Lipotoxicity in adrenoleukodystrophy

Size matters!

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Chapter 7

Summary, general discussion and future research
Summary

X-linked adrenoleukodystrophy (ALD) is the most common peroxisomal metabolic disorder with a birth incidence of 1:14,700. All patients have a mutation in the gene ABCD1 that encodes the peroxisomal transmembrane protein ABCD1. The function of the ABCD1 protein is to transport very long-chain fatty acids (VLCFA, ≥C22:0) as CoA-esters into the peroxisome where they are degraded via beta-oxidation. Due to the defect in the ABCD1 protein, VLCFA cannot enter the peroxisome and accumulate in plasma and the cells of all tissues, including the spinal cord and white matter of the brain (chapter 3 of this thesis). The clinical spectrum in male patients ranges from a relatively mild adrenal insufficiency to the rapidly progressive and devastating cerebral demyelination. Females, and males who do not develop cerebral demyelination, often suffer from myelopathy. Therapy for ALD is currently only available to treat adrenal insufficiency (with adrenal corticosteroid replacement therapy) and very early stage of cerebral demyelination (with hematopoietic stem cell replacement, HSCT). No therapy is available for the myelopathy that affects both males and females.

In cells from ALD patients, the beta-oxidation capacity is decreased while the elongation activity is enhanced due to increased substrate levels. In chapter 2, a method is described that can be used to measure beta-oxidation and elongation activity in living cells by using stable-isotope labeled docosanoic acid. In chapter 3, the identification of a new biomarker for ALD in mice and men, C26:0-carnitine, is reported. C26:0-carnitine was found elevated in tissues from ALD mice, including the spinal cord and brain and in dried blood spots from ALD mice and adult male ALD patients. In chapter 4, a comparison between C26:0-carnitine and C26:0-lysoPC in dried blood spots from newborns is presented. C26:0-carnitine and C26:0-lysoPC levels were measured in 200 dried blood spots from control newborns and 11 dried blood spots from newborns with ALD. In addition, the New York State Newborn Screening program with whom we collaborated in this project measured C26:0-carnitine levels in approximately 270,000 dried blood spots from newborns and in 64 dried blood spots from newborns diagnosed with ALD, Zellweger syndrome or Aicardi Goutières Syndrome. Both the data obtained in the Netherlands and the data obtained in New York State showed that C26:0-carnitine would result in missing/false-negative results in approximately 17% of cases. Therefore, C26:0-carnitine is not a reliable marker to use for the screening of ALD in the newborn screen.

Of all women with ALD, 15-20% have normal C26:0 plasma levels. In chapter 4, the levels of plasma C26:0 and C26:0-lysoPC in dried blood spots from 49 women with ALD were analyzed and the sensitivities of these biomarkers were compared. All women had elevated C26:0-lysoPC levels. This makes C26:0-lysoPC a superior biomarker compared to C26:0 plasma levels for diagnosing women with ALD.

The exact mechanism behind VLCFA toxicity is still unknown. In chapter 5, it is demonstrated...
that there is a direct link between VLCFA and the ER stress response in ALD. When fibroblasts from ALD patients were exposed to VLCFA, increased levels of ER stress markers on mRNA and protein level were found. In addition, exposure to VLCFA caused lipoapoptosis in ALD fibroblasts.

Mono-unsaturated fatty acids are less toxic than saturated fatty acids. In chapter 6 it is shown that LXR agonists activate SCD1. Activation of SCD1 resulted in a redirection of the fatty acid synthesis from the saturated VLCFA towards the mono-unsaturated VLCFA. Importantly, the endogenous levels of C26:0 in ALD fibroblasts were completely normalized.

**General discussion and future research**

The aim of the first three chapters of this thesis was to improve the diagnosis of ALD. The method described in chapter 2 is not only helpful for diagnosing patients with ALD but can be used as well for the diagnosis of other diseases with a defect in the peroxisomal beta-oxidation: Zellweger spectrum disorders, D-bifunctional protein (HSD1B4) deficiency, acyl-CoA oxidase type 1 (ACOX1) deficiency\(^\text{18}\), the "contiguous ABCD1 DXS1357E deletion syndrome" (CADDS)\(^\text{19}\) and acyl-CoA binding domain containing protein 5 (ACBD5) deficiency\(^\text{20}\). The two main advantages of this method compared to the existing methods are that: 1) it uses a stable isotope labeled substrate instead of a radioactive labeled substrate, and 2) with this method both beta-oxidation and elongation activity can be measured while with the existing method only the beta-oxidation activity can be measured.

For ALD patients, early diagnosis is of major importance since cerebral ALD can only be treated in a very early stage and adrenal insufficiency often remains undiagnosed. For these reasons, an increasing number of countries are adding ALD to their newborn screening programs. In chapter 3, C26:0-carnitine was identified as a new biomarker for ALD. The C26:0-carnitine levels were increased in dried blood spots of the ALD mice and adult male patients. It was hypothesized in chapter 3 that the implementation of ALD into newborn screening programs would be faster and easier since C26:0-carnitine does not require a dedicated, separate method, which is needed for the detection of C26:0-lysoPC. In chapter 4, C26:0-carnitine levels were measured in dried blood spots from newborns. The results from the Netherlands showed that the C26:0-carnitine levels of 2 independent samples of 1 newborn ALD patient fell into the control range. This resulted in a sensitivity of 82%, which means that 18% of the cases would be missed in the newborn screen. The results from the New York State Newborn Screening Program showed that in 64 dried blood spots with elevated C26:0-lysoPC, 17% was at risk for being missed if the screening was based on the detection of C26:0-carnitine. From the results obtained in the Netherlands and the results from New York State it is clear that C26:0-carnitine is not a proper biomarker for ALD to use in the newborn screen. These data show that a good biomarker in a mouse or an adult does not necessary have to be a good biomarker for a newborn.
Diagnosis in women with ALD can be difficult since 15-20% have normal C26:0 plasma levels. It is of major importance to find a good, discriminative biomarker to diagnose women with ALD more accurately since ALD is a genetic disorder and can be passed on to the next generation. Furthermore, the majority of women with ALD will develop symptoms as well and not knowing what causes these symptoms can be very disturbing. Indeed, it was reported that women with ALD are often relieved when it is established that their symptoms are caused by ALD\(^6\). In order to identify a more discriminative and sensitive biomarker to diagnose women with ALD, C26:0-carnitine and C26:0-lysoPC levels were measured in dried blood spots of 49 women with ALD. The C26:0-carnitine levels of 35 out of 49 women with ALD fell into the control range, corresponding to a sensitivity of 35%. However, when C26:0-lysoPC was measured, none of the women with ALD fell into the control range, corresponding to a sensitivity of 100%. These results show that C26:0-lysoPC is a superior marker compared to C26:0 concentrations in plasma to diagnose women with ALD. Detection of C26:0-lysoPC in an independent or larger cohort, is needed to establish whether the sensitivity of C26:0-lysoPC is indeed around 100%.

VLCFA are toxic to ALD cells and although many studies have shown that oxidative stress and mitochondrial dysfunction play a role in VLCFA toxicity\(^{21-26}\), the exact mechanism behind VLCFA toxicity has not been resolved at the cellular level. A direct link between VLCFA and the activation of the ER stress response is shown in chapter 5 of this thesis. The combination of ER stress and mitochondrial dysfunction has been reported in various diseases. Mitochondria and ER are linked via mitochondrial associated membranes (MAMs). For that reason, the data of chapter 5 indicate a complicated interaction between VLCFA-induced ER stress, mitochondrial dysfunction and oxidative stress. This provides new insight into the mechanism behind VLCFA toxicity that contributes to a better understanding of the pathophysiology of ALD.

The only treatments available at this moment for ALD are corticosteroid replacement therapy for patients that suffer from an adrenal insufficiency and hematopoietic stem cell transplantation (HSCT) for patients that were diagnosed with a very early stage of cerebral demyelination. For the majority of the patients who suffer from myelopathy, no therapy is available. Since VLCFA levels are not normalized after HSCT and patients who received a HSCT still develop myelopathy as adults\(^7\), it was hypothesized that chronic exposure to VLCFA causes myelopathy\(^2\). For that reason, the focus must be on the normalization of VLCFA levels for the development of a curative therapy for myelopathy. In chapter 6, it is shown that activation of SCD1 with LXR agonists, results in a redirection in the fatty acid synthesis from saturated VLCFA towards mono-unsaturated VLCFA. In addition, endogenous C26:0 levels normalized upon treatment with LXR agonists. The data in chapter 6 is the first demonstration of a small molecule treatment that resulted in a complete normalization of C26:0. Decreased C26:0 levels have been shown in the past for example by using C18:1\(^{28}\),
4-PBA\textsuperscript{29}, bezafibrate\textsuperscript{30} and sobetirome\textsuperscript{31}. The data in chapter 6 show that LXR agonist are extremely interesting compounds and that SCD1 is a target for a therapy for ALD. The effects of LXR agonists on VLCFA metabolism are currently studied in vivo by treating \textit{Abcd1} knockout mice with a LXR agonist. This study is taking place in collaboration with Prof. Johannes Berger and Dr. Sonja Forss-Petter of the Medical University in Vienna. Hopefully the first in vivo results will be obtained soon and matches with the results that were gain in the cell model system.

Before a compound can be tested in a clinical trial, it is of major importance to establish the primary outcome parameters. Outcome parameters can be biochemical, for example an effect on VLCFA levels in blood cells from ALD patients, but also clinical such as for example no or delayed development of symptoms. To measure these clinical outcome parameters, the natural history of the disease course must be known. For this reason, long-term follow-up of male and female patients is required to establish so called “clinical trial readiness”. Without knowledge about the natural disease course, it is impossible to draw conclusions whether or not a drug will prevent the development of symptoms or delay the initiation of disease onset.

Currently an intensive long-term follow-up study of the Dutch ALD cohort (with >120 ALD patients both males and females) is ongoing in the Academic Medical Center in Amsterdam to investigate whether short-term clinical endpoints can be identified.
References