Peroxisome-Mitochondria Interplay in Adrenoleukodystrophy: Effect of Diminished Acetyl-CoA β-Oxidation Product

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Summary
X-Linked adrenoleukodystrophy (X-ALD) results from a mutation in the *ABCD1* gene. This gene codes for a protein that imports very-long-chain fatty acids (VLCFAs) into the peroxisome for degradation via β-oxidation (β-O). Previous research has labeled the loss of function of the ABCD1 protein, and thus, the build-up of VLCFAs as the primary cause of neurodegeneration. However, VLCFA levels do not correlate with disease severity. Therefore, we aim to investigate the downstream effects of a lack of β-O acetyl-CoA (a-CA) product and how this may impact functioning of specific molecules involved in peroxisome-mitochondria interaction. The goal of the present proposal is to investigate how the products of VLCFA β-O are shuttled to the mitochondria from the peroxisome, how lack of product affects the functioning of metabolic and transport proteins Cit2, Cat2, and Pex34, and whether the function of these proteins is altered in X-ALD. We will first examine the relationship between behavioral and cellular markers of disease severity and peroxisomal a-CA levels, both *in vivo* with an established *Drosophila* ALD model and *in vitro* with human fibroblast cell cultures. Peroxisomal a-CA is hypothesized to negatively correlate with behavioral and cellular markers of disease severity in *Drosophila* and human fibroblasts. Next, we will focus our attention on Cit2 and Cat2 functioning and their relationship with diminished a-CA by creating fibroblast groups with Cit2 and Cat2 genes knocked out. We will then measure fatty acid β-O in the peroxisome. Mitochondrial integrity (MI) will also be measured via CO2 levels and mitochondrial membrane potential (MMP). If lack of a-CA impairs Cit2 and Cat2, mitochondrial function will be impaired and further contribute to disease phenotype. Finally, we will look to establish a correlational relationship between Pex34 gene silencing and molecular function/severity in ALD fibroblasts by measuring peroxisomal a-CA levels in a cell culture of human fibroblasts. We aim to show that a-CA is diminished in ALD-Pex34 knockdowns (KD) and that this causes a chain reaction leading to mitochondrial dysfunction and overall neurodegeneration.

Background
X-linked adrenoleukodystrophy (X-ALD) is a progressive and often fatal neurodegenerative disease that affects the white matter of the brain and spinal cord (Gordon et al. 2018). The cause of X-ALD is an autosomal recessive mutation in the *ABCD1* gene which codes for the ABCD1 transporter protein residing in the peroxisomal membrane (Gordon et al. 2018). ABCD1 is responsible for the import of very-long-chain fatty acids (VLCFAs) into the peroxisome for degradation via β-O (Gordon et al. 2018; Kawaguchi et al. 2018). However, the mutations that result in X-ALD are loss of function, and as a result VLCFAs, are not imported into the peroxisome for degradation, resulting in several toxic effects (Kawaguchi et al. 2018). First, due to a lack of VLCFAs being degraded, there a lack of product that would normally be produced by β-O of VLCFAs, which include a-CA (a-CA) and medium-chain fatty acids (MCFAs) (Gordon et al. 2018). Additionally, the accumulation of VLCFAs in fibroblasts of X-ALD patients is a well-known biomarker for this disease and thought to be the first sign of a mutated ABCD1, since they cannot enter the peroxisome to be degraded and have been shown to be substrates for further elongation (Ofman et al. 2010). However, the levels of VLCFAs in cells and tissues do not correlate with phenotype nor with disease severity (Gordon et al. 2018; Stradomska and Tylki-Szymańska 2018; Schirinzi et al. 2019). Therefore, a novel hypothesis of the downstream effects of mutated ABCD1 is that the lack of product of VLCFA peroxisomal β-O is causative of disease rather than a buildup of the VLCFA/VLCFA-CoA substrate (Gordon et al. 2018; Stradomska and Tylki-Szymańska 2018; Schirinzi et al. 2019). Additionally, it has been shown that β-O of fatty acids in the mitochondria and function of the citric acid cycle (CAC) is coupled with β-O in the peroxisome (Violante et al. 2013; Violante et al. 2019; van Roermund et al. 1995; Shai et al. 2018). Indeed, a-CA, a product of peroxisomal β-O, has been shown to be shuttled from the peroxisome to the mitochondria. This is important given the fact that the peroxisomal membrane is impermeable to a-CA and NAD(H), another product of β-O (van Roermund et al. 1995). One of the pathways implicated in shuttling a-CA from the peroxisome to the mitochondria involves conversion of a-CA into citrate by citrate synthase (Cit2) (van Roermund et al. 1995; Visser et al. 2007) in the peroxisome; another pathway involves the conversion of a-CA to acetylcarnitine by carnitine transferase (Cat2) (van Roermund et al. 1995; Visser et al. 2007) in the peroxisome. Then, both citrate and acetylcarnitine seem to be shuttled to the mitochondria by citrate acetyl transferase protein (van Roermund et al. 1995; Visser et al. 2007) or the glyoxylate cycle where they can be converted back into a-CA (Visser et al. 2007). It remains an open question whether citrate and acetylcarnitine are lacking in X-ALD patients because there is a lack of a-CA product from β-O of VLCFAs not occurring. Additionally, Pex34 is a peroxisome-mitochondria tethering protein found on the peroxisomal membrane (Shai et al. 2018). Pex34 has been previously found to be involved in transporting a-CA from the peroxisome to the mitochondria (Shai et al. 2018). However, further investigation is needed to assess how these pathways and contact proteins interact as well as how a lack of product might affect their function. Lack of product from peroxisomal β-O of VLCFAs might cause downstream damage to the mitochondria, among other possibly impacted organelles and cellular functions. The goal of the present proposal is to investigate how the products of VLCFA β-O are shuttled to the mitochondria, the implication of a lack of product on the functioning of Cit2, Cat2, and Pex34, and how the functioning of these proteins are affected in X-ALD.

Significance
Identifying the features of the pathway of ABCD1 dysfunction and alterations in peroxisomal β-O dysfunction in ALD sheds light on the key mechanistic properties that can be targets for future therapeutics and interventions. Furthermore, investigating the role that product loss plays in disease severity would indicate that therapeutics focused on restoring this product loss should be investigated.

Specific Aims
Aim 1: Is lack of a-CA product causative of ALD phenotypic severity?
The goal of this aim is to directly connect lack of peroxisomal product with ALD phenotypes, as a lack of β-O product a-CA has been implied in previous studies to be causative of disease (Gordon et al. 2018); however, a dose-dependent relationship between levels of a-CA product and disease severity has yet to be characterized. Firstly, a dose-dependent relationship between the levels of a-CA product and behavioral and cellular measures of disease severity will be assessed. Disease severity and phenotype will be measured by locomotor activity and retinal degeneration in the *bgm dbb Drosophila* model of ALD (Gordon et al. 2018) as well as peroxisomal a-CA levels and MI in both *bgm dbb Drosophila* and human fibroblasts derived from ALD patients and healthy controls (Morita et al. 2016; Ofman et al. 2010). It is hypothesized that levels of peroxisomal a-CA will correlate with disease severity such that the lower the levels of peroxisomal a-CA, the more severe the disease as measured behaviorally and cellularly. Then, the rescue effects of introduction of MCFA will be assessed both in *Drosophila* and human fibroblasts. This will be done by measuring if introduction of MCFA reduces severity of neurodegeneration, returns a-CA levels to normal, and prevents loss of mitochondrial integrity. Additionally, the level of rescue (assessed by measuring mitochondrial integrity) will be correlated to the level of peroxisomal a-CA restoration to see if there is a correlation between the two. This might suggest that MCFA diet supplementation has rescue effects at least partly due to providing β-O product. It is hypothesized that the magnitude of rescue by treatment with MCFA, measured by mitochondrial integrity, will correlate restoration of peroxisomal a-CA levels, such that the greater the restoration of peroxisomal a-CA, the greater the rescue.
Aim 2: Does lack of a-CA product result in impaired Cit2 and Cat2 functioning or mitochondrial integrity?
The objective of this aim is to explore whether a lack of a-CA product impairs Cit2 and Cat2 functioning, and how this impairment may impact MI. Cit2 functions by converting a-CA into citrate before being shuttled from the peroxisome to the mitochondria (van Roermund et al. 1995). Cat2 is involved in converting a-CA to acetylcarnitine before being transferred to the mitochondria via citrate acetyl transferase protein (van Roermund et al. 1995; Visser et al. 2007). Because VLCFA build-up does not seem to be causative of disease severity (Gordon et al. 2018; Stradomska et al. 2009; Schirinzi

et al. 2019), we will be investigating the possible correlation between lack of a-CA product and Cit2/ Cat2 functioning. There will be five groups of fibroblasts: healthy controls, ALD, KD of Cit2, KD of Cat2, and double KD of Cit2 and Cat2. A-CA levels will be measured in each of the cells after giving pyrene□C12:0 since pyrene□C12:0 is only broken down in the peroxisome. This allows us to measure the peroxisomal activity for fatty acid β-O. Mitochondrial functioning will also be measured by assessing levels of CO2 as well as MMP. Cit2 and Cat2 convert a-CA into citrate and acetylcarnitine, respectively (van Roermund et al. 1995). If there is a lack of a-CA product in ALD, then Cit2 and Cat2 may be impaired in their conversion functioning, further impairing mitochondrial function (van Roermund et al. 1995).

Aim 3: Does Pex34 silencing correlate with a lack of a-CA product, mitochondrial dysfunction, and phenotypic severity?

The objective of this aim is to investigate whether silencing Pex34, a protein involved in transporting a-CA from the peroxisome to the mitochondria (Shai et al. 2018), results in a lack of a-CA product from VLCFA β-O. There will be four groups of fibroblasts: healthy control cells, ALD cells, ALD + Pex34 silenced cells, and healthy + Pex34 silenced cells. We will determine what silencing of Pex34 in the presence or absence of a functional ABCD1 transporter does to levels of a-CA as well as assess MI by measuring levels of CO2 and MMP. We will then compare the groups of cells to see if ALD fibroblasts have similar a-CA levels and MI as healthy cells with Pex34 KD. If the a-CA levels and MI are found to be comparable, this could indicate that Pex34 may be impaired in ALD fibroblasts due to a lack of a-CA product from β-O of VLCFAs in the peroxisome.

Design and Methods

Aim 1: Is lack of a-CA product directly related ALD phenotypic severity?

Rationale. If a lack of product is causative of disease (Gordon et al. 2018), then a direct link between the level of a-CA in the peroxisome and ALD disease severity should exist. Despite previous studies indicating that rescue effects of introduction to MCFA was due to restoration of a-CA product, this was not directly measured (Gordon et al. 2018). Therefore, characterizing the levels of peroxisomal a-CA might establish that a lack of product correlates with disease severity and phenotype; utilizing both *in vivo* and *in vitro* methods will strengthen these findings (Gordon et al. 2018; Morita et al. 2016). Additionally, comparing behavioral and cellular measures of disease severity before and after treatment with MCFA will isolate the effects of MCFA treatment alone on rescue of disease severity measures. This might provide evidence for a causal relationship between restoration of peroxisomal β-O product a-CA and reduction in severity of disease, further supporting that cellular damage in ALD is due to lack of the β-O product.

Methods. To see if the level of a-CA in the peroxisome correlates with disease phenotype *in vivo*, *bgm dbb* double mutant *Drosophila* (a previously established model of ALD (Gordon et al. 2018)) and WT Drosophila would be assessed for behavioral and cellular markers of disease severity as follows. First, levels of locomotion would be recorded for a behavioral marker (Gordon et al. 2018). Then, animals will then be sacrificed, and the level of retinal degeneration will be recorded a cellular marker of disease severity (Gordon et al. 2018). The rest of the bodies of the *Drosophila* will be used to make homogenized tissue samples. The samples will be assessed for levels of peroxisomal a-CA by first isolating the peroxisome by differential and density gradient diffusion (Graham 2001). Then, after isolation, the amount of a-CA will be determined using a commonly-used HPLC and ultraviolet detection procedure (Various Species Acetylcarnitine ELISA Kit). The cell cultures will also be assessed for CO2 levels following protocols outlined by Shai et al. (2018) and MMP following protocols outlined by Baarine et al. (2015). A one-way ANOVA with the one independent variable of disease type with four dependent variables that measure disease severity will be conducted to assess the differences in these measures of pathology in the flies. Specifically, the independent variable will have two levels (WT vs *bgm dbb*) and the four dependent measures of disease severity will be locomotor activity, peroxisomal acetyl Co-A, mitochondrial CO2, and MMP. Within-group comparisons might elucidate if severity of neurodegeneration is related to a-CA and mitochondrial function. Between-group comparisons would elucidate how levels of peroxisomal a-CA differ between WT *Drosophila* and the *bgm dbb* ALD model *Drosophila*. To see if peroxisomal a-CA correlates with severity of disease in human cells, human fibroblasts from ALD patients and healthy controls will be assessed for peroxisomal a-CA, mitochondrial CO2, and MMP, with the same protocols as was used with the *Drosophila* samples. A one-way ANOVA will be conducted with disease type (control vs ALD) as the independent variable, and the following three dependent variables: peroxisomal acetyl Co-A, mitochondrial CO2, and MMP. Within-group comparisons will compare levels of peroxisomal a-CA with MI to see if there is a direct interaction between the two. Between-group comparisons will compare levels of peroxisomal a-CA and MI between control and ALD fibroblasts. To expand on previous findings implicating a relief of ALD symptoms using MCFA supplementation in the *bgm dbb Drosophila* ALD model (Gordon et al. 2018), the effects of MCFA diet supplementation on levels of peroxisomal a-CA and MI will be measured. Then, rescue of disease phenotype with MCFA diet supplementation will be compared to possible restored levels of a-CA, to see if magnitude of rescue correlates with restoration of β-O product. This will be investigated *in vivo* in *Drosophila* fly models and *in vitro* in human fibroblast cell cultures, as follows. *In vivo* assessment of MCFA diet supplementation will include WT and *bgm dbb Drosophila* with three groups within each: standard diet, MCFA diet, and long-chain fatty acid (LCFA) diet. The rescue effect of diet supplementation will i assessed by measuring locomotor activity in live flies, then, after sacrifice, severity of retinal degeneration, levels of peroxisomal a-CA as previously described (Graham 2001, Various Species Acetylcarnitine ELISA Kit), and MI via levels of CO2 (Shai et al. 2018) and MMP (Baarine et al. 2015). Data analysis will include a multivariate analysis of variance (MANOVA) with two independent variables: disease type (WT or *bgm dbb Drosophila*) and diet type (standard, MCFA, LCFA), with four dependent variables: locomotor activity, peroxisomal acetyl Co-A, mitochondrial CO2, and MMP). *In vitro* assessment will involve human fibroblasts from ALD patients and healthy controls. The fibroblasts will be exposed to medium with fatty acids of differing lengths (MCFA, LCFA, standard diet), where the standard diet will consist of an array of fatty acids that represent a well-balanced human diet. The three dependent measures will be peroxisomal a-CA, measured as previously described (Graham 2001, Various Species Acetylcarnitine ELISA Kit), and MI via levels of CO2 (Shai et al. 2018) and MMP (Baarine et al. 2015). To analyze these data, a MANOVA will be run with two independent variables of disease type (ALD vs control) and diet (MCFA, LCFA, standard diet), and three dependent measures of disease severity (peroxisomal a-CA, mitochondrial CO2 levels, and MMP).

Predicted outcomes. Previous research found that diet supplementation with MCFA reduced phenotypic disease severity in *bgm dbb Drosophila*, and the authors speculated if this could be due to a restoration in β-O product that might otherwise be lacking (Gordon et al. 2018). However, an experiment relating ALD to lower levels of β-O products has yet to be done. Additionally, establishing a dose-dependent relationship between peroxisomal a-CA and level of cellular damage can extend previous findings (Gordon et al. 2018) by establishing that a direct relationship between lack of peroxisomal β-O product and disease severity exists in ALD. Severity of disease measured in vivo (locomotor activity and retinal damage of *Drosophila*) and *in vivo* (molecular functioning in cellular assays from both the *Drosophila* and human fibroblasts) in the same study will establish these findings are present both *in vivo* and *in vitro*, as they have been found in separate studies until now (Gordon et al. 2018; Ofman et al. 2010; Various Species Acetylcarnitine ELISA Kit). Then, these findings could be extended by directly relating the behavioral and cellular neurodegeneration measures with abundance of β-O product and mitochondrial integrity. Previous findings assumed that providing MCFA provides an alternate pathway for production of β-O products (Gordon et al. 2018), but directly measuring these products after introduction of MCFA could further establish this causality. Assessing rescue via changes in β-O product and MI upon MCFA diet supplementation will further point to a lack of β-O product as causative of disease while elucidating components of the β-O pathway impacted by ALD product loss. Additionally, assessing if restoration of peroxisomal a-CA levels through MCFA diet supplementation directly relates to MI would further establish that lack of peroxisomal a-CA contributes to cellular damage and neurodegeneration. Furthermore, it may provide insight into possible targets for therapeutic intervention, such as restoring peroxisomal β-O products to slow disease progression.

Aim 2: Does lack of a-CA product result in impaired Cit2 and Cat2 functioning or mitochondrial integrity?

Rationale. Cit2 and Cat2 work by converting β-O product, a-CA, into citrate and acetylcarnitine, respectively (van Roermund et al. 1995). If there is a lack of a-CA product established in aim 1, then Cit2 and Cat2 will have no product to transfer into citrate and acetylcarnitine, two molecules involved in many cellular processes (Violante et al. 2013; van

Roermund et al. 1995; Shai et al. 2018). Thus, a lack of a-CA product may contribute to a lack of citrate and acetylcarnitine, diminishing the effects of Cit2 and Cat2 and resulting in neurodegeneration.
Methods. To elucidate the functional response of the Cit2 and Cat2 signaling pathways, five groups of fibroblasts will be used: healthy controls, ALD, Cit2 knock-down, Cat2 knock-down, and Cit2 Cat2 double knock-down fibroblasts. KD of Cit2 and Cat2 will be achieved by utilizing short hairpin RNAs (shRNAs) to target and silence the Cit2 and Cat2 genes via RNA interference (RNAi), similarly to how genes have been knocked down in previous research (Alsayegh et al. 2015). Then, levels of citrate and acetylcarnitine will be assessed in each of the cells and correlated to measures of mitochondrial CO2 and MMP. Then, the cells will be introduced to MCFAs, and after this, the change in levels of citrate and acetylcarnitine will be assessed to see if the lack of product was restored and could therefore be seen by an increase in production of citrate and acetylcarnitine. This will also be correlated with changes in mitochondrial integrity. To measure levels of citrate, Citrate Assay Kit ab83396 will be used (Citrate Assay Kit). A one-way ANOVA will be conducted to compare levels of citrate between cell groups. In order to assess levels of acetylcarnitine, an ELISA acetylcarnitine detection kit will be utilized in conjunction with methods from Stejskal et al. (2008) to assess acetylcarnitine levels in each group of fibroblasts (Various Species Acetylcarnitine ELISA Kit). A one-way ANOVA will be conducted to compare levels of acetylcarnitine between cell groups. MCFAs will be introduced by being added to the fibroblast medium (Jones et al. 2006). Once MCFAs are introduced, citrate and acetylcarnitine levels will be reassessed using the methods above. A repeated measures ANOVA will be conducted to compare citrate and acetylcarnitine levels in each group before and after the introduction of MCFAs.To assess CO2 levels, protocols previously outlined by Shai et al. in 2018 will be followed. CO2 levels will then be measured as a percentage relative to the rate of oxidation in control groups (Shai et al. 2018). A one-way ANOVA will be conducted to compare the mean levels of CO2 in each group of fibroblasts. MMP will be examined as a measure of mitochondrial dysfunction. Protocols previously described by Baarine et al. (2015) will be followed. A one-way ANOVA will be conducted to compare the mean MMPs of each group of fibroblasts.

Aim 3: Does Pex34 silencing correlate with a lack of a-CA product, mitochondrial dysfunction, and phenotypic severity?

Rationale. Aim 3 attempts to establish a relationship between Pex34 silencing, mitochondrial dysfunction, and phenotypic severity in ALD fibroblasts. Despite a significant amount of research focusing on the impact of a build-up of VLCFAs on neurodegeneration, it has recently been established that phenotypic severity may be due to a lack of β-O product (Gordon et al. 2018). A-CA product is involved in many downstream processes in the mitochondria (Shai et al. 2018), so a lack of this product might negatively impact mitochondrial integrity. If it is found that a-CA levels are lower in ALD and ALD + Pex34 KD fibroblasts compared to control fibroblasts, it can be hypothesized that lack of a-CA product diminishes functionality of Pex34. The diminished function of Pex34 might then contribute to mitochondrial dysfunction due to a decreased ability to transfer a-CA from the peroxisome to the mitochondria. Thus, downstream effects of impaired Pex34 function may add to the neurodegeneration seen in X-ALD patients.
Methods. The current proposal will compare a-CA levels, CO2 levels, and MMP of four experimental groups. Fibroblasts from healthy controls (HC-F) and fibroblasts from ALD patients (ALD-F) will serve as control groups to compare to healthy control fibroblasts + Pex34 silencing (HC-F/Pex34-/-) and ALD fibroblasts + Pex34 silencing (ALD-F/Pex34-/-). Pex34 will be silenced in the latter two experimental groups by utilizing short hairpin RNAs (shRNAs) to target and silence the Pex34 gene via RNA interference (RNAi). Methodology for Pex34 silencing was modeled after a previously described protocol by Alsayegh et al. (2015), although researchers utilized RNAi for KD of another gene in human fibroblasts. First, a-CA levels will be measured in each of the four cell groups to determine whether ALD-F and ALD-F/Pex34-/- groups have lower levels of product compared to both the HC-F and HC-F/Pex34-/- groups. In order to measure a-CA levels, high performance liquid chromatography will be utilized as previously described by Shurubor et al. in 2017. This technique allows for measurement of a-CA in whole cells by analyzing the supernatant left after centrifugation of cells. If there is a lack of a-CA product being transferred to the mitochondria, this should be demonstrated by lower overall a-CA levels in the whole-cell samples. A one-way ANOVA will be conducted to compare the mean levels of a-CA in each group of fibroblasts. To assess CO2 levels in fibroblast samples, the current proposal will follow protocols previously outlined by Shai et al. in 2018. Protocols previously described in aim 2 will be followed. A one-way ANOVA will be conducted to compare the mean levels of CO2 in each group of fibroblasts. Finally, MMP will be examined as a measure of mitochondrial dysfunction. Protocols previously described by Baarine et al. (2015) and outlined in aim 2 will be followed. A one-way ANOVA will be conducted to compare the mean MMPs of each group of fibroblasts.
Predicted outcomes. Gordon et al. (2018) was the first to provide evidence that a lack of β-O product is causative of disease, rather than a build-up of VLCFAs. Thus, we predict lower levels of a-CA in ALD-F and ALD-F/Pex34-/- groups compared to control cells. If it is true that Pex34 functioning is impaired in ALD due to a lack of a-CA product, ALD-F and ALD-F/Pex34 -/- should have similar a-CA levels. Also, HC-F/Pex34 -/- might show decreased A-CA, but levels should not be as low as ALD-F and ALD-F/Pex34-/- because these cells still have an intact ABCD1 protein to bring VLCFAs into the peroxisome for degradation into a-CA. Furthermore, Shai et al. (2018) found that overexpression of Pex34 increased CO2 levels, so it is possible that KD of Pex34 may decrease CO2 levels. Because CO2 is a product of the Krebs cycle in mitochondria, decreased CO2 levels may indicate mitochondrial dysfunction. Correlating with decreased CO2, Pex34 silencing may also contribute to impaired MMP, further suggesting mitochondrial dysfunction. Mitochondrial dysfunction may be attributed to a lack of VLCFA β-O product. These predicted findings would suggest that phenotypic severity in X-ALD is due to a lack of β-O product ultimately impacting downstream mitochondrial processes involving Pex34.