation inhibitors of these fibrates were studied in more detail to gain insight into the inhibitory mechanism. The results of our study show that the CoA esters of bezafibrate and gemfibrozil inhibit chain elongation by specifically inhibiting the first step of the elongation system, catalyzed by ELOVL1. The implications of these findings will be discussed.
Materials and methods

Chemicals
The antibody against human ELOVL1 was generated by Eurogentec (Liege, Belgium). D3-behenic acid (docosanoic acid-22,22,22-D3) was purchased from CDN Isotopes (Québec, Canada). Bezafibrate, fenofibrate, clofibric acid, ciprofibrate and gemfibrozil were from Sigma-Aldrich (St. Louis, MO, USA), fenofibric acid from respectively Biofine international Inc. (Vancouver, Canada) and clofibrate from Janssen pharmaceuticals (Beerse, Belgium). Stock solutions for fibrates and VLCFA were prepared in DMSO. All chemicals used were of analytical grade.

Cell lines and cell culture
Human skin fibroblasts were obtained from X-ALD patients through the Neurology Outpatient Clinic of the Academic Medical Center. Written informed consent was received from each patient. X-ALD diagnosis was confirmed by VLCFA and ABCD1 mutation analysis. Control fibroblasts were obtained from male anonymous volunteers with written informed consent. Hek293 Flp-inTM cells and fibroblasts were cultured in DMEM supplemented with 10% fetal calf serum, 2.5 mM HEPES, 100 U/ml penicillin and 100 U/ml streptomycin. Cells were cultured at 37°C in a humidified 5% CO2 atmosphere.

D3-C22:0 elongation activity in intact cells
The synthesis of D3-C26:0 from D3-C22:0 was measured in cultured skin fibroblasts from controls and X-ALD patients. Cells were seeded at approximately 40% confluency in T75 flasks in DMEM. The next day, medium was replaced with medium containing 30 µM D3-C22:0. After 72h, cells were harvested and VLCFA analyzed as described [30].

Expression of human ELOVL1 in HEK293 cells
The ELOVL1 open reading frame was cloned into the pcDNA5/FRT vector, as described previously [12]. Hek293 Flp-inTM cells (Invitrogen) were grown to 50-60% confluency in a 6-well plate following transfection with either a combination of pOG44 and pcDNA5 (mock) or pOG44 and the ELOVL1 plasmid using lipofectaminTM 2000 (Invitrogen) according to the manufacturer’s manual. After 2 days, transfected cells were trypsinized, taken up in standard culture medium and divided over a 96-well plate. The next day, selection was started by the addition of 200 µg/ml hygromycin. After 3 weeks of culturing in the presence of hygromycin 12 clones were selected. The β-galactosidase activity was measurement in each clone according to the manufacturer’s manual and five independent clones selected. The expression of ELOVL1 was confirmed by immunoblot analysis.

Preparation of microsomes
Microsomes were isolated from HEK293 cells over-expressing ELOVL1 by differential centrifugation [31]. After trypsinization, cells were centrifuged for 5 min at 600g and the pellet was resuspended in homogination buffer containing 250 mM sucrose, 2 mM EDTA, 2 mM DTT and 5 mM MOPS (pH 7.4). Cells were treated with 20 strokes of a dounce homogenizer while kept on ice and a post-nuclear supernatant was produced by centrifugation at 600g for 10 min. The supernatant was centrifuged at 22,500g for 10 min and the pellet was discarded. To obtain a microsomal fraction,
the supernatant was centrifuged for 1h at 100,000g. To remove any residual fatty acids the pellet was resuspended in homogenization buffer containing 10 mg/mL methyl-β-cyclodextrin and sonicated four times for 5 seconds at 7W. The microsomal membranes were collected by 1h centrifugation at 100,000g. All centrifugation steps were performed at 4°C. Finally, the microsomes were resuspended in homogenization buffer and stored at -80°C in 100 μL aliquots until further use. Protein concentration was determined using Pierce® BCA protein assay (Thermo Scientific) with human serum albumin as standard.

**In vitro fatty acid elongation assay**

The fatty acid elongation assay was performed as described by Nagi et al [32] with some modifications. The assay consisted of a reaction mixture containing 50 mM potassium phosphate (pH 6.5), 5 µM rotenone, 20 µM palmitoyl-CoA (Avanti Polar Lipids, Alabaster, AL), 60 µM [2-14C] malonyl-CoA (6.5 dpm/pmol) (American Radiolabeled Chemicals, St Louis, MO), 1 mM NADPH and 10 mg/ml α-cyclodextrin in a total volume of 200 µL. The reaction mixture was pre-incubated for 2 min at 37°C and started by the addition of 100 µg microsomal protein. After 30 min at 37°C the reaction was stopped by adding 200 µL 5 M KOH in 10% methanol and saponified at 65°C for 1 h. After acidification, by adding 200 µL 5 N HCl and 200 µL 96% ethanol, fatty acids were extracted three times with 1 mL hexane and the hexane phases were collected in a scintillation vial. To each scintillation vial 10 mL scintillation cocktail (Ultima-Gold, Perkin Elmer) was added and the radioactivity was counted.

**Analysis of VLCFA elongation products by RP-HPTLC**

Chromatographic analysis of the VLCFA elongation products was performed using reversed-phase high-performance thin-layer chromatography (RP-HPTLC) as described [17]. After the in vitro elongation assay and subsequent hexane extraction, samples were dried under nitrogen and dissolved in 200 µL 1 M methanolic-HCl and 5% 2,2-dimethoxypropane. Subsequently, samples were incubated for 1h at 80°C following the addition of 400 µL 1% KCl, and lipids were extracted three times with 1 mL hexane. Samples were dried under nitrogen, resuspended in 40 µL chloroform and separated on a RP-HPTLC silica gel 60 RP-18 F254s plates with concentrating zone (Merck) using chloroform/methanol/water (5:15:1, v/v/v) as the solvent system. Labelled lipids were detected with a phosphor imager (Fuji Photo Film) followed by densitometric analysis using AIDA image analyzer software (Raytest Isotopenmessgeräte GmbH).

**Synthesis of fibroyl-CoA esters**

Fibroyl-CoA esters were synthesized, purified and quantified as described previously [18,33]. In short, fibroyl-CoA esters were synthesized using 36 µmol of the fibrate or the corresponding fibric acid and 40 µmol CoA trilithium salt (Sigma-Aldrich). Fibroyl-CoA esters were purified with a C18 solid phase extraction (SPE) column (JT Baker) using a gradient of acetonitrile (0 - 50% v/v) and 40 mM ammonium acetate (pH6). After evaporation of the acetonitrile the purity of the final product was checked by RP-HPLC. The fibroyl-CoA ester concentration was quantified using 5,5’-dithiobis-(2-nitrobenzoic) acid (DTNB) (Sigma-Aldrich), method adapted from [34].
Results

Inhibition of VLCFA elongation by fibrates in a cell based assay

We have shown that bezafibrate, an agonist of PPARα, directly inhibits the elongation of very-long-chain fatty acids [18]. However, it is unknown whether bezafibrate is the only fibrate capable of inhibiting VLCFA elongation. Therefore, we incubated skin fibroblast from X-ALD patients with deuterium-labelled behenic acid (D3-C22:0) in the presence or absence of a series of fibrates followed by the measurement of VLCFA elongation products. The results in figure 1, show that bezafibrate, but also gemfibrozil and to a lesser extent ciprofibrate inhibited C26:0 formation whereas clofibrate and fenofibrate did not. It should be noted that the concentration of fenofibrate used in this experiment was lower (100 µM) than that of the other fibrates (400 µM) due to the toxicity of fenofibrate at concentrations above 100 µM (data not shown).

Figure 1. Effect of fibrates on de novo VLCFA synthesis in intact cells. D3-C26:0 synthesis from D3-C22:0 was measured in 3 X-ALD cell lines and a control cell line. To test the effect on the synthesis of D3-C26:0, fibrates were supplemented to the culture medium at the following final concentrations: 400 µM bezafibrate, 400 µM ciprofibrate, 400 µM clofibrate, 100 µM fenofibrate or 400 µM gemfibrozil. Mean from 3 X-ALD cell lines each treated in duplicate is indicated, the error bars represent the SD. *=P<0.05, **=P<0.01 by unpaired student’s t-test.

Enzymatic analysis of ELOVL1 elongation activity in microsomes

ELOVL1 is the key enzyme in the elongation of C22:0 to C26:0 and to study it at the enzyme level, we generated an ELOVL1 over-expression model using HEK293 Flp-in cells. After transfection individual clones were isolated and
characterized by western blot analysis. ELOVL1 overexpressing HEK293 Flp-in cells showed increased levels of ELOVL1 protein (Fig. 2A) and analysis of the endogenous VLCFA levels revealed an approximate 12-fold increase in C26:0 (Fig. 2B). Next, we set out to optimize the VLCFA elongation assay as described previously [20] to measure activity in isolated microsomal fractions. Bovine serum albumin (BSA) or α-cyclodextrin are generally used to solubilize fatty acyl-CoA-esters. We compared the two methods and obtained the highest activity when VLCFA-CoA esters were dissolved in alpha-cyclodextrin (Fig. 2C). Microsomes isolated from clones over-expressing ELOVL1 had a 10-fold increase in the elongation activity of C22:0-CoA compared to clones of mock-transfected cells (Fig. 2D).

Figure 2. Over-expression of ELOVL1 results in increased VLCFA synthesis. Selected clones of stable transfected HEK293 Flp-in cell lines were characterized for the expression of ELOVL1, VLCFA levels and VLCFA elongation activity. (A) Immunoblot analysis of ELOVL1 in mock-transfected and five ELOVL1 over-expressing clones. (B) C26:0 levels in mock-transfected clones (black bar) and five ELOVL1 over-expressing HEK293 cells (white bar). (C) Microsomes isolated from the HEK293 ELOVL1 over-expressing clone (H2) were used to measure fatty acid elongation activity with either BSA (black bar) or α-cyclodextrin (alpha-CD, white bar). Malonyl-CoA and C22:0 concentrations were kept constant at 60 µM and 20 µM, respectively. (D) Microsomal fractions, isolated from a mock-transfected and ELOVL1 over-expressing HEK293 cells were used for the fatty acid elongation assay with C22:0-CoA as substrate. Values represent the mean ±SD.
Effects of fibrates on VLCFA elongation by ELOVL1

We have shown that the CoA-ester of bezafibrate (bezafibroyl-CoA) inhibits VLCFA elongation [18]. We now studied the effect of a series of fibrates and their CoA-esters, i.e. bezafibrate, bezafibroyl-CoA, ciprofibrate, ciprofibroyl-CoA, clofibrate, clofibroyl-CoA, fenofibrate, fenofibroyl-CoA, gemfibrozil and gemfibroyl-CoA on the C22:0-CoA elongation capacity by ELOVL1. At a concentration of 100 µM none of the fibrates had any significant effect on C22:0-CoA elongation (Fig. 3). In contrast, all CoA-esters tested, except clofibroyl-CoA, inhibited C22:0-CoA elongation. At the concentration of 100 µM, gemfibroyl-CoA showed the highest degree of inhibition (70%), followed by fenofibroyl-CoA (58%), ciprofibroyl-CoA (57%) and bezafibroyl-CoA (54%).

Figure 3. Only fibroyl-CoA esters inhibit VLCFA elongation activity.

Microsomes were isolated from HEK293 ELOVL1 over-expressing cells and used to analyze the effect of fibrates (white bars) and fibroyl-CoA esters (black bars) on C22:0-CoA (20 µM) elongation. Final concentration of each fibrate and its corresponding CoA-ester was 100 µM. Reactions were started with 50 µg protein. The enzyme activity is expressed relative to the activity measured in the absence of inhibitor (100%). Error bars indicate variation between duplicate measurements.

Kinetic characterization of ELOVL1 activity and inhibition.

Our data shows that bezafibrate had the strongest effect in the intact cell assay while gemfibroyl-CoA was the strongest inhibitor of VLCFA elongation activity in the
in vitro assay. Therefore, all subsequent experiments were focused on the inhibitory mechanism of these two fibroyl-CoA esters. The complete VLCFA elongation reaction consist of four sequential enzymatic steps catalyzed by four different enzymes and it is unclear which of the enzymatic step is affected by the fibroyl-CoA esters. In the absence of NADPH the VLCFA elongation reaction is blocked directly after the first enzymatic step catalyzed by ELOVL1, i.e. the condensation of malonyl-CoA with the fatty acyl-CoA producing a 3-keto acyl-CoA. In the presence of NADPH all four reactions of the elongation cycle can proceed. This is due to the fact that the second enzymatic step, catalyzed by HSD17B12, is completely NADPH dependent. To test the effect of the fibroyl-CoA esters on the elongation of C22:0-CoA we used HPTLC analysis to identify and quantify the amounts of the different elongation products formed. In the presence of NADPH the elongation reaction was found to produce mainly C24:0 and to a lesser extent C26:0 (Fig. 4A). The production was significantly increased when microsomes isolated from cells overexpressing ELOVL1 were used. When NADPH was omitted from the reaction only a single product corresponding to 3-keto-acyl-CoA was observed, which is in agreement with earlier data obtained from yeast studies [21]. The formation of the 3-keto-acyl-CoA was higher when microsomes from cells overexpressing ELOVL1 were used.

Figure 4. Bezafibroyl-CoA and gemfibroyl-CoA inhibit ELOVL1 directly. Microsomes isolated from a mock-transfected and an ELOVL1 over-expressing HEK293 clone were used for the fatty acid elongation assay with C22:0-CoA as substrate. Reactions were started by the addition of 100 µg microsomal protein.
After 30 min the reaction was terminated and the elongation products were analyzed by RP-HPTLC. The (*) indicates the position of 3-keto acyl-CoA. (A) Analysis of C22:0-CoA elongation products formed in the presence or absence of 1 mM NADPH. (B) Analysis of C22:0-CoA elongation products formed in the presence or absence of 1 mM NADPH and bezafibroyl-CoA (100 µM) or gemfibroyl-CoA (100 µM). (C) Quantification of the elongation products formed in the presence of 1 mM NADPH from Fig. 4B. C24:0-CoA (white bars); C26:0-CoA (hatched bars) and total VLCFA production (black bars). (D) Quantification of the 3-keto-C24:0-CoA formed in the absence of NADPH from Fig. 4B.

Next, we tested the effect of the fibroyl-CoA esters on VLCFA elongation product formation in the presence or absence of NADPH (Fig. 4B and C). Without NADPH both gemfibroyl-CoA and bezafibroyl-CoA showed a significant decrease in the formation of 3-keto-acyl-CoA, amounting to 47% and 34% respectively. In the presence of NADPH, the production of C24:0 and C26:0 from C22:0 decreased with 47% for gemfibroyl-CoA and 45% for bezafibroyl-CoA.

Figure 5. ELOVL1 enzyme kinetics and analysis of product formation. Microsomes from an ELOVL1 over-expressing clone were used to determine the kinetic parameters, Km and Vmax, of ELOVL1 for both C22:0-CoA and malonyl-CoA.
Reactions were started by the addition of 50 µg microsomal protein and terminated after 30 min. (A) Fatty acid elongation activity for an increasing concentration of C22:0-CoA in the presence of 60 µM malonyl-CoA was determined and used to calculate the kinetic parameters with the Michaelis-Menten equation. Insert shows the Lineweaver-Burk plot of the collected data. (B) Fatty acid elongation activity for an increasing concentration of malonyl-CoA in the presence of 20 µM C22:0-CoA was determined and used to calculate the kinetic parameters using the Michaelis-Menten equation. Insert shows the Lineweaver-Burk plot of the collected data, values represent the mean ±SD.

Figure 6. Bezafibroyl-CoA and gemfibroyl-CoA show a different type of inhibition of ELOVL1. Microsomes from an ELOVL1 over-expressing clone were used to determine the inhibitory kinetic parameter, Ki, of bezafibroyl-CoA and gemfibroyl-CoA for both C22:0-CoA and malonyl-CoA. Reactions were started by the addition of 50 µg microsomal protein and terminated after 30 min. (A) Fatty acid
elongation activity, with NADPH, was measured with increasing concentrations of C22:0-CoA in the presence of different concentration of bezafibroyl-CoA: 0 µM (●), 25 µM (■), 50 µM (▲), 100 µM (▼). Malonyl-CoA concentration was kept constant at 60 µM. (B) Fatty acid elongation activity was measured with increasing concentrations of malonyl-CoA in the presence of different concentration of bezafibroyl-CoA: 0 µM (●), 25 µM (■), 50 µM (▲), 100 µM (▼). C22:0-CoA concentration was kept constant at 20 µM. (C) Fatty acid elongation activity, without NADPH, was measured with increasing concentrations of C22:0-CoA in the presence of different concentration of bezafibroyl-CoA: 0 µM (●), 25 µM (■), 50 µM (▲), 100 µM (▼). Malonyl-CoA concentration was kept constant at 60 µM. (D) Fatty acid elongation activity, with NADPH, was measured with increasing concentrations of C22:0-CoA in the presence of different concentration of gemfibroyl-CoA: 0 µM (●), 25 µM (■), 50 µM (▲), 100 µM (▼). Malonyl-CoA concentration was kept constant at 60 µM. (E) Fatty acid elongation activity was measured with increasing concentrations of malonyl-CoA in the presence of different concentration of gemfibroyl-CoA: 0 µM (●), 25 µM (■), 50 µM (▲), 100 µM (▼). C22:0-CoA concentration was kept constant at 20 µM. (F) Fatty acid elongation activity, without NADPH, was measured with increasing concentrations of C22:0-CoA in the presence of different concentration of gemfibroyl-CoA: 0 µM (●), 25 µM (■), 50 µM (▲), 100 µM (▼). Malonyl-CoA concentration was kept constant at 60 µM. Values represent the mean ±SD.

The results thus far show that the fibroyl-CoA esters inhibit the VLCFA elongation pathway at the level of ELOVL1. To study this further, we determined the inhibitory constants (Ki) for both gemfibroyl-CoA and bezafibroyl-CoA specifically, in the absence of NADPH (only involvement of ELOVL1), and in the presence of NADPH (the complete pathway). First, microsomes isolated from cells overexpressing ELOVL1 were used to measure elongation activity at different C22:0-CoA or malonyl-CoA concentrations and the apparent Km and Vmax values were calculated using the Michaelis-Menten kinetics equation and visualized using Lineweaver-Burk plots (Fig. 5A and B). For C22:0-CoA an apparent Km of 11.0 (±1.8) µM with a Vmax of 0.11 (±0.01) nmol/min.mg was calculated and for malonyl-CoA an apparent Km of 38.0 (±4.9) µM with a Vmax of 0.17 (±0.01) nmol/min.mg. Next, we used our assay to determine the Ki values for bezafibroyl-CoA and gemfibroyl-CoA using different C22:0-CoA concentrations. Results are presented as Lineweaver-Burk plots (Fig. 6). The calculated Ki values for ELOVL1 specifically and for the complete elongation pathway were comparable: 118 µM vs 101 µM for bezafibroyl-CoA and 50 µM vs 48 µM for gemfibroyl-CoA. Additionally we determined the Ki values for the two fibroyl-CoA esters using different malonyl-CoA concentrations and calculated a Ki value of 116 µM for bezafibroyl-CoA and a Ki value of 63 µM for gemfibroyl-CoA (Fig. 6). The type of inhibition of bezafibroyl-CoA and gemfibroyl-CoA was determined using the VisualEnzymics software (Table 1). The results obtained revealed that bezafibroyl-CoA inhibits non-competitive with respect to C22:0-CoA as indicated by the intersection of the lines with the various inhibitor concentrations at the X-axis (Fig. 6A). In contrast, bezafibroyl-CoA was found to be a competitive inhibitor with malonyl-CoA as indicated by the intersection of the lines with the various inhibitor concentrations at the Y-axis (Fig. 6B). Gemfibroyl-CoA inhibition was competitive with both C22:0-CoA and malonyl-CoA.
Table 1. Inhibition kinetics of bezafibroyl-CoA and gemfibroyl-CoA for C22:0-CoA and malonyl-CoA. Data shown in Fig. 6 were used to calculate the inhibitory kinetic parameter, Ki, of bezafibroyl-CoA and gemfibrozil-CoA for both C22:0-CoA and malonyl-CoA with NADPH and additionally for C22:0-CoA without NADPH. SE indicates the standard error of each analysis.

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<th>Type of inhibition</th>
<th>Ki [µM]</th>
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Discussion

VLCFA are synthesized from LCFA by elongation in a four step cycle, which involves the concerted action of ELOVL, HSD17B12, HACD and TECR [16,22–24]. In humans, seven different ELOVL proteins have been identified each with its own substrate specificity [12,17]. The first step in the elongation of C22:0 is catalyzed exclusively by ELOVL1 [12] and it has been shown that bezafibrate is able to reduce the synthesis of C26:0 from C22:0 by direct inhibition of the elongation pathway [18]. Recently, bezafibrate was used in a clinical study to determine whether it could reduce VLCFA levels in X-ALD patients [19]. Unfortunately, bezafibrate was unable to lower VLCFA levels in plasma or lymphocytes of patients and thus was concluded to have little therapeutic benefit in X-ALD. In spite of this result bezafibrate could still serve as a good starting point in our search for better ELOVL1 inhibitors.

Initial experiments using intact skin fibroblasts showed that next to bezafibrate, gemfibrozil also reduced C26:0 synthesis while clofibrate, fenofibrate and ciprofibrate had no significant effect. Interestingly, results from an earlier study on the influence of fibrates on serum lipoproteins showed that both bezafibrate and gemfibrozil had a greater effect than fenofibrate and clofibrate [25]. Our studies with isolated microsomes from HEK293 cells over-expressing ELOVL1 revealed that while none of the fibrates had an effect on C22:0-CoA elongation, their corresponding CoA esters significantly inhibited activity. Hence, all fibrates must be activated within the cell before they can act as inhibitor. Only with clofibroyl-CoA we could not observe any inhibition on elongation activity. Historically, clofibrate was one of the first fibrates used in the treatment of hyperlipidemia [26,27] and it was later used as lead compound for the development of better antihyperlipidemic agents. The first improved fibrate was fenofibrate that had better pharmacokinetic and pharmacological properties [28]. Subsequently, bezafibrate, ciprofibrate and gemfibrozil were developed. This could indicate that the modifications that made these fibrates more potent antihyperlipidemic agents also improve their ability to inhibit VLCFA elongation. Indeed, when bezafibrate, clofibrate and gemfibrozil were compared for their ability to decrease palmitoyl-CoA elongation in rat liver microsomes bezafibrate and gemfibrozil were more potent inhibitors than clofibrate [29].
While fenofibrate and ciprofibrate had no significant effect on elongation activity in intact cells, both fenofibroyl-CoA and ciprofibroyl-CoA did inhibit fatty acid elongation effectively in our assay using isolated microsomes. This strongly suggests that the intracellular CoA-ester concentration of these fibrates in intact cells never reached sufficient levels to affect elongation activity. This could explain the fact that bezafibrate at low concentrations had no significant effect on de novo D3-C26:0 synthesis in intact cells [18].

Bezafibroyl-CoA and gemfibroyl-CoA inhibit elongation in the presence and absence of NADPH, which is required for the second and fourth step of the elongation pathway and indicates inhibition at the level of ELOVL1. Detailed analysis of the kinetic characteristics of the elongation pathway using C22:0 as a substrate revealed that the Ki of the fibroyl-CoA ester is relatively high compared to the Km values of the substrates, C22:0-CoA and malonyl-CoA. Both fibroyl-CoA esters showed competitive inhibition kinetics with malonyl-CoA indicating that the malonyl-CoA concentration will modulate inhibition by the fibrates. The inhibition of bezafibroyl-CoA is non-competitive with C22:0-CoA which is in contrast to gemfibroyl-CoA that showed competitive inhibition. From this we conclude that gemfibroyl-CoA interacts with ELOVL1 in a manner that comprises the binding sites for both malonyl-CoA and VLCFAcyl-CoA while bezafibroyl-CoA only interacts with the malonyl-CoA binding site.

In conclusion, we demonstrated that gemfibrozil and bezafibrate, as CoA-esters, are direct inhibitors of ELOVL1. However, bezafibrate showed no clinical benefit in X-ALD patients and our data indicates that this is the consequence of its low affinity for ELOVL1. Of course, fibrates are primarily designed for their potency as PPARα agonists in order to treat hyperlipidemia and not as inhibitors for VLCFA synthesis. Nevertheless, they still could serve as lead compounds for the development of more potent and specific inhibitors for ELOVL1.

Acknowledgements

The authors thank Henk van Lenthe and Femke Stet for technical assistance with the electrospray ionization mass spectrometry. This research was supported by the Netherlands Organization for Scientific Research (VIDI-grant number 91786328 to SK) and the European Union Framework Programme 7 (grant number LeukoTreat 241622 to RW and SK). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript. The authors declare no conflicts of interest.
References


Chapter 4

Pathogenicity of novel ABCD1 variants: the need for biochemical testing in the era of advanced genetics

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Published in:
Molecular Genetics and Metabolism, Volume 118, Issue 2, June 2016, Pages 123–127

Running title:
Pathogenicity of novel ABCD1 variants
Highlights

- Confirming the diagnosis ALD in women can be challenging.
- A normal VLCFA profile does not exclude heterozygosity for ALD.
- The clonal method enables biochemical studies within a physiological intact system.

Abstract

X-linked adrenoleukodystrophy (ALD), a progressive neurodegenerative disease, is caused by mutations in ABCD1 and characterized by very-long-chain fatty acids (VLCFA) accumulation. In male patients, an increased plasma VLCFA levels in combination with a pathogenic mutation in ABCD1 confirms the diagnosis. Recent studies have shown that many women with ALD also develop myelopathy. Correct diagnosis is important for management including genetic counseling. Diagnosis in women can only be confirmed when VLCFA levels are elevated or when a known pathogenic ABCD1 mutation is identified. However, in 15-20% of women with ALD VLCFA plasma levels are not elevated. Demonstration that a novel sequence variant is pathogenic can be a challenge when VLCFA levels are in the normal range. Here we report two women with a clinical presentation compatible with ALD, an ABCD1 variation (p.Arg17His and p.Ser358Pro) of unknown significance, but with normal VLCFA levels. We developed a diagnostic test that is based on generating clonal cell lines that express only one of the two alleles. Subsequent biochemical studies enabled us to show that the two sequence variants were not pathogenic, thereby excluding the diagnosis ALD in these women. We conclude that the clonal approach is an important addition to the existing diagnostic array.

Keywords: Carrier testing; metabolic disease; peroxisome; diagnosis
Introduction

X-linked adrenoleukodystrophy (ALD) (MIM #300100), the most common peroxisomal disorder, is caused by mutations in the ABCD1 gene that codes for ALDP [1], the peroxisomal half-transporter for very long-chain fatty acids (VLCFA) [2,3]. A defect in ALDP results in deficient peroxisomal beta-oxidation of VLCFA [4], and consequently VLCFA accumulation in plasma and tissues [5]. Virtually all men with ALD develop adrenal insufficiency and progressive myelopathy in adulthood [6,7]. Additionally, males with ALD can develop rapidly progressive cerebral demyelination (cerebral ALD). The onset of cerebral ALD is unpredictable, and can occur during childhood but also in adulthood [7–9]. Women with ALD develop signs of myelopathy in adulthood [10]. Disease onset is usually not before the age of 40, and thereafter the incidence of myelopathy increases sharply with age to more than 88% when over 60 years of age [10]. It is known that only 85% of women with ALD have abnormal VLCFA levels in plasma [11], and in fibroblasts [10]. Assessment of peroxisomal beta-oxidation capacity in fibroblasts from women with ALD revealed normal activity in 40% [10]. Therefore, it is recommended to perform ABCD1 mutation analysis in women suspected of ALD. However, if this yields a sequence variation with unknown significance in combination with a normal VLCFA profile in plasma or fibroblasts a diagnostic dilemma as described in the following two cases can ensue.
Materials and Methods

Cell lines and culture conditions

Human skin fibroblasts were obtained from ALD patients through the Neurology Outpatient Clinic of the Academic Medical Center and the Medical Center Alkmaar. Written informed consent was received from each patient. Control fibroblasts were obtained from male anonymous volunteers. Cells were grown in DMEM supplemented with 10% fetal bovine serum, 25 mM HEPES, 100 U/ml penicillin and 100 µg/ml streptomycin, 2.5 µg/ml fungizone and maintained at 37ºC in a humidified atmosphere at 5% CO2. When fatty acids were added DMSO concentration in the culture medium was <1%.

Measurement of peroxisomal VLCFA beta-oxidation capacity and de novo C26:0 synthesis activity in intact cells

The activity of peroxisomal beta-oxidation in intact cells was determined by measuring the formation of D3-hexadecanoic acid (D3-C16:0) from D3-docosanoic acid (D3-C22:0; CDN Isotopes) essentially as described [12]. Cells were seeded in T75 flasks at approximately 40% confluency. The next day, medium was replaced with medium containing either 30 µM D3-C22:0 for the primary cells, or 60 µM D3-C22:0 for the transformed clonal cell lines. D3-C22:0 was added to the culture medium from a 6 mM stock solution in DMSO. After 72h, cells were harvested and the VLCFA levels measured as described [13]. Peroxisomal beta-oxidation activity was expressed as the D3C16:0/D3C22:0 ratio. De novo C26:0 synthesis was determined by analysis of the amount of D3-C26:0 formed from D3-C22:0 [10].

Immunoblot analysis

Cell lysates were prepared by sonication at 8 W for 20 sec on ice in PBS containing a protease inhibitor mix (Roche). Protein concentration was determined using BCA Protein Assay Reagent (Thermo Fisher Scientific). Protein samples (40 µg) were separated on a 10% SDS-polyacrylamide gel and subsequently transferred to nitrocellulose by semi-dry blotting. The blots were probed with a monoclonal antibody against human ALDP (ALD-1D6-As, Euromedex) as described [14]. IRDYE 800CW (LI-COR Biosciences) goat anti-mouse IgG was used as a secondary antibody. Visualization was done with the Odyssey IR imaging system (LI-COR Biosciences).

SV40 transformation and clonal selection of fibroblasts

To generate clonal lines from primary fibroblasts cells were transfected with the pRSV-SV40LT plasmid using the Amaxa Nucleofection system, following the manufacturer’s instructions. To this end, 5x105 cells were resuspended in 100 µl of Nucleofector (NHDF) solution with 3 µg pRSV-SV40LT plasmid and subjected to electroporation using the U-23 program. Immediately after electroporation, cells were diluted in 20 ml DMEM medium and plated in multiple 96-well culture plates at a density of <10 cells per well. Cells were cultured under standard conditions until foci became
visible. Clones were expanded and sequenced at the mRNA level. Total RNA was isolated with TRIzol® reagent (Invitrogen) and cDNA was synthesized using the first strand cDNA synthesis kit (Roche). The ABCD1 open reading frame was amplified and sequenced using the BigDye Terminator cycle sequencing kit (Applied Biosystems) to determine whether the normal allele or the variant allele was expressed. For each ABCD1 variant, three independent clones were maintained for further studies.
Results and Discussion

Case 1:

A 36-year-old woman was referred to the neurology outpatient clinic with a slowly progressive gait disorder and urinary urgency. On examination there was paraparesis, impaired vibration sense and the plantar sole responses were extensor. These findings were considered to be compatible with myelopathy. MRI of brain and spinal cord was unremarkable, as was routine laboratory work-up including vitamin B12, copper, ACE, lysozyme, anti-SS-A and anti-SS-B, and serologic tests for syphilis and Borrelia burgdorferi. An initial lumbar puncture was normal, there was no pleiocytosis or elevated protein. ABCD1 mutation analysis was performed which revealed a NM_000033.3(ABCD1):c.50G>A (p.Arg17His) sequence variation unknown significance. Plasma and fibroblast VLCFA levels were in the normal range. ALDP expression as assessed by immunofluorescence in fibroblasts was also normal. This raised a dilemma because the clinical presentation was compatible with ALD, but the pathogenicity of the ABCD1 sequence variation in this woman with a negative family history for ALD was unclear given the normal biochemical parameters. DNA analysis was performed in both parents; her mother was found to carry the variant and her father was not. Neurologic examination in the mother showed had no clinical signs, and plasma VLCFA levels were in the normal range. The p.Arg17His variation was previously reported, but in combination with a certainly disease-causing mutation p.Ile474Serfs*84 [15]. We used several bioinformatics tools and web-based software programs to assess the nature of the alteration at the protein level. p.Arg17His was predicted to affect protein function by SIFT (score 0.05), to be disease-causing (probability value: 0.509) by MutationTaster, but predicted to be benign by PolyPhen-2 (score 0.00). Alternative options like hereditary spastic paraparesis or primary progressive multiple sclerosis (PP-MS) were also considered. To this end, a lumbar puncture was repeated and was positive for oligoclonal bands this time, further supporting a diagnosis of PP-MS. A second opinion from an neurologist with special expertise in MS was obtained. He revised the MRI scan and noted subtle diffuse signal hyperintensity in the cervical and thoracic spinal cord and several small white matter hyperintensities in the juxtacortical and periventricular white matter on T2-weighted images. He considered a diagnosis of PP-MS likely, but could not make a definitive diagnosis because according to the revised McDonald criteria other causes for the observed neurological symptoms have to be excluded and ALD was still an option. For this patient who had a child wish the distinction between an inheritable disease (ALD) and an acquired disease (PP-MS) was highly relevant in light of genetic counseling.
Case 2:

A 60-year old woman was referred to our clinic because of painful burning feet and increasing trouble with walking distances. The family history was negative for rapidly progressive brain disease or endocrine disorders; the only remarkable finding was that her mother developed difficulties with walking in her 6th or 7th decade. On neurological examination, plantar dysaesthesia was found, plantar reflexes were extensor, but otherwise there were no signs of polyneuropathy or pyramidal tract involvement. We concluded that her symptoms and signs most probably were the result of a spinal cord disorder. Routine laboratory investigations, including plasma levels of vitamin B1, vitamin B6 and vitamin B12 were normal. Antibodies against Borrelia burgdorferi were absent. Electroneurography and electromyography were unremarkable, just as MRI investigation of brain and spinal cord. A lumbar puncture was not performed. Adrenoleukodystrophy was considered in the differential diagnosis. Plasma and fibroblast VLCFA levels were in the normal range. ALDP expression as assessed by immunofluorescence in fibroblasts was also normal. ABCD1 mutational analysis revealed a NM_000033.3(ABCD1):c.1072T>C (p.Ser358Pro) sequence variation not previously described. DNA analysis was performed in her mother and son, who both were found not to carry the variant. Her father was no longer alive, her daughter and other relatives preferred not to be tested. The p.Ser358Pro variant was predicted to affect protein function by SIFT (score 0.06), to be disease-causing (probability value: 0.998) by MutationTaster, but predicted to be benign by PolyPhen-2 (score 0.00). We questioned the pathogenicity of the variant found, as all biochemical tests were normal and the bioinformatics tools undecided. We offered additional diagnostic procedures to find a cause of her signs and symptoms, which she declined.
Figure 1. Peroxisomal VLCFA beta-oxidation and de novo C26:0 synthesis activity in primary skin fibroblasts. Fibroblasts from controls (n=25), male ALD patients (n=33), female ALD patients (n=46), peroxisomal biogenesis disorder (PBD) patients (n=12), and the two females, case 1 (p.Arg17His) and case 2 (p.Ser358Pro), were cultured for three days in DMEM supplemented with 30 µM D3-C22:0. (A) Peroxisomal beta-oxidation activity is expressed as the ratio of D3-C16:0 (product) over D3-C22:0 (substrate). (B) de novo C26:0 synthesis is measured as the level of D3-C26:0 (nmol/mg protein) produced from D3C22:0. Data are the mean ± SD. The results for the two women are presented as the mean ± SD from three independent tests. *** P <0.001 by unpaired student’s t-test.

Peroxisomal VLCFA beta-oxidation and de novo C26:0 synthesis activity in primary fibroblasts

We measured the peroxisomal beta-oxidation activity and de novo C26:0 synthesis in primary fibroblasts derived from controls, male and proven female ALD patients, peroxisome biogenesis disorder (PBD) patients and the two females. To this end, cells were incubated with D3-C22:0. In male ALD patients the peroxisomal beta-oxidation activity was decreased significantly compared to controls. An even more pronounced decrease was found in PBD patients (Fig. 1A). These data are in agreement with earlier data using D3-C24:0 as substrate [12]. Proven female ALD carriers showed a peroxisomal beta-oxidation activity ranging from normal levels to those found in male ALD patients. The peroxisomal beta-oxidation activity in fibroblasts derived from the two women, however, was in the lower normal (case 1: p.Arg17His) and normal (case 2: p.Ser358Pro) range, respectively (Fig.1A). As 40% of women with ALD have been shown to have normal VLCFA beta-oxidation activity in fibroblasts (Fig. 1A) [10], these data were inconclusive and
warranted the need for additional diagnostic approaches. Analysis of de novo C26:0 synthesis demonstrated enhanced elongation activity in male ALD patients and PBD patients (Fig. 1B), which is in line with earlier reports [16]. Women with ALD showed enhanced VLCFA elongation activity ranging from normal levels to those found in ALD males (Fig. 1B). The C26:0 levels present in fibroblasts derived from the two women, however, were in the normal range (Fig. 1B).

**Peroxisomal beta-oxidation in clonal cells enables identification of pathogenicity**

In women with ALD, biochemical abnormalities in fibroblasts correlate with the X-inactivation pattern [10]. To rule out the contribution of the normal allele on the overall biochemical read-out we generated clonal cell lines expressing only one of the two alleles. As a control we included fibroblasts from four women with ALD with proven pathogenic mutations (p.Met1Val; p.Arg389His; p.Pro543Leu; p.Leu654Pro) [10], three normal controls and three male ALD patients. The fibroblasts were transfected with pRSV40 and subjected to clonal selection. Twelve clonal cell lines were validated by western blot analysis for ALDP expression (Fig 2) and by ABCD1 mRNA sequencing to determine which of the two alleles was active. For p.Arg17His, four clones were p.17Arg and eight clones were p.17His. For p.Ser358Pro, three clones were p.358Ser and nine clones were p.358Pro. For each ABCD1 variant we selected three individual clones expressing the normal ABCD1 allele and three clones expressing the mutant or variant ABCD1 allele.

As expected, immunoblot analysis showed that cells expressing the mutant ABCD1 alleles (p.Met1Val, p.Pro543Leu or p.Leu654Pro) had strongly reduced protein expression compared to the cells derived from the same patient expressing the normal allele (Fig. 2). Cells expressing the mutant ABCD1 p.Arg389His allele showed normal ALDP levels compared to the normal allele, which is in line with previous reports [17] (Fig. 2). Clonal cells derived from the p.Arg17His and p.Ser358Pro fibroblasts showed comparable ALDP expression levels for both alleles indicating that the amino acid substitution did not affect protein level.

Subsequently, for all mutations investigated three independent clones from each allele were used to measure the peroxisomal beta-oxidation activity (Fig 3). All clonal cells derived from the female ALD patients expressing the mutant ABCD1 allele showed a significantly reduced beta-oxidation activity. Whereas all clonal cells expressing the normal ABCD1 allele showed normal activity (Fig 3). The clonal cell lines generated from fibroblasts of the two women expressing either the p.Arg17His variant or the p.Pro358Ser variant showed a similar peroxisomal beta-oxidation activity when compared to the cell lines expressing the normal ABCD1 allele (Fig 3). Based on these results we conclude that both p.Arg17His and p.Pro358Ser are non-pathogenic variants.
Figure 2. Immunoblot analysis of ALDP in transformed clonal cell lines. The position of the variant amino acid residue is indicated. For each ABCD1 genotype three independent clones were analyzed. As a control, we included independent clones generated from transformed fibroblasts of three controls and three ALD male patients. Beta-actin was used as loading control.
Figure 3. Peroxisomal beta-oxidation in transformed clonal cell lines. To determine the peroxisomal beta-oxidation activity cells were cultured with 60 µM D3-C22:0 for three days. Clones expressing the normal allele are indicated with white bars, and clones expressing the variant allele are indicated with black bars. Independent clones generated from transformed fibroblasts of two controls and three male ALD patients (gray bars) were included as controls. Peroxisomal beta-oxidation activity is measured as the ratio of D3-C16:0/D3-C22:0. The average beta-oxidation activity of normal ALDP expressing clones was set as 100%. Each bar indicates three independent tests for three independent clones. Data are mean ± SD. **** P <0.0001 by unpaired t-test.
Concluding remarks

In this paper we developed a method to investigate the pathogenicity of two new ABCD1 variants identified in women with clinical features compatible with ALD. The conventional method for studying the pathogenicity of a novel ABCD1 sequence variant of unknown pathogenicity is transfection of ALD cells with either a construct expressing the normal allele or a construct expressing the variant allele followed by peroxisomal beta-oxidation analysis [18,19]. Despite the fact that the clonal method is time-consuming, the major advantage of the approach is that it allows the analysis of the effect of a variant allele within a physiological intact system (no over-expression system) in the absence of the normal allele. We conclude that the clonal approach is an important addition to the existing diagnostic array. New diagnostic tools like whole exome sequencing have found their way into the routine diagnostic process. The problem described here will therefore occur with increasing frequency. This new diagnostic test is helpful in these cases.

Acknowledgements

This research was supported by a grant from the European Leukodystrophy Association (ELA 2011-02411 to SK). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.
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Chapter 5

Function of peroxisomes in phospholipid homeostasis

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Manuscript in preparation
Abstract

Peroxisomes are small subcellular organelles. Their importance is underlined by the existence of a group of genetic diseases in man caused by defects in peroxisome function or biogenesis. The metabolic characteristics of peroxisomes can vary between organs. However, the metabolic pathway that is common to all peroxisomes is the β-oxidation of fatty acids. In this study, we investigated the oxidation of different FAs in peroxisomes with particular emphasis on the role of ABCD1 and ABCD3 in the oxidation process and also studied the metabolism of these fatty acids in a broader context including their elongation, unsaturation and incorporation into phospholipids. We show that the long-chain fatty acids C16:0, C18:0, and C20:0 can also be β-oxidized in peroxisomes next to their well-known degradation in mitochondria and that long-chain fatty acids are mainly transported by ABCD3 whereas very long-chain fatty acids are primarily transported by ABCD1. Our data indicate that C16:0 and C18:0 are the main end-products of fatty acid β-oxidation in peroxisomes which can either enter the mitochondrion for full oxidation or be used for subsequent lipid biosynthesis. Furthermore we show that a substantial fraction of the C16:0 and C18:0 derived from peroxisomal beta-oxidation is converted into mono-unsaturated fatty acids.
Introduction

Peroxisomes are subcellular organelles surrounded by a single membrane. They are present in all eukaryotes and are involved in a variety of physiological processes that differ among species. The importance of peroxisomes is underlined by the existence of a group of genetic diseases in man caused by defects in peroxisome function or biogenesis [1][2]. The enzymatic properties of peroxisomes may vary among organisms and even between organs from the same organism. The metabolic pathway that is common to all species, however, is the β-oxidation of fatty acids. Prior to peroxisomal β-oxidation, free fatty acids must be converted to acyl-CoAs [3,4], followed by their transport across the peroxisomal membrane [5]. This is mediated by one of three ATP-binding cassette (ABC) half-transporters. The peroxisomal ABC transporters belong to subclass D of the ABC protein superfamily and are referred to as adrenoleukodystrophy protein (ABCD1/ALDP), ALDP-related protein (ABCD2/ALDRP), and the 70-kDa peroxisomal membrane protein (ABCD3/PMP70) [6–8]. Within the peroxisome, the first β-oxidation step is catalyzed by different acyl-CoA oxidases (ACOX1 and ACOX2) [9]. The second and third step in peroxisomal β-oxidation are catalyzed by two so-called bifunctional proteins (LBP/MFE1 and DBP/MFE2) harboring two separate enzymatic functions: enoyl-CoA hydratase and 3-hydroxy-acyl-CoA dehydrogenase (for reviews see [10,11]). DBP/MFE2 has been shown to play an indispensable role in the oxidation of very long-chain fatty acids (VLC-FA), pristanic acid, and di- and trihydroxycholestanoic acid whereas LBP/MFE1 is involved in the oxidation of dicarboxylic acids [12]. The final step is catalyzed by two different thiolases, a straight-chain 3-oxoacyl-CoA thiolase (ACAA1) and sterol carrier protein X (SCPx) [9,11]. In addition to the role of peroxisomes in fatty acid β-oxidation, peroxisomes also have important functions in a number of metabolic pathways including: (1.) the biosynthesis of etherphospholipids which are phospholipids characterized by an ether bond at the sn-1 position of the glycerol backbone (2.) the α-oxidation of fatty acids; and (3.) the detoxification of glyoxylate [10,11]. Peroxisomes are also involved in the formation of omega-3 fatty acids like docosahexaenoic acid (C22:6 ω-3) which occurs by β-oxidation of C24:6 ω-3. Following their β-oxidation, it is not fully understood how end-products exit the peroxisome, although it is generally believed that it is not in the form of the CoA-ester. Current evidence holds that acyl-CoAs are either converted to carnitine esters by carnitine acyltransferases [13] or hydrolyzed to the corresponding free fatty acids by one of several acyl-CoA thioesterases identified in peroxisomes [14]. These products are then transported across the peroxisomal membrane [15]. Peroxisomal β-oxidation is impaired in a number of different peroxisomal disorders including the Zellweger spectrum disorders (ZSD) which includes patients affected by Zellweger syndrome, Infantile Refsum disease, and neonatal adrenoleukodystrophy (X-ALD) as well as in patients with X-linked adrenoleukodystrophy (XALD). Although much information has been gathered about the enzymology of the peroxisomal β-oxidation system, much less is known about the fate of the substrates of peroxisomal β-oxidation in case peroxisomal β-oxidation is impaired and the same is true for the end products of peroxisomal β-oxidation. What is known is that when VLCFA β-oxi-
In order to shed more light on the role of peroxisomes in whole cell fatty acid homeostasis we have conducted the current study making use of deuterated fatty acids. These fatty acids were used to study the metabolic fate of long-chain (C16:0) up to very long-chain (C26:0) fatty acids in control and peroxisome deficient cells from ZSD patients. This not only revealed which fatty acid was most actively β-oxidized but also which fatty acid was most actively elongated. Next we determined which LCFA and/or VLCFA were transported by ABCD1 and/or ABCD3 using deuterium labelled fatty acids. These studies revealed that LCFA are primarily transported by ABCD3 but that with increasing chain length ABCD1 starts to take over and that VLCFA are only transported by ABCD1. Finally, we used a new method to measure de novo phospholipid formation in control, X-ALD and ZSD fibroblasts which makes use of C23:0 as the substrate. This method allowed us to specifically measure the C23:0 derived phospholipids thus providing information on which phospholipid groups are affected by an impairment in the peroxisomal β-oxidation system.
Materials and Methods

Fatty acid substrates
Deuterium-labelled free fatty acid 16,16,16-D3-hexadecanoic acid (D3-C16:0), 17,17,18,18,18-D5-octadecanoic acid (D5-C18:0), 17,17,18,18-D4-eicosanoic acid (D4-C20:0), 22,22,22-D3-docosanoic acid (D3-C22:0) and 12,12,13,13-D4-hexacosanoic acid (D4-C26:0) were obtained from CDN Isotopes, (Québec, Canada). Deuterium-labelled free fatty acid 24,24,24-D3-tetracosanoic acid (D3-C24:0) and tricosanoic acid (C23:0) were obtained from Larodan Fine Chemicals AB (Malmö, Sweden). Stock solutions for the fatty acids were prepared in dimethyl sulfoxide (DMSO).

Cell lines and culture conditions
Written informed consent was received from each patient. Control fibroblasts were obtained from male anonymous volunteers. Human skin fibroblasts from X-ALD patients were obtained through the Neurology Outpatient Clinic of the Academic Medical Centre. Human skin fibroblasts from patients with an ABCD3 mutation or a severe ZSD (PEX1, PEX5 and PEX19) [35] were used in this study. Cells were grown in DMEM supplemented with 10% fetal bovine serum, 2.5 mM HEPES, 100 U/ml penicillin and 100 U/ml streptomycin and maintained at 37ºC in a humidified 5% CO2 atmosphere. DMSO concentrations were kept at 1% for all experiments.

Peroxisomal fatty acid metabolism measurements
Cells were seeded at 40% confluency in T75 flasks. The next day, medium was refreshed and the appropriate deuterium-labelled fatty acids were added. After 72h, cells were harvested and VLCFA analysed as described (Valianpour et al. 2003).

Phospholipid measurements
Six X-ALD, six ZSD and six control fibroblast cell lines were seeded at 50% confluence in T162 flasks. The next day, medium was refreshed with either 30 µM C23:0 or an equal volume DMSO (final DMSO concentration <1%). After 72h, cells were harvested and resuspended in milli-Q and 1 mg total protein was used for further analysis. Internal standard mix containing; CL(14:0)4 0.20 nmol, BMP(14:0)2 0.60 nmol, PC(14:0)2 8.00 nmol, PG(14:0)2 0.10 nmol, PS(14:0)2 5.00 nmol, PE(14:0)2 1.00 nmol, PA(14:0)2 0.50 nmol, SM(12:0) 2.00 nmol, LPG(14:0) 0.02 nmol, LPE(14:0) 0.10 nmol, LPC(14:0) 0.50 nmol, LPA(14:0) 0.10 nmol were added. The samples were sonicated for 5 min, followed by centrifugation at 13200 rpm for 5 min. The organic layer was transferred to a glass vial and dried under nitrogen at 60ºC. The samples were then resuspended in 200 µL of chloroform/methanol/water (50:45:5, v/v/v) containing 0.01% of NH4OH. Of the sample 10 µL was injected into the high-performance liquid chromatography-mass spectrometry (HPLC-MS) system. The HPLC system consisted of a Surveyor quaternary gradient pump, a vacuum degasser, a column temperature controller, and an autosampler (Thermo Electron, Waltham, MA, USA). The column temperature was maintained at 25 ºC. The lipid extract was injected onto a LiChrospher 2 _ 250-mm silica-60 column, 5 lm particle diameter.
(Merck, Darmstadt, Germany). Separation took place using a linear gradient between solution B (chloroform/methanol, 97:3, v/v) and solution A (methanol/water, 85:15, v/v). Solutions A and B contained 1 and 0.1 ml of 25% (v/v) aqueous ammonia per liter of eluent, respectively. The gradient (0.3 ml/min) was as follows: 0–10 min, 20% A–100% A; 10–12 min, 100% A; 12–12.1 min, 100% A–0% A; and 12.1–17 min, equilibration with 0% A. All gradient steps were linear, and the total analysis time, including the equilibration, was 17 min. A TSQ Quantum AM (Thermo Electron) was used in the negative electrospray ionization mode. Nitrogen was used as the nebulizing gas. The source collision-induced dissociation collision energy was set at 10 V. The spray voltage used was 3500 V, and the capillary temperature was 300 °C.

In negative mode, mass spectra of phospholipid molecular species were obtained by continuous scanning from m/z 380 to m/z 1500 with a scan time of 2 s. In positive mode, mass spectra of PC, LPC and SM were obtained by continuous scanning for product ions with m/z 184 from m/z 400 to 1000. Mass spectra for PE and LPE were obtained by scanning on neutral losses of 141 from m/z 400 to 1000.

**Phospholipid computational analysis**

The raw LC/MS data were converted to mzXML format using ms-Convert [36] for the Negative Scan data and ReAdW for the Positive Specific Scan data (PR184 and CNL141). The dataset was then processed using a semi-automated metabolomics pipeline written in the R programming language [http://www.r-project.org], consisting of:

Pre-processing using the R package XCMS [37] for peak finding per sample, retention time correction for optimal alignment of peaks from different samples, grouping of peaks over all samples and filling in of missing peaks. Various XCMS parameters (e.g. for Negative scan mode: fwhm=11, snthresh=7, mzdiff=2, bw=4 and mzwid=2) were optimized to best capture the IS peaks, and the peak finding function (filtermethod=matchedFilter) was adapted to enable separation of neighboring peaks with the same m/z but similar, or even partly overlapping, RT.

Identification of metabolites: the IS peaks were used to locate each particular PL peak cluster of interest, and assignments were made based on theoretical molecular mass and expected RT per PL class. For the assignment of peaks from doubly-charged molecular species (CL and mlCL), only peaks that had isotope peaks at m/z ± 0.5 were considered. The identification step also incorporated the pattern of the peaks; checking for systematic patterns in lines of compounds with the same chain length or degree of saturation. Classes that were identified included: PE, LPE, PS, PC, LPC and SM.

Isotope correction: using the chemical composition of the compound corresponding to the assigned peak labels, the expected relative intensities of isotope peaks were calculated using the R package Rdisop [38]. Intensities for observed isotope peaks were then corrected using the method described by Liebisch [39] in order to obtain semi-quantitative peak intensities.

Normalization and scaling: Intensities of peaks belonging to a certain PL class (based on m/z and RT range) were scaled to the response of the IS of that class. Thus, the intensity of the IS peak was set to the corresponding concentration of that IS that was added to each sample, and the intensities of
the other peaks of the same PL class could be estimated semi-quantitatively, relative to that of the IS peak.

Statistical analysis: a two-way ANOVA was calculated per peak with main factors Health (levels: Control, ALD, ZSD) and Medium (levels: DMSO, C23), followed by post-hoc comparisons between relevant groups, as matching the design of the experiment where there were a total of 6 groups all containing 6 samples each.

Details on the metabolic data pre-processing pipeline will be described elsewhere (manuscript in preparation). The resulting Excel tables contain all measured peaks, characterized by their m/z and RT values and, where possible, their identification, including isotope-corrected intensities and statistics based on the normalized and corrected values. EIC plots and boxplots were included in the tables, to allow for quick visual assessment of the peaks.
Results

Peroxisomal metabolism of long and very long-chain fatty acids in human fibroblasts

To gain insight into the chain-length specificity of peroxisomal β-oxidation, we incubated control and cells without functional peroxisomes, derived from patients with a defect in peroxisome biogenesis, with increasing concentrations of deuterium-labelled fatty acids ranging from C16:0 (D3-C16:0) up to C26:0 (D4-C26:0) and analysed the deuterium-labelled β-oxidation products. When D3-C22:0 was used as substrate, control cells produced D3-C20:0, D3-C18:0, D3-C16:0 and D3-C14:0 (see Fig. 1). However, none of these products were detectable in ZSD cells. This is in line with the notion that C22:0 is an exclusive substrate for peroxisomal β-oxidation (see [21]). Incubation of ZSD cells with D3-C22:0 resulted in higher amounts of the elongation products D3-C24:0 and especially D3-C26:0 when compared with control cells (Fig. 1). Similar experiments were done with fatty acids ranging from deuterium-labelled C16:0 to C26:0 (Fig. S1-S5). For all these fatty acid substrates the products of peroxisomal β-oxidation were clearly identifiable in control cells, but not in ZSD fibroblasts. In line with the D3-C22:0 results, ZSD fibroblasts produced significantly higher levels of fatty acid elongation products for all substrates tested (Fig. S1-S5) when compared to control fibroblasts. Taken together, we detected peroxisomal β-oxidation products with both long-chain (C16:0 – C20:0) and very long-chain (22:0 – C26:0) fatty acid substrates in control fibroblasts, but not in ZSD fibroblasts. These data indicate that peroxisomes can degrade both long-chain fatty acids as well as VLCFA.

Subsequently, we determined the optimal concentrations for each fatty acid substrate. Based on the results obtained (see Fig. S1-S5) we selected the following optimal concentration for each individual fatty acid: 100 µM D3-C16:0, 40 µM D5-C18:0, 50 µM D4-C20:0, 40 µM D3-C22:0, 40 µM D3-C24:0 and 30 µM D4-C26:0. Next, we analysed the balance between fatty acid elongation, degradation and desaturation for deuterium-labelled substrates ranging from C16:0 up to C26:0 (Fig. 2). To this end, control and ZSD fibroblasts were incubated with the deuterium-labelled fatty acids for three days at their optimal concentration which were different for each fatty acid as determined in the previous experiments (see Fig. 1 & S1-S5) and the deuterium labelled products were analysed after an incubation of 3 days. The results depicted in Fig. 2 show that among the different fatty acids investigated C20:0 and C22:0 were most effectively metabolized both in control as well as in ZSD cells. Furthermore, in control cells each fatty acid substrate did not only undergo beta-oxidation but also chain elongation and desaturation whereas in ZSD cells there was only chain elongation and desaturation.

Interestingly, when studying the elongation percentages in both the control and ZSD cells we observed that with D5-C18:0 as substrate the total elongation observed was less than 1% of the total recovered deuterium labelled fatty acids. However, in both control and ZSD fibroblasts using D5-C18:0 as substrate, high amounts of the mono-unsaturated fatty acid (MUFA) D5-C18:1 were found, both in control and in ZSD fibroblasts. The amounts
were 17% and 19% of total deuterium labelled fatty acids respectively. When D3-C16:0 was used as substrate two MUFAs were found including D3-C16:1 (5.5% in control and 6% in ZSD fibroblasts) and D3-C18:1 (6.5% in control and 5.5% in ZSD fibroblasts) (Fig. 2B). For both D4-C20:0 and D3-C22:0 we detected deuterium labelled C18:1 in control cells which amounted to 7.5% and 6% of total deuterium labelled fatty acids respectively whereas no C18:1 was found in ZSD cells (Fig. 2B). This indicates that C18:1 is the main desaturation product from C18:0, C20:0, and C22:0 in control cells.
Figure 1. Metabolism of D₃-C22:0 in control and ZSD cells. Fibroblasts from ZSD patients (●) and control subjects (○) were cultured for three days in the presence of D₃-C22:0 at indicated concentrations (x-axis). Deuterium-labelled metabolites were analysed using mass spectrometry as described. Levels of D₃-C22:0 (E), products from peroxisomal β-oxidation: D₃-C14:0 (A), D₃-C16:0 (B), D₃-C18:0 (C) and D₃-C20:0 (D) or fatty acid elongation: D₃-C24:0 (F), D₃-C26:0 (G) and D₃-C28:0 (H) were quantified. Each point indicates 2 cell lines measured in duplicate. Data are mean ± SD in nmol/mg protein.
Figure 2. Balance between fatty acid β-oxidation, elongation and desaturation in control and ZSD cells. Fibroblasts from ZSD patients and control subjects were cultured for three days in the presence of different deuterium-labelled fatty acids each at their optimal concentration. Metabolites were analysed using mass spectrometry as described. For each fatty acid the sum of products from β-oxidation, elongation or desaturation was calculated and expressed as percentage of the total recovered deuterium-labelled metabolites. (A) Metabolism of deuterium-labelled C16:0, C18:0, C20:0, C22:0, C24:0 and C26:0 in control and ZSD cells. Substrate is indicated on the x-axis. (B) Analysis of deuterium-labelled unsaturated fatty acids produced from each indicated substrate. Each bar indicates 3 cell lines measured in triplicate. Data is given as mean ± SD.

The role of ABCD1 and ABCD3 in peroxisomal β-oxidation

To determine the contribution of ABCD1 and ABCD3 in the transport and metabolism of LCFA and VLCFA in fibroblasts we incubated ABCD1 deficient, ABCD3 deficient, ZSD and control fibroblasts with the different deuterium-labelled fatty acids at their optimal concentration. Fig 3A shows that the formation of D3-C14:0 from D3-C16:0 was completely deficient in ABCD3 deficient cells as well as in ZSD cells whereas in ABCD1 deficient cells oxidation of D3-C16:0 was comparable to control. With D5-C18:0 as substrate, formation of D3-C16:0 was reduced to 40% of control in ABCD3 defi-
icient cells whereas with D3-C20:0 as substrate both the ABCD1 and ABCD3 deficient cell lines showed a decreased peroxisomal β-oxidation capacity compared to controls amounting to 55% and 25% respectively (Fig 3C). With D3-C22:0 as substrate peroxisomal β-oxidation activity in ABCD3 deficient cells was normal whereas in ABCD1 deficient cells activity was decreased by 55% (Fig. 3D). Peroxisomal β-oxidation of D3-C24:0 (Fig 3E) and D3-C26:0 (Fig 3F) was completely deficient in ZSD cells, reduced in ABCD1 deficient cells (48.5% for C24:0 versus 62.5% for C26:0, respectively) but normal in ABCD3 deficient cells.

Next we studied the elongation of the various fatty acids in these cell lines using the same methodology. Using D3-C16:0 as substrate, formation of D3-C26:0 was 3-fold higher in ABCD1 deficient cells as well as in ZSD cells when compared to control (Fig 3G). When D5-C18:0 was used as substrate D5-C26:0 formation was only observed in ZSD cells (Fig 3H). With D4-C20:0 as substrate D4-C26:0 formation was not significantly increased in either ABCD1 deficient or in ABCD3 deficient cells (Fig 3I). With D3-C22:0 and D4-C24:0 as substrates there was a significant increase in the formation of deuterium labelled C26:0 in ABCD1 deficient cell lines but not in ABCD3 deficient cells (Fig 3J&K). When D¬4-C26:0 was used as substrate D4-C28:0 formation was clearly increased in ABCD1 deficient and ZSD cells but not in ABCD3 deficient cells (Fig 3L). Taken together, we conclude that ABCD1, but not ABCD3, catalyses the transport of VLCFAs like C24:0 and C26:0. On the other hand, the substrate C16:0 is only handled by ABCD3 whereas both ABCD1 and ABCD3 are involved in the uptake and subsequent oxidation of C18:0, C20:0 and C22:0.
Figure 3. Role of peroxisomal transporters ABCD1 and ABCD3 in fatty acid metabolism. Fibroblasts from control subjects (black), ZSD pa-
tients (white), patients with a deficiency in ABCD1 (grey) or ABCD3 (hatched) were cultured for three days in the presence of 100 µM D3-C16:0, 40 µM D5-C18:0, 50 µM D4-C20:0, 40 µM D3-C22:0, 40 µM D3-C24:0 or 30 µM D4-C26:0. Deuterium-labelled metabolites were analysed using mass spectrometry as described. Production of D3-C14:0 from D3-C16:0 (A), D5-C16:0 from D5-C18:0 (B), D4-C18:0 from D4-C20:0 (C), D3-C18 from D3-C22:0 (D), D3-C18:0 from D3-C24:0 (E) and D3-C18:0 from D3-C26:0 (F). Production of D3-C26:0 from D3-C16:0 as a result of fatty acid elongation (G), D5-C26:0 from D5-C18:0 (H), D4-C26:0 from D4-C20:0 (I), D3-C26:0 from D3-C22:0 (J), D4-C26:0 from D4-C24:0 (K) and D4-C28:0 from D4-C26:0 (L). All bars indicate 3 different cell lines except for ABCD3 only one cell line was available. Each cell line was measured in triplicate. Data is given as mean ± SD. Statistical analysis was performed using one-way ANOVA followed by Dunnett’s multiple comparison test. * = P<0.05, ** = P<0.01 and *** = P<0.001.

**Effect of peroxisomal β-oxidation deficiency on de novo phospholipid biosynthesis**

Next, we studied de novo phospholipid biosynthesis in cells with and without a functional peroxisomal β-oxidation system in more detail. Initial experiments with the deuterium-labelled fatty acids as used in the experiments described above resulted in data where the masses of the deuterium-labelled and endogenous lipids could not be distinguished. To circumvent this problem, we decided to explore the feasibility of using fatty acids with an uneven chain length especially since the endogenous levels of uneven chain fatty acids in human fibroblasts were found to be very low. Previous experiments had shown (see Fig. 2) that of all VLCFAs tested D3-C22:0 is best metabolized in control cells. Therefore we tested C23:0 as candidate substrate. In order to compare the metabolism of C23:0 with that of D3-C22:0 we incubated control and ZSD fibroblasts with increasing concentrations of C23:0 and analysed the products of the two different fatty acid substrates using mass spectrometry. In control cells we observed formation of C15:0, C17:0, C19:0 and C21:0 as products of peroxisomal β-oxidation as well as C25:0 as product of elongation (Fig. 4). In ZSD cells incubated with C23:0 there were no degradation products were found. Instead, ZSD cells showed increased levels of C25:0, C27:0 and C29:0. The metabolic properties of D3-C22:0 and C23:0 were further analysed by comparing the products of elongation, peroxisomal β-oxidation as well as the MUFAs derived from D3-C22:0 and C23:0, respectively. In control cells peroxisomal β-oxidation of D3-C22:0 produced D3-C18:0 and D3-C16:0 which are 4 and 6 carbon atoms shorter compared to C22:0 whereas in ZSD cells C24:0 and C26:0 were produced by elongation which are 2 (D3-C24:0) and 4 (D3-C26:0) carbon atoms longer (Fig 5A). The MUFAs that were formed include D3-C16:1 and D3-C18:1 (Fig 5B). With C23:0 as substrate the formation of β-oxidation products in control cells are 4 (C19:0) and 6 (C17:0) carbon atoms shorter whereas the products of elongation in ZSD cells are 2 (C25:0) and 4 (C27:0) carbon atoms longer (Fig 5C). The MUFAs that were formed include C17:1, C19:1 and C21:1 (Fig 5B). Comparison of the results obtained with D3-C22:0
and C23:0 reveals that the two substrates are metabolically very similar albeit with two minor differences. The first is that there is less product formation that is 4 carbon atoms shorter with C23:0 as substrate (Fig 5A compared to 5C). The second is that there is a larger variety of MUFAs formed with C23:0 as substrate (Fig 5B compared to 5D). Based on the results described above we selected C23:0 for subsequent experiments.

Figure 4. Metabolism of tricosanoic acid in control and ZSD fibroblasts

Fibroblasts from ZSD patients (●) and control subjects (◦) were cultured for three days in the presence of tricosanoic acid (C23:0) at indicated concentrations and metabolites analysed using mass spectrometry as described. Products from peroxisomal β-oxidation: C15:0 (A), C17:0 (B), C19:0 (C) and C21:0 (D), substrate C23:0 (E), and from fatty acid elongation: C25:0 (F), C27:0 (G) and C29:0 (H) were quantified. Each point indicates two independent cell lines measured in triplicate. Data is given as mean ± SD.
Figure 5. Profiling of D3-C22:0 and C23:0 metabolism in control and ZSD fibroblasts. Fibroblasts from two controls and two ZSD patients were cultured in the presence of 40 µM D3-C22:0 or 40 µM C23:0 for three days and all metabolites analysed as described. Measurement of deuterium-labelled saturated (A) and unsaturated (B) fatty acids derived from D3-C22:0 in control (white bar) and ZSD (black bar). Measurement of saturated (C) and unsaturated (D) fatty acids derived from C23:0. The total sum of recovered metabolites for each substrate was set at 100%. Each cell line was measured in duplicate and data is given as mean ± SD.

Next, we cultured ABCD1 deficient, ZSD and control fibroblasts in the presence and absence of C23:0 and subsequently measured the amounts of all uneven chain fatty acids incorporated in phosphatidylcholine (PC), lysophosphatidylcholine (LPC), phosphatidylethanolamine (PE), lysophosphatidylethanolamine (LPE), phosphatidylserine (PS) and sphingomyelin (SM). Figure 6 shows the results obtained with C23:0 as substrate. When PC was measured in ZSD cells there was significantly more PC in the C23:0 treated cells than there was in the DMSO treated cells (Fig 6A). Addition of C23:0 did not significantly change the amount of PC measured in control or ABCD1 deficient cells. The phospholipids LPC (Fig 6B), PE (Fig 6C), LPE (Fig 6D) and PS (Fig 6E) had similar results compared those of PC as there was an significant increase of these phospholipids in ZSD cells but not in control and ABCD1 deficient cells. However, when SM was measured there was no significant increase when comparing the C23:0 treated to the DMSO treated ZSD cells (Fig 6F).
Figure 6. Changes in total phospholipid pools upon C23:0 supplementation. Fibroblasts from control (black), ABCD1 deficient (white) and ZSD (grey) were cultured for 3 days in the presence or absence of 30 µM C23:0 and all phospholipids analysed using LC-MS and quantified as described. Subsequently, the differences resulting from C23:0 supplementation were calculated for each phospholipid pool. Depicted are PC (A), LPC (B), PE (C), LPE (D), PS (E), and SM (F). Each bar indicates the absolute change in a phospholipid pool that was calculated from eight different cell lines. Data is given as mean ± SD. Statistical analysis was performed using one-way ANOVA followed by Dunnett’s multiple comparison test. * = P<0.05, ** = P<0.01 and *** = P<0.001.
We continued with a more detailed analysis of the de novo synthesized phospholipids. The results are shown in Figure 7 in which only the results with the most significant changes when compared to control cells are shown. Quantitative analysis of PC revealed a decrease in the incorporation of the C23:0 β-oxidation product C17:0 for both ZSD and ABCD1 deficient cells (Fig 7A). In contrast, incorporation of C23:0 and the elongation product C25:0 were increased in both the ZSD and ABCD1 deficient cell lines (Fig 7A). The results for LPC show a decrease in the incorporation of the β-oxidation product C15:0 in ZSD cells whereas incorporation of the other β-oxidation product C17:0 was decreased in both ZSD and ABCD1 deficient cells (Fig 7B). The incorporation of C23:0 and the elongation product C25:0 was increased in ZSD and ABCD1 deficient cells (Fig 7B). Similar results were obtained for PE and LPE with a reduced incorporation of the β-oxidation product C17:0 in ZSD and ABCD1 deficient cells while incorporation of C23:0 and the elongation products C25:0 and C27:0 were increased in these cells (Fig. S6). Analysis of PS revealed a decrease in ZSD and ABCD1 deficient cells for the incorporation of the β-oxidation products C17:0 and C19:0 (Fig 8A). The incorporation of C23:0 and elongation product C25:0 are only significantly increased in ZSD cells (Fig 8A). Measurements of SM revealed a significant decrease in the incorporation of the β-oxidation product C15:0 in ABCD1 deficient cells whereas the incorporation of the β-oxidation product C17:0 was found to be decreased in both ZSD and ABCD1 deficient cells (Fig 8B). Interestingly, there was no increase in the incorporation of C23:0 or the elongation product C25:0 in either the ZSD or the ABCD1 deficient cells in contrast to all the other sphingolipid species (Fig 7, 8 and S6).
Figure 7. Incorporation of C23:0 and metabolites in phosphatidylcholine and lysophosphatidylcholine. Fibroblasts from control (black bar), ABCD1 deficient (white bar) and ZSD (grey bar) were cultured for 3 days in the presence or absence of 30 µM C23:0. All phosphatidylcholine and lysophosphatidylcholine species were analyzed using LC-MS and quantified as described. The amounts of C23:0 and its metabolites incorporated in phosphatidylcholine (A) and lysophosphatidylcholine (B) in control (black bar), ABCD1 deficient (white bar) and ZSD (gray bar) cells were quantified. Only phospholipid species with a significant (P<0.05 using a two-way ANOVA) increase in the levels of uneven chain fatty acids are depicted. Each bar indicates the measurement of eight different cell lines. Data is given as are mean ± SD. * = P<0.05, ** = P<0.01 and *** = P<0.001.
Figure 8. Incorporation of C23:0 and metabolites in phosphatidylserine and sphingomyelin.
Fibroblasts from control (black bar), ABCD1 deficient (white bar) and ZSD (grey bar) were cultured for 3 days in the presence or absence of 30 µM C23:0. Analysis and quantification of C23:0 and its metabolites incorporated in phosphatidylserine (A) and sphingomyelin (B) in control (black bar), ABCD1 deficient (white bar) and ZSD (gray bar) cells was performed as described for fig.8. Each bar indicates the measurement of eight different cell lines. Data is given as mean ± SD. * = P<0.05, ** = P<0.01 and *** = P<0.001.
Discussion

The peroxisomal β-oxidation system is not only involved in the metabolism of VLCFA [21] but also in the degradation of dihydroxycholestanolic acid (DHCA), trihydroxycholestanolic acid (THCA) [22], and pristanic acid [23]. Short- medium- and long-chain fatty acids on the other hand are predominantly β-oxidized by mitochondria [24]. Here we show that the long-chain fatty acids C16:0, C18:0 and C20:0 can also be β-oxidized by peroxisomes. The results point to C22:0 as the optimal substrate to study peroxisomal β-oxidation as it is not only actively β-oxidized in control cells but also elongated in peroxisome deficient cells. Interestingly, C18:1 (oleic acid) is the main MUFA formed, by stearoyl CoA desaturase, during saturated fatty acid metabolism as the metabolism of C16:0, C18:0, C20:0 and C22:0 all resulted in high amounts of C18:1.

Recently a patient with a defect in the peroxisomal ABC half transporter ABCD3 was described [25]. This patient had severe hepatosplenomegaly, cholestasis, liver dysfunction and increased levels of the peroxisomal C27-bile acid intermediates. Previous studies using X-ALD fibroblasts had shown that C22:0 and C26:0 are mainly transported by ABCD1. Furthermore, the same group reported that C26:0 is also transported by ABCD3 albeit at low efficiency [26]. Subsequently work in the PMP70 (-/-) mouse pointed to the involvement of ABCD3 in the peroxisomal metabolism of DHCA and THCA, phytanic and pristanic acid. Earlier reports had already indicated that overexpression of ABCD3 can stimulate C16:0 β-oxidation activity in CHO cells [27]. Studies in the yeast Saccharomyces cerevisiae have revealed that ABCD3 (PMP70) has a role in dicarboxylic acid (DCA) oxidation and that it can transport (2R)-methyl fatty acids [20]. The results described in this paper indicate that ABCD3 is also involved in the transport of LCFA across the peroxisomal membrane notably of C16:0 and to a lesser extend C18:0. As there is no accumulation of elongation products in ABCD3 deficient cells we suggest that after the elongation of LCFA in ABCD3 deficient cells the VLCFA synthesized by chain elongation, are rapidly degraded in the peroxisome after transport across the peroxisomal membrane by ABCD1.

Having gained insight into the chain-length specificity of the peroxisomal β-oxidation system and the involvement of ABCD1 and ABCD3 therein, we studied the effect of a peroxisomal β-oxidation deficiency on phospholipid biosynthesis. Phosphatidylcholine and phosphatidylethanolamine are the most abundant phospholipid species in human cells [28]. We selected C23:0 for this purpose since comparison of peroxisomal β-oxidation products and elongation products formed indicate that C22:0 and C23:0 are metabolized in a very similar manner. However, there is a clear difference in the MUFA formation with C22:0 or C23:0 as substrate. The main product from C22:0 is C18:1 whereas the MUFAs produced from C23:0 range from C17:1 to C21:1. Although there are differences in MUFA formation this method still allowed to study the effect of a peroxisomal β-oxidation deficiency which revealed that the amounts of PC, LPC, PE, LPE and PS were increased in ZSD cells incubated with C23:0. One explanation would be that the increased levels of VLCFA could lead to increased levels of these phospholipids which in turn could lead to improper membrane function and signaling [29].
terestingly, the amount of SM did not increase. This could indicate that SM synthase is more specific than the other synthases. However, it could also indicate that sphingomyelinase [30] or another regulatory mechanism exerts more tight control over the SM species synthesized. The Kennedy pathway is involved in the de novo synthesis of both PC an PE [31]. Hence it was not unexpected to find that C23:0 and its metabolites were incorporated in a similar fashion into these lipid species. More specifically, PC can be synthesized by the CDP-choline branch and PE by the CDP-ethanolamine branch of the pathway [28]. PS is synthesized by phosphatidyl transfer from PE or PC in humans [32]. In yeast PS is synthesized by CDP-diacylglycerol but despite extensive investigation, no enzymatic activity for an enzyme with this activity was found in mammals [33]. For this reason, it is remarkable that in control cells there was no incorporation of C23:0 into PC and PE whereas C23:0 was incorporated into PS. A possible explanation could be that both PC and PE species are more strictly regulated through degradation than the PS species. As PC is also a direct substrate for SM synthesis via sphingomyelin synthases [34], another explanation for the finding that C23:0 was not incorporated into PC and PE, could be that they are rapidly converted into other species like PS and SM. The specific conversion of PC and PE species to SM could also explain the relatively high amounts of C23:0 and C25:0 found in the SM species among the three different cell lines. This could also explain the increased C17:0 found in SM control cell lines.
References


Figure S1 Metabolism of D3-C16:0 in control and ZSD cells. Fibroblasts from ZSD patients (●) and control subjects (◦) were cultured for three days in the presence of D3-C16:0 at indicated concentrations (x-axis). Deuterium-labelled metabolites were analysed using mass spectrometry. The levels of the product from peroxisomal β-oxidation D3-C14:0 (A), substrate D3-C16:0 (B) and products from fatty acid elongation D3-C18:0 (C), D3-C20:0 (D), D3-C22:0 (E), D3→-C24:0 (F) and D3→-C26:0 (G) were determined. Each point indicates two independent cell lines with measured in duplicate. Data is given as mean ± SD in nmol/mg protein.
Figure S2 Metabolism of D5-C18:0 in control and ZSD cells. Fibroblasts from ZSD patients (●) and control subjects (◦) were cultured for three days in the presence of D5-C18:0 at indicated concentrations (x-axis). Deuterium-labelled metabolites were analysed using mass spectrometry. The levels of the products from peroxisomal β-oxidation D5-C14:0 (A) and D5-C16:0 (B), substrate D5-C18:0 (C) and products from fatty acid elongation D5-C20:0 (D), D5-C22:0 (E), D5-C24:0 (F) and D5→C26:0 (G) were determined. Each point indicates two independent cell lines with measured in duplicate. Data is given as mean ± SD in nmol/mg protein.
Figure S3 Metabolism of D4-C20:0 in control and ZSD cells. Fibroblasts from ZSD patients (●) and control subjects (○) were cultured for three days in the presence of D4-C20:0 at indicated concentrations (x-axis). Deuterium-labelled metabolites were analysed using mass spectrometry. The levels of the products from peroxisomal β-oxidation D4-C14:0 (A), D4-C16:0 (B) and D4-C18:0 (C), substrate D4-C20:0 (D) and products from fatty acid elongation D4-C22:0 (E), D4-C24:0 (F) and D4-C26:0 (G) were determined. Each point indicates two independent cell lines with measured in duplicate. Data is given as mean ± SD in nmol/mg protein.
Figure S4 Metabolism of D₃-C₂₄:₀ in control and ZSD cells. Fibroblasts from ZSD patients (●) and control subjects (◦) were cultured for three days in the presence of D₃-C₂₄:₀ at indicated concentrations (x-axis). Deuterium-labelled metabolites were analysed using mass spectrometry. The levels of the products from peroxisomal β-oxidation D₃-C₁₄:₀ (A), D₃-C₁₆:₀ (B), D₃-C₁₈:₀ (C), D₃-C₂₀:₀ (D) and D₃-C₂₂:₀ (E), substrate D₃-C₂₄:₀ (F) and products from fatty acid elongation D₃-C₂₆:₀ (G) and D₃-C₂₈:₀ (H) were determined. Each point indicates two independent cell lines with measured in duplicate. Data is given as mean ± SD in nmol/mg protein.
Figure S5 Metabolism of D4-C26:0 in control and ZSD cells. Fibroblasts from ZSD patients (●) and control subjects (◦) were cultured for three days in the presence of D4-C26:0 at indicated concentrations (x-axis). Deuterium-labelled metabolites were analysed using mass spectrometry. The levels of the products from peroxisomal β-oxidation D4-C16:0 (A), D4-C18:0 (B), D4-C20:0 (C), D4-C22:0 (D) and D4-C24:0 (E), substrate D4-C26:0 (F) and products from fatty acid elongation D4-C28:0 (G) and D4–C30:0 (H) were determined. Each point indicates two independent cell lines with measured in duplicate. Data is given as mean ± SD in nmol/mg protein.
Figure S6 Incorporation of C23:0 and metabolites in phosphatidylethanolamine and lysophosphatidylethanolamine.
Fibroblasts from control (black bar), ABCD1 deficient (white bar) and ZSD (grey bar) were cultured for 3 days in the presence or absence of 30 µM C23:0. Phosphatidylethanolamine (A) and lysophosphatidylethanolamine (B) species were analyzed using LC-MS and quantified as described. The amounts of C23:0 and its metabolites incorporated in phosphatidylethanolamine and lysophosphatidylethanolamine in control (black bar), ABCD1 deficient (white bar) and ZSD (gray bar) cells were quantified. Only phospholipid species with a significant increase (P<0.05 using a two-way ANOVA) in levels of uneven chain fatty acids are depicted. Each bar indicates the measurement of eight different cell lines. Data is given as are mean ± SD. * = P<0.05, ** = P<0.01 and *** = P<0.001.
Chapter 6

Summary

Future prospects

Nederlandse samenvatting
Summary

The first case of X-linked adrenoleukodystrophy (X-ALD) was reported over a century ago [1]. Since then much progress has been made. An important finding was the identification of very long-chain fatty acids (VLCFA) as a biochemical marker [2] for X-ALD. Normally, VLCFA are degraded by peroxisomal β-oxidation [3] classifying X-ALD as a peroxisomal disease. Another important step was when X-ALD was genetically mapped to the Xq28 chromosomal band [4] followed by the discovery of the gene involved (ABCD1) [5] coding for adrenoleukodystrophy protein (ALDP) which transports VLCFA across the peroxisomal membrane in their CoA-ester form [6]. The finding that most VLCFA are the result of endogenous synthesis [7] has led to the development of a dietary therapy in which a mixture of trioleate (C18:1) and trierucate (C22:1) (Lorenzo’s oil (LO)) was administered to patients. Although plasma VLCFA-levels in patients usually decrease, treatment is contested at best and does not cure the disease.

VLCFA are synthesized from LCFA, via a process called chain elongation [7]. Chain elongation involves four consequential reactions of which the initial reaction is catalyzed by a transferase that allows the condensation of an acyl-CoA ester with malonyl-CoA to produce 3-ketoacyl-CoA. This transferase is part of a family of elongase proteins known as the elongase of very long-chain (ELOVL) fatty acid-like proteins. ELOVL1 has been identified as the elongase responsible for the elongation of C22:0 to C24:0 and C26:0 [8].

Chapter 1 gives an introduction to X-ALD, describing the clinical aspects, biochemistry and treatment of the disease. Chapter 2 and 3 of this thesis are dedicated to the reduction of VLCFA synthesis by inhibition of elongation. In chapter 2 it is shown that bezafibrate lowers VLCFA levels by direct inhibition of ELOVL1 activity [9]. Bezafibrate is a well-known ligand of PPARα which could explain the results. However, evidence is presented that the effects shown are not mediated by PPARα activation or increased peroxisomal fatty acid beta-oxidation capacity. In contrast, bezafibrate was shown to directly inhibit fatty acid elongation activity thus lowering VLCFA synthesis. Based on these results, an open-label pilot study was performed to evaluate the effect of bezafibrate on VLCFA accumulation in blood cells of AMN patients. Unfortunately, bezafibrate failed to lower VLCFA levels in blood cells of X-ALD patients. Most likely this is attributable to the inability to reach adequate drug levels in vivo [10]. As ELOVL1 still seems a valuable therapeutic target, the kinetic characteristics of ELOVL1 were studied and the effects of fibrates on fatty acid chain elongation were investigated further in chapter 3. The results show that the CoA-esters of fibrates are responsible for the specific inhibition of ELOVL and that not only the CoA-ester of bezafibrate but also those of gemfibrozil and ciprofibrate can inhibit elongation [11]. The CoA-esters of bezafibrate and gemfibrozil inhibit chain elongation by specifically inhibiting the first reaction of the elongation system, catalyzed by ELOVL1. The results of these 2 chapters combined with the data from the clinical trial show that fibrates are not potent enough as inhibitors of ELOVL to be useful in a clinical setting. Nevertheless, they could serve as lead compounds for the development of more potent and specific inhibitors for ELOVL1.

Heterozygous female carriers of X-ALD are known to develop symptoms. Eventually up to 80% of female X-ALD carriers develop neurological symptoms [12,13]. It is recommended to perform ABCD1 mutation analysis in women suspected of X-ALD. However, if this yields a sequence variation that has not previously been
identified and if VLCFAs are normal in plasma or fibroblasts, a diagnostic dilemma can ensue. Resolution of the question whether the sequence variation is pathogenic or not, is of great importance for future treatment and correct counselling of the patient. However, in the absence of a male relative with the same mutation, pathogenicity cannot be determined [14]. In chapter 4 two females both with a novel sequence variation in ABCD1 are reported. The chapter further describes an approach which can be used to determine the pathogenicity of the sequence variation. It makes use of clonal cell lines, which have been generated from primary fibroblasts of the patient, to select for either of the sequence variations. Then these cells are used to measure peroxisomal β-oxidation activity. This approach identifies sequence variants as being pathogenic or not making it possible to identify the pathogenicity of novel ABCD1 mutations. The sequence variations found in the female patients were both found to be non-pathogenic.

In chapter 5 the intracellular metabolism of a range of FAs was studied with emphasis on the role of peroxisomes therein. The results show that C18:0 up to C26:0 are substrates for peroxisomal β-oxidation with C22:0 as optimum. Our data indicate that C16:0 and C18:0 are the main end-products of fatty acid β-oxidation in peroxisomes which can either enter the mitochondrion for complete oxidation and energy production or be used for subsequent lipid biosynthesis. While studying the effect of C16:0 up to C26:0 in ABCD1 and ABCD3 deficient cell lines it was found that transport of C16:0 into peroxisomes strictly depends on ABCD3 while C18:0 and C20:0 are transported by ABCD1 as well as ABCD3. The transport of C22:0 and longer FA is strictly dependent on ABCD1. The effects of VLCFA on lipid biosynthesis were studied using C23:0. The metabolism of C23:0 turned out to be very similar to that of even chain FAs, especially C22:0. Phospholipids were studied by incubating control, peroxisome biogenesis disorder (PBD) and X-ALD fibroblasts with C23:0. In PBD cell lines phospholipids were increased across most of the studied lipid species. These increases can lead to improper membrane function and signaling [15] and may negatively affect the survival of the cells over time. When comparing lipids between phosphatidylcholine (PC) and phosphatidylethanolamine (PE) it was found that there was no incorporation of C23:0 [16]. However, in control cells there was incorporation of C23:0 into phosphatidylserine (PS). Possible explanation could be that both PC and PE species are more strictly regulated through degradation than the PS species. As PC is also a direct substrate for sphingomyelin (SM) synthesis via sphingomyelin synthases [17], another explanation for the finding that C23:0 was not incorporated into PC and PE, could be that they are rapidly converted into other species like PS and SM.
Future prospects

The search for treatment options to help and ultimately cure patients suffering from X-ALD is still ongoing. For young boys, a hematopoietic stem cell transplant (HSCT) is curative, provided that procedure is performed in an early stage of the disease. However, the finding that transplanted boys can still develop adrenomyeloneuropathy (AMN) in adulthood indicates that HSCT only halts the inflammatory component of cerebral ALD without addressing the underlying biochemical defect [18]. Indeed plasma C26:0 levels were still in the X-ALD range. This indicates that the chronic myelopathy that affects all men and most women with X-ALD is related to chronic VLCFA toxicity [19,20]. Therefore, reducing VLCFA synthesis could very well be the best way to treat X-ALD patients and so halt progression of symptoms in patients. In chapter 2 it is shown that it is possible to directly inhibit elongation and that this will lower the amount of very long-chain fatty acids in X-ALD fibroblasts. The most potent inhibitor identified was bezafibrate that is known for its capacity to lower LDL cholesterol and triglyceride levels in humans. In order to achieve a better understanding of how bezafibrate inhibits fatty acid chain elongation the study of this compound and others in the fibrate family was continued in chapter 3. It turned out that bezafibrate was not the only fibrate to inhibit FA chain elongation. In chapter 3 it is shown that gemfibrozil and to a lesser extent clofibrate have a similar inhibitory effect. Another important finding is that these fibrates only inhibit elongation when they are in their CoA-ester form. It was found that bezafibrate was unable to lower VLCFA levels in X-ALD patients. Only with a more potent inhibitor would a therapy based on inhibition of chain elongation be feasible for X-ALD.

To identify a more potent inhibitor multiple strategies can be followed. One of these strategies makes use of the finding that bezafibrate or rather the CoA-ester of bezafibrate can inhibit ELOVL1. A program can be envisioned where the structure of bezafibrate is taken as a model (lead) compound which then needs to be modified using synthetic chemistry. This would yield multiple compounds that would all need to be tested for their potency at the level of ELOVL1. Testing of these new compounds could be achieved using a method derived from the bezafibrate assay described in chapters 2 and 3. Another possible strategy would be to take an unbiased approach to search for compounds able to inhibit ELOVL1. To this end the Prestwick library, which contains > 1200 pharmaceutical compounds which have been administered to human patients already for many years to combat different diseases, may be useful. The compounds identified should then be tested in an intact cell model by measuring the chain elongation of D3-C16:0 to D3-C26:0 before testing the compound in the X-ALD mouse and ultimately in human beings. Use of either of the two strategies might well be successful in generating an improved, more potent inhibitor for ELOVL1. This may open up a new perspective in terms of generating a therapy for X-ALD patients based on the reduced formation of very long-chain fatty acids.

Identification of patients with a mutation in ABCD1 will greatly increase in the near future as both neonatal screening together with whole exome sequencing will quickly identify them. This will lead to far more requests for diagnosis. For males this has been done for some time now by measuring peroxisomal β-oxidation in fibroblasts, analyze ALDP-protein expression in fibroblasts by immunofluorescence and/or immunoblot analysis, and especially measuring the VLCFAs in plasma. In plasma (and fibroblasts) of obligate heterozygous females VLCFAs may be entirely normal.
which points to the need of a different diagnostic method. The method described in chapter 4 may well be of great value in this respect, as for now this is the only known way to identify whether a new genetic variant will be pathogenic or not. If the request for diagnosis truly increases as expected it would be a good idea to look into faster methods as the current one is very time intensive. It would be ideal if a new screening method could be achieved by directly measuring a biomarker in the cell of the patient. There is a good possibility that such a marker exists in the form of a phospholipid. Although the experiments in chapter 5 have not identify such a marker yet it is very well possible that new more sensitive equipment allows identification of such a marker.

The phenotype of X-ALD cannot be predicted based on the genotype. However, it could very well be that phospholipids or a phospholipid profile holds the key to determine the phenotype. Our understanding of the role phospholipids play in X-ALD is still very limited. It could very well be that when the phospholipids involved with X-ALD are studied in more detail this could lead to better or faster diagnostics or even new potential targets for treatment. In the coming years phospholipids will probably be a key focus in the research of X-ALD and will hopefully shed light on at least some aspects of X-ALD.

In conclusion X-ALD being a genetic disease will probably only be cured through gene therapy. In the near future, transplantation with genetically corrected autologous hematopoietic stem cells might become an alternative to allogeneic HSCT, once the highly encouraging results reported in the first two treated patients [21] will have been extended to a larger number of patients with cerebral ALD. This study has been initiated as a trial with 18 boys diagnosed with CCALD, aged 17 and younger have been treated with gene therapy (see starbeamstudy.com for more information). However, the long lasting effects of gene therapy will have to be tested (efficacy and long-term safety) and developed further. Therefore it is probably some years off before it can be used as a regular treatment option for X-ALD. So until that time increasing our knowledge on the biochemistry and pathophysiology of X-ALD and using this to identify novel therapeutic targets remains a necessary approach.
References


Mensen bestaan uit cellen en deze cellen vormen onze organen en deze organen voeren allerlei functies uit, maar binnen in onze cellen zelf gebeurt ook heel veel. Zo zitten er veel soorten eiwitten in onze cellen, deze zijn het gereedschap van de cel en hebben uiteenlopende functies. Onze cellen bevatten ook verschillende celorganellen, zeg maar verschillende ruimtes met specifieke functies. Een daarvan is de nucleus met daarin onze chromosomen. Chromosomen bestaan uit DNA (desoxyribonucleinezuur) wat eigenlijk een soort van blauwdruk is waarin staat hoe alles van het lichaam, elk eiwit in elke cel en ieder orgaan, gemaakt moet worden. Een ander celorgan is het zogenaamde mitochondrion. Deze breekt vetten (vetzuren) en suiker af om energie te produceren dat onze cellen nodig hebben. Een ander celorgan dat niet zo bekend is, is het peroxisoom. Peroxisomen hebben verschillende functies; een daarvan is het afbreken van specifieke vetzuren waaronder zeer lang keten vetzuren die niet door het mitochondrion kunnen worden afgebroken. Vetzuren bestaan uit koolstofatomen (C) die als een keten aan elkaar zijn gebonden. Aan ieder van deze koolstofatomen zitten een of meer waterstofatomen (H) gebonden, dit vormt het vet gedeelte. Aan het einde van deze keten zit de zuur groep, dit is een koolstofatoom (C) waar een zuurstofatoom (O) en een hydroxide (OH) aan vast zitten. Vetzuren kunnen sterk variëren in lengte van heel kort met maar 2 koolstofatomen tot ketens met 26 of zelfs meer koolstofatomen. De vetketen kan verzadigd of onverzadigd zijn. In een verzadigde keten is er geen ruimte meer in de keten, dit betekent dat de vier bindingsplaatsen die een koolstofatoom heeft allemaal zijn gebruikt. Als er twee koolstofatomen zijn die nog een bindingsplaats over hebben wordt er een dubbele binding tussen deze twee koolstofatomen gemaakt: dit wordt een onverzadigd vetzuur genoemd. Als er vier of meer koolstofatomen zijn die een bindingsplaats over hebben, worden er meer dubbele bindingen gemaakt en het vetzuur wordt dan ‘meervoudig onverzadigd’ genoemd. Om makkelijker over vetzuren te praten wordt er een simpele code gebruikt. Als er wordt gesproken over een verzadigd vetzuur met een keten van 22 koolstofatomen dan wordt er C22:0 geschreven, is deze éénmalig onverzadigd wordt er C22:1 geschreven. Het getal achter de ‘C’ geeft dus het aantal koolstofatomen aan en het getal achter de ‘:’ geeft het aantal dubbele binding aan. Peroxisomen breken de zeer lang keten vetzuren (ZLKVZ, C22:0 en langer) af en mitochondriën breken de kortere vetzuren af. Vetzuren zijn dus nodig voor energie voor de cel maar dat is niet het enige. Ze zijn ook belangrijke bouwstenen voor een cel en maken bijvoorbeeld deel uit van membranen (de muren van de verschillende ruimtes in een cel) en bepaalde grote structuren van een cel. Het soort vetzuur, de verzadiging en de lengte van het vetzuur bepaalt sommige eigenschappen van een cel. Als er iets mis is, kan dit bijvoorbeeld bepaalde hersen en zenuw ziektes veroorzaken.

X-chromosoom gebonden adrenoleukodystrofie (X-ALD) is de meest voorkomende peroxisomale stofwisselingsziekte en komt voor bij 1 op de 17.000 pasgeboren. De symptomen van de ziekte zijn zeer variabel. Zelfs binnen één familie met dezelfde mutatie kan de ziekte zich op verschillende manieren presenteren. De manier waarom een ziekte zich presenteert wordt een fenotype genoemd. Eén zo’n fenotype is “childhood” ‘cerebrale adrenoleukodystrofie’ (CCALD), wat ontstaat bij jongens tussen het 3e en 18e levensjaar. De ziekte kenmerkt zich door een heftige ontstekingsreactie in de hersenen en onherstelbare beschadiging van het myeline wat fatale hersenbeschadiging veroorzaakt. Een ander fenotype is adrenomyeloneuropathie (AMN). Hierbij is er een geleidelijke achteruitgang van.
de functie van het ruggenmerg en de zenuwen in de benen. De levensverwachting kan normaal zijn maar op elke leeftijd kan zich toch de cerebrale vorm van ALD ontwikkelen zoals bij CCALD. Verder is er een fenotype dat Addison-only wordt genoemd. Hierbij is er een verminderde functie van de bijnieren waardoor er onvoldoende bijnierschorshormonen worden geproduceerd. Symptomen kunnen worden overkomen door medicatie die deze hormonen bevat. Addison-only kan zich ook ontwikkelen tot een andere vorm van ALD. Ondanks dat X-ALD een X-gebonden aandoening is, kunnen vrouwen er ook door worden aangedaan. Behalve dat draagsters van X-ALD het genetische defect door kunnen geven aan hun kinderen, ontwikkelt zeker 80% van de vrouwen ook AMN-achtige verschijnselen. Bij patiënten met X-ALD zit er een fout in het genetische materiaal, om precies te zijn in het ABCD1 gen. Als wordt verondersteld dat een vrouw X-ALD heeft, bijvoorbeeld vanwege bepaalde klachten die ze heeft, wordt aangeraden om een ABCD1 mutatie analyse te doen. Als een tot dusver onbekende mutatie wordt gevonden en de ZLKVZ niveaus in haar plasma normaal zijn ontstaat er een diagnostisch probleem. Hoewel er tot op heden nog geen bewezen effectieve behandeling is voor patiënten met X-ALD, is het vaststellen of de mutatie de symptomen van de patiënt veroorzaakt van groot belang voor verdere symptoom bestrijding en begeleiding van de patiënt. Ook is het belangrijk te weten wat de gevolgen van de mutatie zullen zijn als de patiënt kinderen wil, zodat eventueel prenatale diagnostiek kan worden aangeboden.

Het ABCD1 gen bepaalt hoe het adrenoleukodystrofie eiwit (ALDP) moet worden gemaakt. ALDP is het transport eiwit dat in het membraan van het peroxisoom zit en ervoor zorgt dat ZLKVZ het peroxisoom in kunnen. Eenmaal daar, worden ZLKVZ afgebroken door een proces dat β-oxidatie wordt genoemd. Bij X-ALD wordt het ALDP eiwit dus helemaal niet, of met een fout erin, gemaakt waardoor de ZLKVZ het peroxisoom niet meer in kunnen om te worden afgebroken. Hierdoor stapelen de ZLKVZ zich op in het plasma en cellen. Doordat er veel te veel van deze ZLKVZ zijn bij deze patiënten kunnen de verkeerde vetzuren worden gebruikt als bouwstenen bij het maken van bepaalde celstructuren. Dit kan dan uiteindelijk leiden tot de neurologische ziekte verschijnselen zoals die te zien zijn bij X-ALD patiënten. De meeste vetzuren komen het lichaam binnen via het dieet. Echter, de ZLKVZ worden voornamelijk door het lichaam zelf gemaakt vanuit lang keten vetzuren. Dit gebeurt door een proces dat ‘keten verlenging’ wordt genoemd. Keten verlenging verloopt in vier opeenvolgende reacties, de eerste reactie wordt mogelijk gemaakt door een transferase dat ervoor zorgt dat een acyl-CoA ester en een malonyl-CoA samen een 3-keto-CoA kunnen vormen. Deze transferase hoort bij een familie van elongatie eiwitten die bekend staat als de elongase van zeer lang keten (ELOVL) vet zuurachtige eiwitten. ELOVL1 is geïdentificeerd als de elongase die verantwoordelijk is voor de verlenging van C22:0 naar C24:0 en C26:0.

In hoofdstuk 1 wordt X-ALD geïntroduceerd en worden de klinische aspecten, biochemie en de behandelingen van de aandoening beschreven. Hoofdstukken 2 en 3 van dit proefschrift gaan over de verlaging van de ZLKVZ productie door het remmen van de elongatie. In hoofdstuk 2 is te zien dat bezafibraat de ZLKVZ niveaus verlaagt door de activiteit van ELOVL1 te remmen. Gebaseerd op deze resultaten is een open-label klinisch onderzoek gestart om de effecten van bezafibraat op de stapeling van ZLKVZ in bloedcellen van AMN patiënten te onderzoeken. Helaas bleek bezafibraat niet in staat de ZLKVZ niveaus in patiënten te verlagen. Een mogelijke verklaring hiervoor is dat er niet genoeg bezafibraat in de cellen van patiënten komt om effectief te zijn. Omdat inhibitie van ELOVL1 nog steeds een goede manier lijkt
om ZLKVZ niveaus te verlagen zijn de kinetische eigenschappen van ELOVL1 en de effecten van fibraten op vetzuur keten elongatie verder bestudeerd in hoofdstuk 3. De resultaten laten zien dat niet de fibraten zelf maar hun corresponderende CoA-esters verantwoordelijk zijn voor de specifieke remming van ELOVL1. Ook is te zien dat niet alleen de CoA-ester van bezafibraat maar ook die van gemfibrozil en ciprofibraat elongatie kunnen verminderen. De CoA-esters van bezafibraat en gemfibrozil verminderen keten elongatie door specifiek de eerste reactie van het elongatie systeem, dat wordt gekatalyseerd door ELOVL1, te remmen. De resultaten van deze 2 hoofdstukken samen met de informatie van het klinische onderzoek laten zien dat fibraten niet goed genoeg werken als remmers van ELOVL1 om te helpen in de behandeling van X-ALD patiënten. Deze fibraten kunnen echter wel dienen als beginpunt in de ontwikkeling van geheel nieuwe remmers van ELOVL1 die sterker en meer specifiek zijn.

In hoofdstuk 4 wordt het effect van 2 niet eerder gevonden genetische variaties in het ABCD1 gen beschreven. Deze variaties werden gevonden in 2 vrouwen met klachten die zouden kunnen wijzen op X-ALD. Wij hebben een methode ontwikkeld waarmee bepaald kan worden of nieuwe ABCD1 variaties pathogeen zijn of niet. De methode maakt gebruik van klonaal gemaakte patiënten cellijnen die zijn geselecteerd op de aanwezigheid van het gemuteerde ABCD1 gen. Deze cellen zijn gebruikt om de peroxisomale β-oxidatie activiteit te meten waarmee kan worden vastgesteld of een nieuwe ABCD1 variatie pathogeen is of niet. De twee beschreven ABCD1 variaties bleken beide niet pathogeen te zijn. De beiden vrouwen hadden dan ook geen X-ALD. Bij 1 vrouw werd uiteindelijk de diagnose multiple sclerose gesteld, de tweede vrouw heeft een onbekende aandoening.

In hoofdstuk 5 wordt het intracellulaire metabolisme van een reeks van verzadigde vetzuren en de rol die peroxisomen hierin beschreven. Peroxisomale β-oxidatie is betrokken bij het metabolisme van ZLKVZ, de degradatie van dihydroxycholestaanzuur (DHCA), trihydroxycholestaanzuur (THCA) en pristaanzuur. De resultaten laten zien dat C18:0 tot en met C26:0 substraten zijn voor de peroxisomale β-oxidatie en dat het optimum ligt bij C22:0. Ook laten de resultaten zien dat C18:1 het voornaamste onverzadigde vetzuur is dat wordt gevormd tijdens het metabolisme van verzadigde vetzuren. Tijdens het bestuderen van de effecten van C16:0 tot en met C26:0 in ALDP (gecodeerd door ABCD1) en PMP70 (gecodeerd door ABCD3) deficiënte cellijnen werd duidelijk dat het transport van C16:0 over het peroxisomale membraan afhankelijk is van PMP70 terwijl C18:0-C20:0 door zowel PMP70 als ALDP kunnen worden getransporteerd. Het transport van C22:0 en langere vetzuren is volledig afhankelijk van ALDP. De effecten van ZLKVZ op de lipide biogenese werden bestudeerd door gebruik te maken van C23:0 als substrate voor controle-, PBD- (cellen zonder peroxisomen) en X-ALD-fibroblasten gevolgd door fosfolipiden analyse. De PBD cellijnen vertoonden significante verhogingen in het aantal fosfolipiden van de meeste soorten ZLKVZ. Verder werden er lichte verhogingen gevonden in X-ALD cellen maar deze waren niet significant. Deze verhoogde hoeveelheden kunnen leiden tot fouten in het functioneren van membranen en in de signalering wat negatieve effecten kan hebben op het functioneren en overleven van cellen. Al met al levert het onderzoek dat beschreven in dit proefschrift een belangrijke bijdrage in het vinden van een therapie voor X-ALD die gebaseerd is op het remmen van de ZLKVZ synthese, een nieuwe methode om vast te kunnen stellen of een variatie in het ABCD1 gen pathogeen is en vergroot het onze kennis op het gebied van ZLKVZ metabolisme.
Acknowledgements

Na een lange tijd bezig te zijn geweest met mijn promotie en proefschrift is het nu tijd om het leukste hoofdstuk te schrijven. Het lastigste wordt nog om niemand te vergeten. Dus wil ik beginnen om maar gewoon iedereen te bedanken voor hun steun tijdens de afgelopen promotie jaren. Verder wil ik iedereen geluk wensen die nog bezig is met zijn of haar promotie/studie en andere werkzaamheden. De eersten die ik wil bedanken zijn mijn promotor en copromotor.

Beste Ronald, het is ongeloofelijk hoeveel ik van je heb geleerd, eerst al tijdens mijn hoger laboratorium onderwijs (HLO) stage waar Jasper Komen mijn begeleider was. Dit was echt ook een geweldige tijd. Na het behalen van mijn master heb je me de mogelijkheid gegeven om bij het laboratorium GMZ mijn promotie onderzoek te doen. Jouw support tijdens deze periode was onmisbaar. Daarvoor wil ik je heel hartelijk bedanken.

Stephan wij zijn ontzettend verschillend en ik wil je bedanken dat je me de kans hebt gegeven om mee te werken aan dit project; ik heb zeer veel geleerd. Ik weet zeker dat het onderzoek naar X-ALD vooruitgang zal blijven boeken en wens je dan ook het allerbeste.

Beste Rob, jij bent zeker een van de belangrijkste mensen die ik hier wil bedanken, zonder jou was dit proefschrift nooit afgekomen. Jouw ideeën, inzichten en motivatie waren ongelooflijk belangrijk voor mij. Het enthousiasme dat je hebt als onderzoeker is geweldig en ik hoop dat dit nooit verandert.

Catherine, als beide PhD student in de X-ALD groep hebben we veel gepraat en ik heb veel steun aan je gehad. Zo was er altijd iemand om mee te delen als de promotie niet helemaal zo verliep als gehoopt. Bedankt voor de steun die je me hebt gegeven. Natuurlijk niet te vergeten de andere leden van de X-ALD groep, Inge en Clair bedankt voor de altijd positieve stemming en invloed tijdens het werk op het lab. Claire, nog even volhouden en veel geluk met de afronding van je PhD. Marc en Bwee-Tien bedankt voor de input tijdens de werkbesprekingen en de kansen die dat hebben gegeven om het onderzoek verder voort te zetten.

Veel dank gaat ook naar de mensen in de AIO kamer. Zonder jullie was deze tijd niet hetzelfde geweest. Lodewijk, altijd kritisch maar op een positieve manier en met een goed gevoel voor humor. Carlo, altijd vol enthousiasme en ideeën. Olga Pougovkina, samen met Catherine hebben wij het langst met elkaar gewerkt en we hebben dan ook heel wat gepraat en koffie gedronken.

Ja, Olga, jou spreek ik in het Nederlands toe en niet in het Engels aangezien jouw Nederlands meer dan goed is. Sandra, jij was een gezellige buurvrouw met altijd wat te vertellen en meestal heerlijk positief. Olga Meijer en Eveline het was zeker gezellig en wat chaotisch om tussen jullie beiden in te zitten. Merel, toen ik stage liet op het lab was jij net als PhD student begonnen en niet lang nadat ik begon als PhD student ben jij gepromoveerd. Kevin, kwam op het lab als student, was al snel PhD-student en in geen tijd gepromoveerd. Gewoon ongelooflijk hoe jij je erdoor heen hebt geslagen.

Now, an important switch to English. Iliana and Arwen I wanted to make sure you would know that I thank you for the great time we had in the PhD room. I wish you both the best of luck finishing your PhD. Then the ex-roommates that have already finished their PhD my initial neighbor Sara and Paula I wish you both the best with your careers. Marco, initially you seemed a little lost in the AMC but it was not long that you found
your place and finished your PhD to great results. Now I’m taking a side step to the other AIO room Katrine and Kim we had some great times and I am sure you will finish your PhD’s soon.

Nu ik in het Engels al in de andere AIO kamer ben, dan nu ook maar in het Nederlands. Eerst maar de ex-PhD studenten. Michel, weer terug in het AMC nu ik er weg ben heb ik gehoord, veel succes! Eugiënä een PhD onderzoek doen op twee plaatsen laat jij gemakkelijk lijken. Dan de postdocs. Vincent, Naomi en Riekelt altijd gedreven in het doen van onderzoek, ik wens jullie het allerbeste voor de toekomst en met de projecten waar jullie aan werken.

Dan moet hier eigenlijk een heel lange lijst komen met al de andere mensen van het lab die ervoor hebben gezorgd dat ik niet alleen het onderzoek heb kunnen uitvoeren, maar die het ook nog eens heel gezellig hebben gemaakt. Omdat ik bang ben iemand te vergeten wil ik jullie graag als groep bedanken. Weet dat een ieder van jullie zeer belangrijk is geweest. Ik weet dan ook zeker dat zonder deze geweldige groep mensen er niet veel promoties zouden plaatsvinden! Dus nogmaals heel erg bedankt.

Broeder, paranimf, Ron, beide in het onderzoek en beide gepromoveerd, wat zijn de kansen!! Wat kan ik zeggen; motivatie, afleiding, goede gesprekken en veel plezier hebben we samen. Ondanks dat je mijn “kleine” broertje bent toch ook wel een voorbeeld. De tijd die we samen in Utrecht woonden was geweldig. Stefanie, de vriendin van mijn broer, altijd aardig en gezellig. Ron is wel wat veranderd nadat hij jou leerde kennen maar dan voornamelijk ten goede! Het was super gezellig in Utrecht terwijl we toen nog alle drie als Aio bezig waren.

Hee ouders, pap en mam, oneindige support en steun, wat meer kan een persoon zich wensen, vooral tijdens deze laatste periode van mijn promotie traject. Het is niet gemakkelijk geweest en zonder jullie oneindige geduld was het ook nooit afgekomen. Beide zoons zijn nu gepromoveerd, wie had dat ooit gedacht. Dat is niet iets dat veel ouders kunnen zeggen. Dit zegt vooral veel over jullie als mens en als ouders. Woorden schieten tekort, bedankt!
Curriculum vitae

List of publications


