



Fasting induces prominent proteomic changes in liver in very long chain Acyl-CoA dehydrogenase deficient mice



Wei Wang^{a,b,*}, Johan Palmfeldt^c, Al-Walid Mohsen^d, Niels Gregersen^c, Jerry Vockley^{b,d}

^a Department of Medical Genetics, Shanghai Children's Hospital, Shanghai Jiaotong University, Shanghai, China

^b Department of Human Genetics, Graduate School of Public Health, University of Pittsburgh, Pittsburgh, USA

^c Research Unit for Molecular Medicine, Institute of Clinical Medicine, Faculty of Health Sciences, Aarhus University, Aarhus, Denmark

^d Department of Pediatrics, School of Medicine, University of Pittsburgh, Pittsburgh, USA

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ABSTRACT

Very long chain acyl-CoA dehydrogenase (VLCAD) deficiency (VLCADD) is a clinically heterogeneous disorder of mitochondrial fatty acid β-oxidation usually identified through newborn screening. Genotype-phenotype correlations have been defined, but considerable clinical heterogeneity still exists. Symptoms are often induced by physiological stress such as fasting or intercurrent illness, setting it as an important example of environmental effects altering clinical course in an individual with a genetic disease. However, neither the cellular changes that predispose to this phenomenon nor the alterations it induces are well characterized. We examined the effects of fasting in a knockout mouse model to explore changes in global mitochondria protein profiles in liver and to investigate the physiologically relevant changes that lead to the clinical presentations. An isobaric tags for relative and absolute quantification (iTRAQ) labeling approach was employed to examine mitochondrial proteome changes in VLCAD deficient compared to wild type mice in the fed and fasted states. We identified numerous proteomic changes associated with the gene defect and fasting within relevant metabolic pathways. Few changes induced by fasting were shared between the VLCAD deficient and wild type mice, with more alterations found in the deficient mice on fasting. Particularly, fasting in the deficient mice could reverse the protective response in oxidative phosphorylation pathway seen in wild type animals. In addition, we found that changes in chaperone proteins including heat shock protein 60 (HSP60) and 10 (HSP10) during fasting differed between the two genotypes, highlighting the importance of these proteins in VLCAD deficiency. Finally, the effects on the liver proteome imposed by changes in fasted VLCAD deficient mice indicates that this environmental factor may be an inducer of both cellular and physiological changes.

1. Introduction

Mitochondrial fatty acid β-oxidation plays a major role in energy production in the body, especially during fasting conditions. Fatty acids are transferred across the mitochondrial membrane as carnitine esters by carnitine palmitoyltransferase 1 and 2 in conjunction with the carnitine-acylcarnitine translocase [1–3]. In the mitochondrial matrix, the fatty acid β-oxidation cycle is initiated by five different acyl-CoA dehydrogenases (ACADs), leading to sequential removal of two carbons in the form of acetyl-CoA molecules with each cycle (4–5). Electrons are transferred from the ACADs to the respiratory chain through complex I of the electron transfer chain or directly to coenzyme Q (ubiquinone) by electron transfer flavoprotein (ETF) and ETF ubiquinone oxidoreductase. Very long-chain acyl-CoA dehydrogenase (VLCAD) is the primary ACAD active in the metabolism of straight

long chain acyl-CoAs, fueling early rounds of ketogenesis during periods of high energy demand after hepatic glycogen depletion (6). It is responsible for the catalysis of acyl-CoAs of 12–20 carbon in chain length in liver, heart, and muscle.

VLCAD deficiency (VLCADD) has been identified in more than 400 patients world-wide. It can present with a variety of clinical symptoms and a spectrum of severity that ranges from severe life threatening illness in the newborn period to relatively mild disease [3,4]. It is now diagnosed through newborn screening in many countries [5,6]. Patients who survive their initial presentation may exhibit progressive cardiomyopathy and have a reported 75% mortality rate in the first few years of life [7,8]. Children with later onset symptoms can have repeated episodes of rhabdomyolysis but are at lower risk of developing cardiomyopathy. Sequence analysis of the *ACADVL* gene has revealed correlation of mutation genotype with phenotype; however, this

* Corresponding author at: Department of Medical Genetics, Shanghai Children's Hospital, Shanghai Jiaotong University, Shanghai, China.
E-mail address: wangwei@shchildren.com.cn (W. Wang).

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relationship is imperfect [3,4,9]. The molecular mechanisms leading to such heterogeneity remain unknown [3,4]. We have shown that the enzymes involved in fatty acid oxidation are arranged in a multifunctional fatty acid oxidation complex that functionally and physically interacts with the respiratory chain supercomplexes [10]. Thus secondary changes in other fatty acid oxidation or respiratory chain proteins could play a role in determining clinical heterogeneity, as could other unanticipated alterations.

The effect of environmental factors on phenotype is usually measured through epidemiological studies of individuals with some genetic susceptibility to an exposure, but such studies are usually not tenable in patients with rare metabolic disorders. For example, the full scope of biological changes induced by VLCADD with or without fasting has been impossible to characterize directly in patients. To overcome these difficulties, mouse models of VLCADD offer an opportunity to explore more complicated questions than is possible in patients with these disorders [11–16]. Two independently VLCAD deficient mouse models biochemically resembling the human deficiency have been developed (16–17). Severe hypoglycemia, hypothermia, skeletal myopathy, and death in one third of mice were observed in fasting mice (18). Additional studies have documented the development of hepatosteatosis in response to fasting with death after prolonged fasting (16). An impairment in amino acid metabolism has also been shown to lead to a reduction in gluconeogenic precursors in VLCAD deficient mice, contributing to hypoglycemia (19). A broader examination of the biological impact of fasting on these animals at the proteomic level should provide additional insights into the underlying pathophysiological mechanism of gene-environmental interactions in this disorder.

2. Material and methods

Mice. VLCAD deficient (C57BL/6+129svJ) and corresponding wild type mice were purchased from Jackson Laboratory (Bar Harbor, Maine) at age 4–5 weeks. At least 6 male mice of each genotype were used for each paired study. For proteomics experiments in fasted mice, each mouse was fasted 16 h prior to sacrifice. All animals were sacrificed at 6–8 weeks of age by CO₂/O₂ asphyxiation according to standard protocols approved by the University of Pittsburgh IACUC. Liver was harvested immediately after death.

2.1. Preparation of mitochondrial protein

Freshly harvested liver mitochondria were isolated on Percoll gradients as previously described (21). 100 µg of protein were precipitated by addition of pre-chilled acetone and collected after air drying.

2.2. iTRAQ labeling and paired studies

Five proteomic studies were performed as previously described to identify mitochondrial proteome changes due to either genetic deficiency, fasting, or both (21). Biological replicates were labeled in a random manner to minimize possible labeling bias (Supplemental Table 1). Labeling procedures were performed according to the manufacturer's protocol (Applied Biosystems, Foster City, CA).

2.3. Nano-LC mass spectrometry (MS)

These experiments were performed as previously described (26). Peptide samples were purified with strong cation exchange (SCX) cartridges, Strata 55 µm, 70 Å (Phenomenex, Torrance, CA).

2.4. Database searches and statistics

The MS/MS spectra (RAW files) were performed as previously described [21,22]. The MS/MS spectra (RAW files) were processed

using extract_msn.exe, February 15, 2010 (Thermo Fischer Scientific). The MS/MS data from 10 different fractions of peptides for each experiment were combined to search against the IPI-mouse database (version 3.65 with 56,775 sequences, released 10/16/2009) using Mascot version 2.2.04 (Matrix Science, London, UK, www.matrixscience.com) using the multidimensional protein scoring algorithm (MudPIT) with information from *Mus* species only, allowing up to two missed trypsin cleavages per protein. The full scan tolerance was 5 ppm and MS/MS fragment tolerance was 0.75 Da. Searches were performed allowing variable oxidation of methionine residues (16 Da) and iTRAQ labels on tyrosine, fixed modification of iTRAQ labels on lysine and N-terminus, and methylthionine modification of cysteine residues. The threshold of protein identification was set to a significance of $p \leq 0.001$ corresponding to a false discovery frequency of 0.002 searching against a decoy database. iTRAQ values were reported for proteins with four or more measured iTRAQ values and each included peptide scan had an expectation value of 0.02 or below. When the identification of a protein in an analysis resulted in several possible protein isoforms, all of them were considered for quantification. In the quantitative calculations, only protein isoforms with iTRAQ values in all analyses were included. Proteins with a minimum of 2 labeled peptides and a mascot score of over 60 were selected for further data analysis. In each study the average of the eight iTRAQ values was normalized to 1.0 for all proteins and then the data set was log (2) transformed to obtain data normally distributed around unity. The mean value of the biological and technical replicates for each genotypic mouse was calculated to obtain the average fold change of a protein's production. For an initial screening student's *t*-test was used to assess significance ($p \leq 0.10$) of the differentially regulated proteins in each study. As a second criterion was a fold change of > 1.33 fold increase or < 0.75 fold decrease applied. Proteins that met both criteria in 2 experiments (experiment 1 and 2) or in one experiment (experiment 3, 4 or 5) were included for further bioinformatics analyses.

2.5. Pathway analysis and prediction of toxicity

Pathway associations of differentially expressed proteins in deficient mice in either the fed or fasted state were analyzed with the Ingenuity Pathways Analysis software (IPA; Ingenuity Systems, Redwood City, CA; www.ingenuity.com) (21). The taxonomy was set to "mouse". The filters and general settings for the core analysis were set to consider all molecules including endogenous chemicals, as well as both direct and indirect relationships. All data sources except cell lines were considered. Scores of 2 or higher (Fisher's exact test, $p < 0.05$) having at least a 99% confidence were considered as significant. In this analysis, we selected the top 2 networks as the most significant. The Global Functional Analysis (GFA) and Global Canonical Pathways (GCP) analysis were utilized to identify the canonical pathways and associated diseases that were related to the differentially expressed proteins. Toxic analysis of the IPA was designed to characterize specific organ system changes related to exposure to suspected toxins. This function was utilized to identify patterns of altered protein expression in VLCAD deficient mice in both the fed and fasting states to assess the secondary physiologic impact of the primary *ACADVL* gene defect and fasting.

3. Results

To examine the biological impact of fasting in VLCAD deficient mice, we first characterized liver mitochondrial proteome alterations in VLCAD deficient mice while on an *ad lib* diet. We next examined liver mitochondrial proteome alterations in fasting mutant and wild type mice. In combination, these experiments allowed us to explore the relative contribution of the VLCADD and the environmental condition of fasting to global proteome changes.

3.1. VLCAD deficiency induced different changes in different feeding states

In the fed state, 43 proteins (as defined by at least 2 peptides with $p \leq 0.001$) were classified as having at least a 30% alteration in level between VLCADD and wild type mice ($p \leq 0.05$). A detailed description of the identification and quantification information is provided in Supplemental Table 2. If a protein has been assigned to multiple subcellular locations by literature, a mitochondrial location for the specific location was assumed. Comparing fasted VLCAD deficient mice to wild type animals, 54 proteins were initially screened as significantly altered (≤ 0.75 fold or ≥ 1.3 fold change; $p \leq 0.05$, 1 sided) out of 286 unambiguous proteins that met the identification criteria ($p \leq 0.001$, $FDR \leq 0.002$) (Supplemental Table 3). The level of 28 proteins was decreased, while 24 were increased. Of note, the repertoire of proteins differing between VLCADD and wild type mice differed in the fed and fasted state, with only a few proteins altered in common under both conditions.

3.2. Fasting effects on deficient and wild type mice

To explore the differential effects of fasting on wild type and VLCADD mice, we compared the proteome of mitochondria from deficient mice in the fasted vs. the fed state (Supplemental Table 4). Then, the same comparison was done for wild type mice (Supplemental Table 5). We found that fasting induced significant alterations (> 1.3 -fold increase or < 0.77 -fold decrease; Student's test, 1 sided, $p \leq 0.05$.) in 35 proteins in VLCADD mice. In wild type mice, 41 proteins changed with fasting (data not shown). Fasting induced a different pattern of alterations in VLCADD mice than was seen in wild type animals, though a limited number of changes were correlated in both genotypes.

3.3. Fasting induced changes in pathways and networks

In fed VLCAD deficient vs fed wild type and fasted VLCAD deficient vs fasted wild type mice, the most highly altered Ingenuity Pathway

Analysis (IPA) network was “functions of lipid metabolism, molecular transport and small molecule chemistry” (Figs. 1 and 2). Notably, even though the same altered network was identified for both pairs of analyses, the proteins comprising the network differed. Two proteins, encoded by *SCP2* and *NUDT7*, were found decreased in deficient mice regardless of feeding status (Supplemental Figs. 1 and 2). A few proteins, including those encoded by *HSPA5*, *HADHA* and *GPX1*, were identified by network analysis to be common between fed and fasted wild type and VLCAD deficient animals, though not directly detected in our experiments. The latter category included the nuclear receptor *PPARA*, the transcription regulator *PPARGC1A*, and the growth factor leptin (Figs. 1A and B, 2). These important linker proteins were also inferred as part of the top-rated network of altered proteins in deficient mice in both feeding states (Fig. 1A and B; see Discussion). Analysis of the canonical pathways associated with altered proteins revealed that most of the affected pathways in VLCADD mice (fed or fasted) were defined by down-regulated proteins. The pathways of fatty acid metabolism, bile acid biosynthesis, and fatty acid elongation were affected in mitochondria from deficient mice under both feeding conditions (Supplemental Tables 1 and 2).

3.4. Gene-fasting interactions in VLCADD

Fasting in the context of VLCADD led to a different pattern of proteomic changes than either condition alone (Summarized in Table 1). In wild type animals, chaperones were up-regulated by fasting. However, in VLCADD, they were down-regulated. A compensatory up-regulation of the oxidative phosphorylation (OXPHOS) pathway in VLCADD mice in the fed state was abrogated by fasting (Figs. 2 and 3). The pathway of “mitochondrial dysfunction” was induced by VLCADD but not fasting. In contrast, the pathways of “branched chain amino acid metabolism including valine, leucine and isoleucine metabolism” and “amino acid metabolism including arginine, proline, tyrosine, phenylalanine and β -alanine were altered more by fasting than the gene deficiency. “Synthesis and degradation of ketone bodies” was not significantly altered in the either fasted or fed

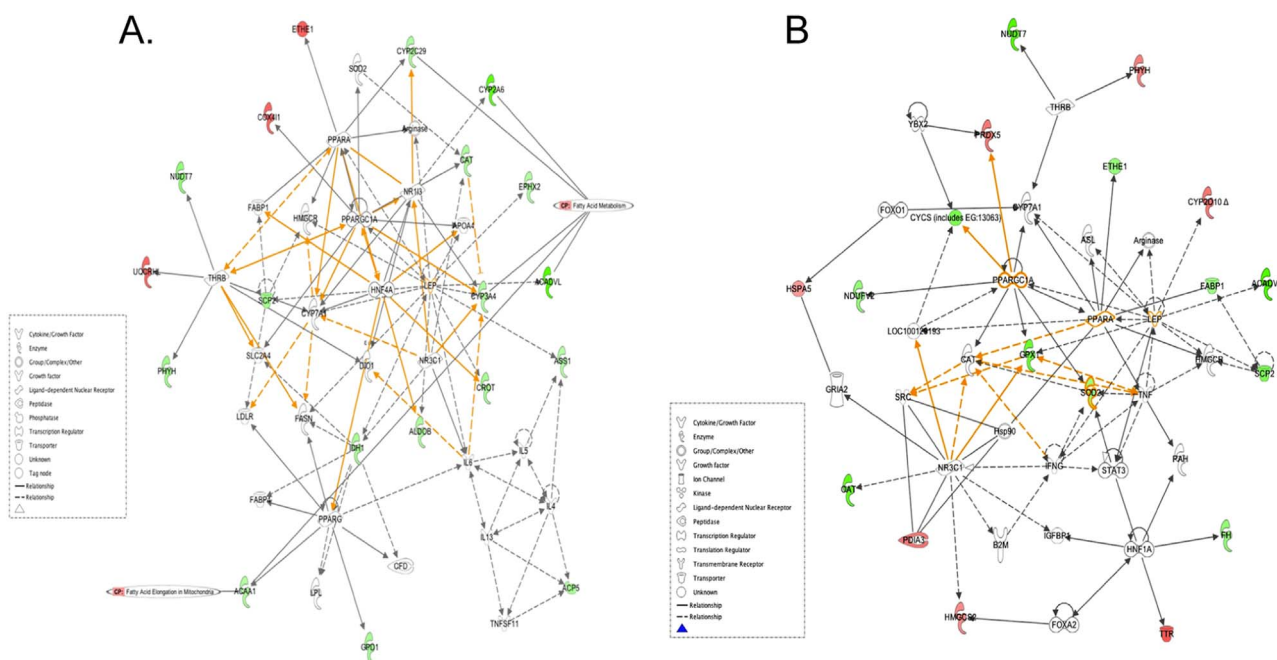


Fig. 1. Top-rated network of altered proteins in VLCAD deficient mice under feeding and fasting states. A. Top-rated network of altered proteins in VLCAD deficient mice in the fed state. B. Top-rated network of altered proteins in VLCAD deficient mice in the fasted state. This network (lipid metabolism, molecular transport and small molecule chemistry) was generated by IPA. Red nodes indicate that the protein is up-regulated in fed VLCAD deficient mice compared to fed wild type mice. Green indicates that the protein is down-regulated. The color intensity corresponds to the degree of abundance. Proteins in white are those inferred from the Ingenuity Pathways Knowledge Base. The shapes denote the molecular class of the protein. A solid line indicates a direct molecular interaction, and a dashed line indicates an indirect molecular interaction.

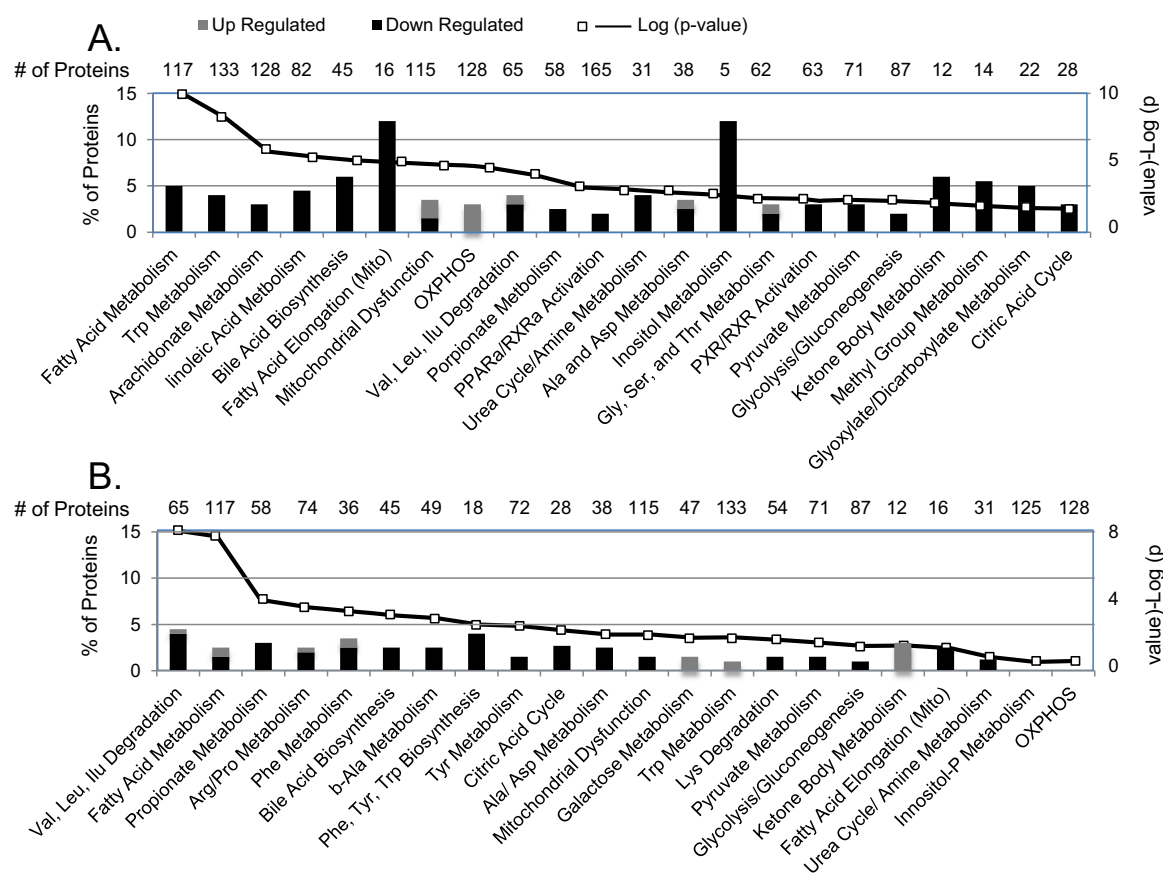


Fig. 2. Canonical pathways associated with altered proteins in VLCAD deficient mice in the fed (A) and fasted states (B). Black indicates that the protein is down-regulated. Gray indicates that the protein is up-regulated. The X-axis denotes the associated functions. The Y-axis represents the percentage of altered proteins among all available molecules in Ingenuity Knowledge database associated with the functional group denoted by X-axis.

Table 1

Altered proteins in paired experiments suggestive of gene-fasting interactions.

Protein/ Enzyme (gene symbol)	Chaperonin 10 (<i>Hspe1</i>)	Cytochrome b-c1 complex subunit 1 (<i>Uqcrc1</i>)	Cytochrome b-c1 complex subunit 2 (<i>Uqcrc2</i>)	3- ketoacyl- CoA thiolase (<i>Acaa1</i>)
*VLCADD (fed)	unchanged	1.37	1.23 ($p=0.027$)	unchanged
vs **WT (fed)				
VLCADD (fasted) vs WT (fasted)	-1.99	No significant change	No significant change	-1.61
VLCADD (fasted) vs VLCADD (fed)	1.38	-1.96	-1.82	1.67
WT (fasted) vs WT (fed)	1.65	No significant change	No significant change	1.48

Numbers indicate fold change, with positive ratio indicating increased fold changes of protein production and negative ratio indicating decreased fold changes of protein production.

p -values shown (Student's t test) in the corresponding experiments.

* VLCADD-very long chain acyl-CoA dehydrogenase deficient mice.

** WT-wild type mice.

VLCADD mice as compared to wild type, in which fasting induced changes. In the fasted state, 3-hydroxy-3-methylglutaryl-CoA synthase 2 (HMGCS2), an enzyme that catalyzes the condensation of acetyl-CoA and acetoacetyl-CoA to form 3-hydroxy-3-methylglutaryl-CoA (HMG-

CoA) was increased in VLCADD mice in the fasted state. However, the pathway “ketone bodies production” was not globally affected as determined by IPA analysis. Proteomic changes suggestive of liver steatosis were seen in fed VLCAD deficient mice but not in fasted animals (Fig. 4A and B).

4. Discussion

The full clinical spectrum of most diseases is determined by the interplay of genetic and environmental factors. In this study, a mouse model of a fatty acid oxidation defect, VLCADD, was used to characterize changes in the liver mitochondrial proteome in mice that are genetically identical except for one mutation and exposure to a specific stressor, fasting, to identify the interaction of genes and environment in shedding molecular insight into clinical symptoms.

The underlying mechanism of clinical and genetic heterogeneity in VLCADD is unknown, and likely includes genetic and environmental factors, as well as their interaction. The novelty of this study lies in the use of animal models to screen for global biological changes related to genotypic and environmental changes and to explore the relevance of alterations in specific functions and pathways using well defined control groups. Multiple proteins were altered in fasted VLCAD deficient mice compared to fasted wild type mice, consistent with an earlier report that VLCAD deficient animals exhibit specific phenotype, pathological and biochemical, under this condition [11]. However, the relative effects of fasting vs VLCADD were not well delineated. The studies revealed that fasting had significantly different effects in VLCAD deficient animals than in wild type ones, and furthermore that gene-environment interactions existed, as indicated by the observations of several proteins and some associated functional groups and

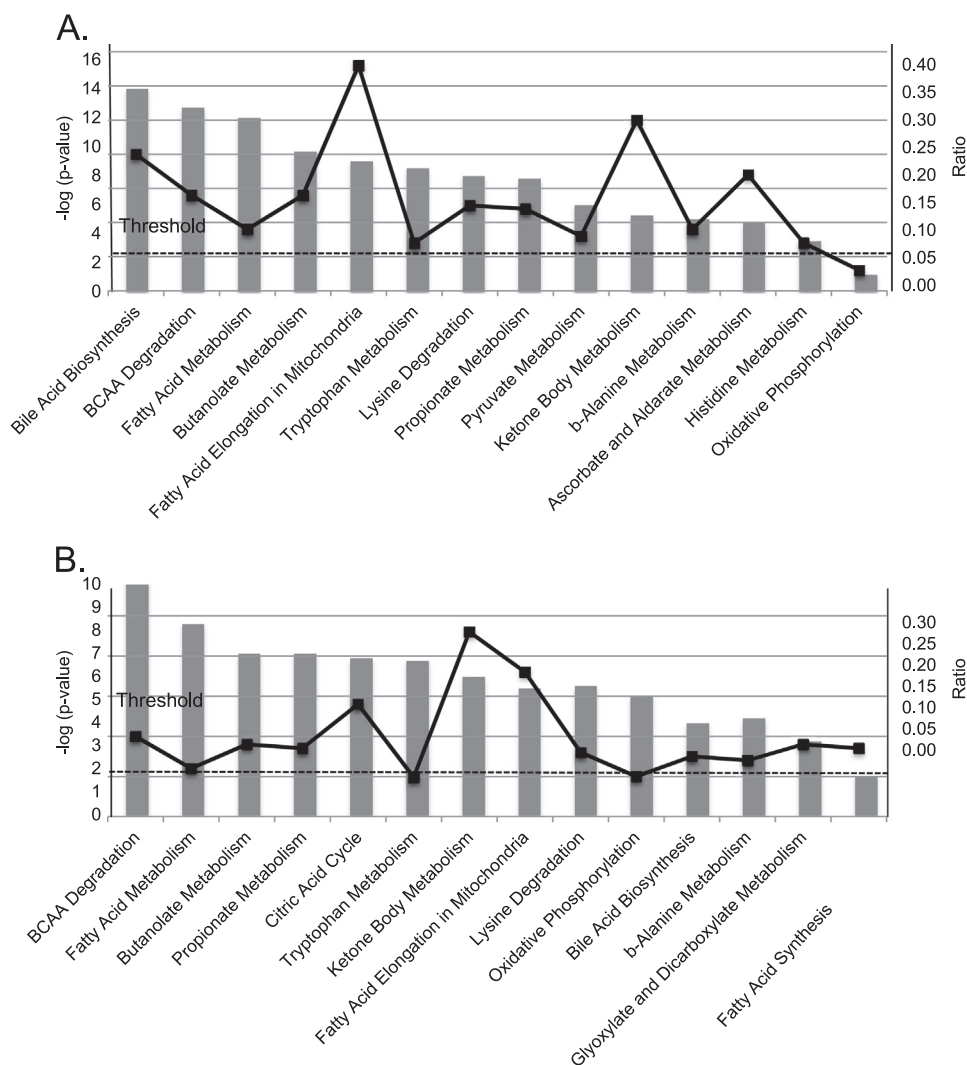


Fig. 3. Canonical pathways associated with fasting in VLCAD deficient mice and wild type mice. A. Wild type and B. VLCAD deficient mice. This bar chart shows the significance of canonical pathways associated with fasting. The left Y-axis displays the significance and corresponds to the height of the bars; the right Y-axis displays the ratio and represents the percentage of altered proteins among all available molecules in Ingenuity Knowledge database associated with the functional group denoted by X-axis.

canonical pathways (Supplemental Table 3).

An apparent gene-environment interaction was demonstrated in the expression of chaperonin HSP10 and Cytochrome b-c1 complex (Supplemental Table 3). Chaperonin 10 (HSPE1), a mitochondrial chaperonin protein, was up-regulated in the fasting state in both VLCAD deficient and wild type animals as compared to the fed state. However, in VLCADD, the down-regulation of chaperonin 10 was seen in fasted VLCAD deficient mice when compared to fasted wild type. Thus, mitochondrial protein folding machinery appears to adjust to environmental conditions. A similar phenomenon was seen in acetyl-CoA acyltransferase 2 (ACAA2; also known as 3-ketoacyl-CoA thiolase.) ACAA2 catalyzes the last step of mitochondrial fatty acid oxidation and valine metabolism, and was up regulated in wild type and mutant mice with fasting. However, it was down-regulated in fasted VLCAD deficient as compared to fasted wild type mice. This suggests that fasting induced compensation in this enzyme that leads to the increased production of acetyl-CoA for citric acid cycle activity under conditions of energy was depletion was abrogated by VLCADD.

Changes in cytochrome b-c1 complex subunits (*QCRr1* and *QCR6* in Supplemental Table 2; *QCR7* in Supplemental Table 3) or the respiratory chain were also induced by fasting in VLCADD. Fasting only impacts cytochrome b-c1 levels in the VLCADD mice. Cytochrome b-c1 complex is the intrinsic membrane protein that catalyzes the oxidation of ubiquinone and the reduction of cytochrome c in

mitochondrial respiratory chains. Fasting only impacts cytochrome b-c1 levels in the VLCAD deficient mice, suggesting a compensatory effect for the primary deficiency induced by fasting. This finding further emphasizes a more pronounced effect on energy metabolism in response to the defect in fatty acid oxidation. It should be noted that additional changes could still be present in the fasted, VLCAD deficient animals due to the difficulty in detecting proteins with low levels of expression with iTRAQ.

In total, our findings indicate that an *ACADVL* gene defect leads not only to alterations in the proteome compared to wild type animals, but also how that proteome changes in response to environmental factors (Fig. 5). Specifically, VLCADD mice respond differently to fasting than do wild type animals. Canonical pathway analysis identified significant changes in fasting animals of both genotypes. In contrast, fewer differences were seen between the changes in deficient mice and in wild type mice, indicating that the changes in these pathways were related to fasting but not genotype. The results from this proteomic study confirm that the normal ketogenic response to fasting remains intact in the face of VLCADD. Although expression of some individual proteins changed, association analysis showed that in total, the pathway of ketone bodies production was not significantly up regulated in fasting deficient animals as it was in wild type animals. The pathways of amino acid metabolism, especially branched chain amino acid metabolism, as well as carbohydrate and fatty acid metabolism were more

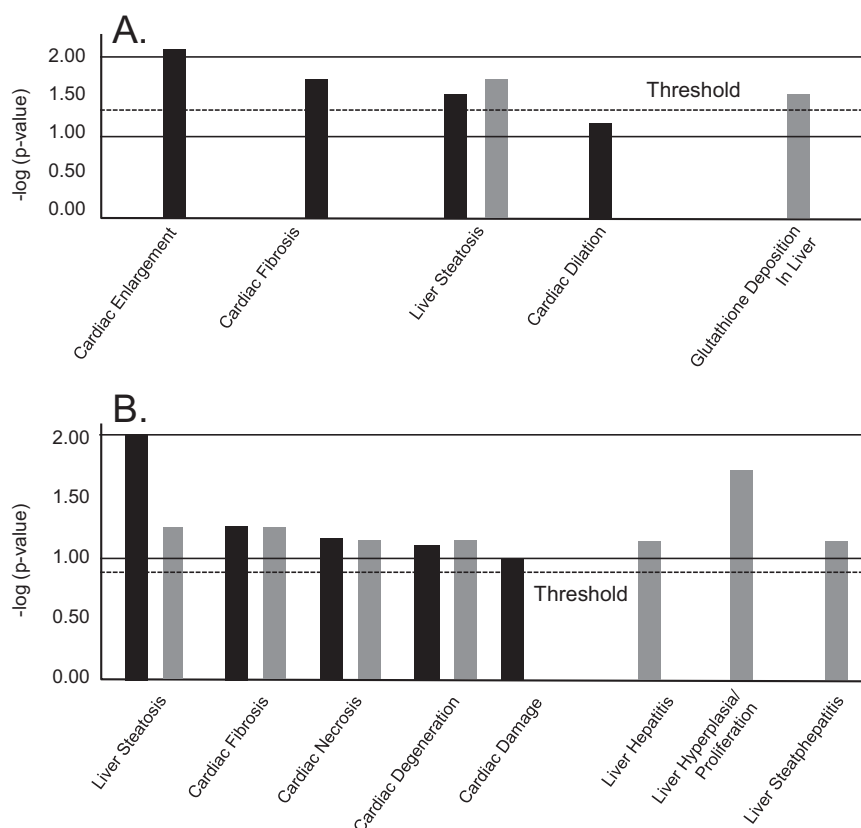


Fig. 4. A. Prediction of pathological changes related to VLCAD deficiency in fasted and fed states in mice. Black denotes toxicity predicted by changed proteins in fasted VLCAD deficient mice compared to fasted wild type mice. Gray shows toxicity predicted by changed proteins in fed VLCAD deficient mice compared to fasted wild type mice. Y-axis: significance of toxicity arising from the changed proteins ($p \leq 0.05$). B. Prediction of pathological changes related to fasting in both VLCAD deficient and wild type mice. Gray shows toxicity predicted by changed proteins in VLCAD deficient mice due to fasting. Black denotes toxicity predicted by changed proteins in wild type mice because of fasting.

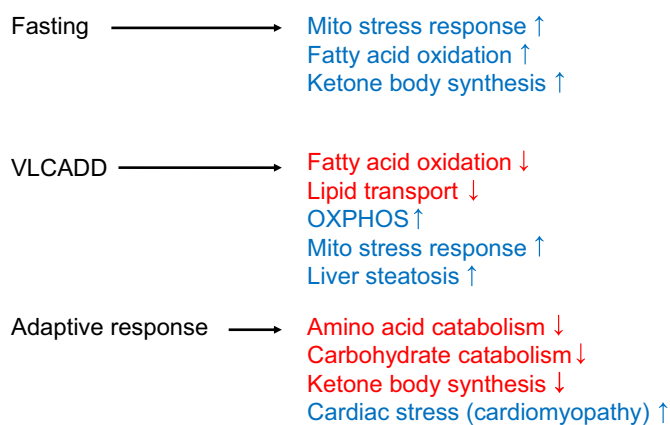


Fig. 5. Summary of the functional effects of fasting and VLCAD deficiency in mice. Up-regulated functions are depicted in red, and down-regulated functions are in blue. Fasting induced a shared upregulation of the mitochondrial stress response in wild type and deficient animals. Compensatory changes in fatty acid oxidation in wild type animals was not seen in deficient animals, which instead showed increased oxidative phosphorylation (OXPHOS) and a pattern of protein changes consistent with liver steatosis. The adaptive response of deficient animals was different compared to wild type with decreases in a variety of intermediary metabolic pathways and a pattern consistent with cardiac stress and cardiomyopathy.

significantly altered in the fasted state than in the fed state in VLCAD deficient mice suggesting a more global derangement in cellular metabolism in fasting mutant animals. These findings are consistent with recent metabolic studies on VLCADD animals [16–21]. The greatest differences were in the OXPHOS and citric acid cycle pathway and bile acid synthesis. An increased pattern in OXPHOS identified in fed VLCAD deficient mice disappeared when they were fasted. The

OXPHOS pathway was unchanged when wild type mice were fasted. However, fasting induced the down-regulation of several proteins in OXPHOS in mutant animals. Thus, a presumed compensatory change in the mutant animals appears to be negated during fasting, and may play a role in the development of symptoms.

Proteins in the most significantly altered constructed network in fed and fasted VLCAD deficient mice were linked by several well described transcription factors inferred from the IPA knowledge database. They included nuclear receptors PPARA (peroxisomal proliferator-activated receptor A), PPARG, PPARGC1A, and NR3C1 (nuclear receptor subfamily 3, group C). These nuclear receptors in the molecular play a more general role in regulating energy metabolism and their upregulation suggests a compensatory response to a secondary energy deficiency in VLCADD. A similar relationship has been demonstrated in one previous study that showed that expression of the *PPARA* and *PPARG* genes were increased in VLCAD deficient mice in the fasted state, but was unchanged or slightly reduced in the fed state (30). 3-Hydroxy-3-methylglutaryl-CoA reductase (HMGCR) is the rate-limiting enzyme for cholesterol synthesis and is regulated via a negative feedback mechanism mediated by sterols and non-sterol metabolites (31). Leptin (*LEP*) is secreted by white adipocytes and plays a major role in the regulation of body weight, but has been reported not to affect fatty acid oxidation (32). Both molecules were inferred on the basis of relationships with other molecules incorporated in the IPA knowledge database to participate in regulating changes in the function of lipid metabolism in VLCADD mice. Further studies are needed to demonstrate their roles in determining clinical symptoms in FAODs.

The clinical relevance to secondary changes associated with a *Acadl* gene defect was further evaluated through the toxicity analysis function in the IPA software to provide additional insight into the pathologic alterations induced by *Acadl* gene defect and fasting. Mice

deficient for VLCAD develop hepatic steatosis upon fasting (16). They also accumulate microvesicular lipids and demonstrate marked mitochondrial proliferation in heart (17). Both phenotypes are seen in the human deficiency to some extent (7). Our toxicity analysis using the IPA software indeed identified a pattern of protein changes predictive of liver damage (steatosis) in VLCAD deficient mice under both feeding states. While, the liver steatosis through the prediction of the pathological changes of secondary proteomic changes was suggestive in VLCAD deficient mice and wild type mice due to fasting. Further analysis of fasting effects alone on deficient mice and wild type mice indicates that the liver steatosis was slightly more exaggerated in wild type mice (Supplemental Tables 1 and 2). However, fasting is also associated with liver hyperplasia/hypersteatohepatitis in VLCAD deficient mice suggesting that the occurrence of liver steatosis in VLCAD may not be solely due to fasting but as a result of the interaction of *Acadvl* gene defect and fasting. This pattern of protein changes suggesting liver dysfunction implies that avoidance of fasting may not alter the liver dysfunction in those patients with VLCAD. The correction of liver dysfunction may be achieved only or at least by treatment of the gene deficiency. The physiologic implication of *ETHE1* (ethylmalonic encephalopathy) gene expression is unclear; however, it has been reported to be altered in response to metabolic state in obese rats (25).

In conclusion, some of the changes of fasting in VLCAD reflect known physiologic and pathologic alterations previously characterized in VLCAD deficiency and fasting. Others provide novel insights into possible new secondary effects of the defect and mechanisms for fasting-gene defect interaction in this disease. The results identify an aberrant fasting response in VLCAD deficient mice and the secondarily altered proteins in these mice define interactions of both genes and specific environmental interactions with the *Acadvl* gene.

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Appendix A. Supplementary material

Supplementary material associated with this article can be found in the online version at <http://dx.doi.org/10.1016/j.bbrep.2016.08.014>.

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