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# ENERGY METABOLISM AND CLINICAL SYMPTOMS IN BETA-OXIDATION DEFECTS, ESPECIALLY LONG-CHAIN 3-HYDROXYACYL-COENZYME A DEHYDROGENASE DEFICIENCY

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Glencoe, 2016

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Published by Karolinska Institutet.
Printed by Eprint AB 2016

ISBN 978-91-7676-227-1

# Energy Metabolism and Clinical Symptoms in Beta-oxidation Defects, Especially Long-Chain 3-Hydroxyacyl-Coenzyme A Dehydrogenase Deficiency

THESIS FOR DOCTORAL DEGREE (Ph.D.)

By


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*To Magnus, Ella and Carl*

*As our circle of knowledge expands, so does the circumference of darkness surrounding it.*

*— Albert Einstein*

# ABSTRACT

Long-chain 3-hydroxyacyl-CoA dehydrogenase deficiency (LCHAD) is a severe inborn error in the beta-oxidation of long-chain fatty acids. The disease presents during the first years of life. Hypoglycemia, hepatic manifestations, muscle hypotonia and episodes of rhabdomyolysis, cardiomyopathy and even sudden death are common symptoms. Despite life-long complicated treatment with a low fat diet and fasting avoidance, episodes of rhabdomyolysis and liver abnormalities may still occur. Patients with LCHAD develop chorioretinopathy, not seen in any other beta-oxidation deficiencies.

The aim of this thesis was to describe the clinical outcome for patients with LCHAD, and investigate the energy metabolism with particular emphasis on the dynamics of fasting. Ten patients were included in the studies.

The patients had rapid weight gain after diagnosis and initiation of dietary treatment. The nutritional surplus caused overweight and accelerated linear growth in the majority of the children, however not affecting final height.

Patients with LCHAD had a decreased fasting tolerance with increased lipolysis. Fat and carbohydrate metabolism during fasting was investigated by stable isotope technique, microdialysis, and biochemical measurements. Despite normal blood glucose and normal glucose production rate (19.6 ± 3.4 umol/kg/min), lipolysis was induced after 3–4 hours, shown by increased glycerol production rate (7.7 ± 1.6 umol/kg/min). Fatty acid intermediates, plasma and microdialysate glycerol levels were increased. Indirect calorimetry showed increased respiratory quotient, indicating mainly glucose oxidation. Our results imply that frequent meals are essential in order to avoid lipolysis and diminish accumulation of the incompletely degraded toxic fatty acid metabolites.

All patients developed ocular changes with retinal pigmentations and chorioretinopathy. Early diagnosis and treatment may delay but not prevent the ocular outcome.

Neuropsychological deficits were more common than expected, and demonstrated a specific cognitive pattern. The patients either had normal IQ scores with a particular weakness in auditive verbal memory and executive functions, or developmental delay and autistic behaviors.

In conclusion, this thesis shows that patients with LCHAD have an increased lipolysis with considerably impaired fasting tolerance. Shorter fasting intervals than has been advocated are thus crucial to reduce the accumulation of fatty acid metabolites and improve the metabolic control. The shorter fasting tolerance should be weighed against the increased the risk for overweight. All patients develop retinal and cognitive symptoms; however, these symptoms may be improved with good adherence to the complicated diet. Neuropsychological screening is important for the identification of special needs early on.

# LIST OF SCIENTIFIC PAPERS

I. **Haglind CB**, Stenlid MH, Ask S, Alm J, Nemeth A, Döbeln U, Nordenström A. Growth in Long-Chain 3-Hydroxyacyl-CoA Dehydrogenase Deficiency. JIMD Rep. 2013;8:81-90.

II. **Haglind CB**, Nordenström A, Ask S, von Döbeln U, Gustafsson J, Stenlid MH. Increased and early lipolysis in children with long-chain 3-hydroxyacyl-CoA dehydrogenase (LCHAD) deficiency during fast. J Inherit Metab Dis. 2015 Mar;38(2):315-22. Erratum in: J Inherit Metab Dis. 2015 Mar;38(2):377.

III. Fahnehjelm KT, Holmström G, Ying L, **Haglind CB**, Nordenström A, Halldin M, Alm J, Nemeth A, von Döbeln U. Ocular characteristics in 10 children with long-chain 3-hydroxyacyl-CoA dehydrogenase deficiency: a cross-sectional study with long-term follow-up. Acta Ophthalmol. 2008 May;86(3):329-37.

IV. Strandqvist A, **Haglind CB**, Zetterström RH, Nemeth A, von Döbeln U, Stenlid MH, Nordenström A. Neuropsychological Development in Patients with Long-Chain 3-Hydroxyacyl-CoA Dehydrogenase (LCHAD) Deficiency. JIMD Rep. 2015 Nov 7.

# CONTENTS

# LIST OF ABBREVIATIONS

| | |
|---|---|
| ABAS | Adaptive Behavior Assessment System® |
| ACAD | Acyl-CoA dehydrogenases |
| AFLP | Acute fatty liver of pregnancy |
| ASD | Autism Spectrum Disorder |
| ATP | Adenosine triphosphate |
| BMI | Body mass index |
| BRIEF | Behavior Rating Inventory of Executive Function® |
| CACT | Carnitine acylcarnitine translocase |
| CK | Creatine kinase |
| CPT 1 | Carnitine palmitoyl transferase 1 |
| CPT II | Carnitine palmitoyl transferase 2 |
| DHA | Docosahexaenoic acid |
| ERG | Electroretinography |
| $FADH_2$ | The Reduced form of flavin adenine dinucleotide. |
| FAO | Fatty Acid Oxidation |
| FAOD | Fatty acid oxidation defect |
| FH | Final Height |
| GAC | General adaptive composite |
| GC-MS | Gas Chromatography Mass Spectrometry |
| GEF | Global executive function |
| GH | Growth hormone |
| GW | Gestational week |
| HELLP | Hemolysis elevated liver enzymes and low platelets |
| IGF I | Insulin growth factor I |
| IQ | Intelligence Quotient |
| LCAD | Long-chain acyl-CoA dehydrogenase |
| LCEH | Long-chain 2, 3-enoyl-CoA hydratase |
| LCHAD | Long-chain 3-hydroxyacyl-CoA dehydrogenase deficiency |
| LCT | Long-chain triglyceride |

| | |
|---|---|
| LKAT | Long-chain 3-ketoacyl-CoA thiolase |
| LCFA | Long-chain fatty acid |
| MCAD | Medium-chain acyl-CoA dehydrogenase deficiency |
| MCT | Medium-chain triglyceride |
| MKAT | Medium-chain 3-ketoacyl-CoA thiolase |
| MS/MS | Tandem mass spectrometry |
| NADH | Reduced form of nicotinamide adenine dinucleotide |
| NEFA | Non-esterified fatty acids |
| OCT | Optical coherence tomography |
| PPAR | Peroxisome proliferator activated receptor |
| REE | Resting energy expenditure |
| RPE | Retinal pigment epithelium |
| RQ | Respiratory Quotient |
| SCAD | Short-chain acyl-CoA dehydrogenase |
| SCEH | Short-chain 2,3-enoyl-CoA hydratase |
| SCHAD | Short-chain 3-hydroxyacyl-CoA dehydrogenase |
| SD | Standard deviation |
| SDS | Standar deviation score |
| SKAT | Short-chain 3-ketoacyl-CoA thiolase |
| TAG | Triacylglycerides |
| TFP | Mitochondrial trifunctional protein |
| TH | Target Height |
| VLCAD | Very long-chain acyl-CoA dehydrogenase deficiency |

# 1 INTRODUCTION

## 1.1 ENERGY METABOLISM

Glucose homeostasis is maintained and energy provided to all tissues by energy rich molecules that are either ingested through our diet or processed from the body's own supplies. They are broken down into amino acids, fatty acids and glucose which are further degraded and converted into energy. Fatty acids are the body's main energy reserves, and are stored as triacylglycerides (TAGs) in the adipose tissue.

The organs in the body have different substrate preferences. Glucose is the dominant fuel for the brain. Ketone bodies are important energy substrates that the brain and skeletal muscle can use as alternative energy fuel. In skeletal muscles, there is a different situation, since the utilization of glucose increases as exercise intensity increases and fat oxidation reaches its maximum at moderate and prolonged exercise intensities (1, 2). The healthy heart mostly utilizes fatty acids for energy supply at all times (3). After a carbohydrate-rich meal, glucose levels are high and excess glucose is utilized for fatty acid-, glycogen- and amino acid-synthesis. However, the glycogen stores in the muscles can only be used by the muscle itself. If no more exogenous glucose is delivered, levels decline and the glycogen stores in the liver and skeletal muscles serve as glucose-providers. The glycogen reserves may last for up to 24 hours in adults, but only for a few hours in children who, consequently, are more prone to develop hypoglycemia. Fatty acids are stored as triacylglycerols (TAGs) in the adipose tissue. Fasting induces breakdown of the TAGs, thereby supplying energy substrates to heart and skeletal muscle and energy for hepatic gluconeogenesis and ketone body synthesis. The different metabolic pathways are tightly regulated by hormonal signals. A high insulin/glucagon ratio stimulates anabolic pathways, while a decreased energy supply results in a lowered insulin/glucagon ratio, changing the metabolic reactions to catabolism.

## 1.2 FATTY ACIDS

Fatty acids have many central functions in the body, particularly for energy production but also as structural phospholipids and glycolipids of cell membranes, with involvement in cell signaling, inflammatory responses and gene expression (4). They represent the major bodily energy reserve, since complete fatty acid oxidation yields 9 kcal/g of fat (37 kJ/g), compared to 4 kcal/g of carbohydrates (17 kJ/g), and 4 kcal/g of protein (917kJ/g).

In plasma, fatty acids exist as free, non-esterified fatty acids, NEFAs, bound to albumin, and are also found as fatty acyl esters in triacylglycerols (TAGs), transported by plasma lipoproteins.

A fatty acid consists of a chain of carbon atoms with maximum number of hydrogen atoms attached to the carbon (Figure 1). Some of the most physiological fatty acids are long-chain fatty acids with an even number of carbons such as, palmitate (C16:0), stearate (C18:0), oleate (C18:1), and linolate (C18:2). Saturated fatty acids do not contain any double bonds, while mono- or poly- unsaturated fatty acids contain one or more double bonds. The structure

is described in terms of the number of carbons and double bonds, e.g. palmitate is 16 carbons long and has no double bonds. The carbons may also be counted from the other end, and are then referred to as ω- (omega-) fatty acids. Another nomenclature for linolate (C18:2) is ω- 3 fatty acid, since the first double bond is 3 carbons from the ω-carbon. In general long-chain polyunsaturated ω- 3 fatty acids are associated with positive health effects, while saturated and trans-fatty acids are associated with cardiovascular disease (4). To optimize biological function and health effects, the balance between ω- 3 and ω- 6 fatty acids is crucial (5). All polyunsaturated fatty acids derived from linolenic acid (C18:3) are ω- 3 fatty acids, and those derived from linoleic acid (C18:2) are ω- 6 types. Both linoleic and linolenic types are essential fatty acids and cannot be synthesized endogenously, but need to be provided by the diet. Linoleic acid is the precursor for prostaglandin synthesis, while linolenic acid is the precursor for docosahexaenoic acid (DHA), synthesized in the peroxisomes (6). DHA is important for brain development and visual acuity (7), and is rather abundant in Western diets.

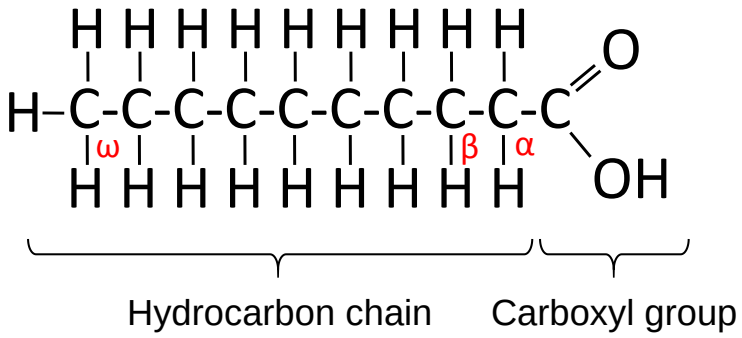



**Figure 1. Schematic illustration of a fatty acid**

Fatty acids are constructed of long chains of carbon atoms, and are named in different ways. The carboxyl-reference system counts the carboxyl carbon as number 1, while the omega-reference system counts from the omega “ω” -carbon, which is always the last carbon regardless of chain length. The carbon next to the carboxyl group is labelled the alfa “α”-carbon (carbon number 2), and the carbon next to the α is the beta ”β”-carbon (carbon number 3).

## 1.3 INTRODUCTION TO FATTY ACID OXIDATION DEFECTS

Fatty acid oxidation defects (FAODs) are a group of inborn diseases affecting the final degradation of fatty acids within the cells. Very long-chain fatty acids (> C20) and branched-chain fatty acids are oxidized by the peroxisomes, while long-chain fatty acids (< C20) are transported into the mitochondria via a carnitine-dependent pathway. Medium- and short-chain fatty acids do not require carnitine to enter the mitochondria. Chain-length specific enzymes facilitate sequential removal of two-carbon fragments by the beta-oxidation process, thus yielding acetyl-CoA, NADH, and $FADH_2$. The first cases of defective fatty acid oxidation (FAO) were reported in the 1970s (8, 9), and, to date, more than 20 different deficiencies have been described. The defects have overlapping symptoms, often occurring in connection with fasting, and may involve hypoglycemia without ketone body production,

muscle hypotonia, rhabdomyolysis, cardiomyopathy and liver involvement. The clinical range is wide, varying from asymptomatic patients to severe symptoms with arrhythmias, lactic acidosis, seizures and sudden death (10-12). Many countries have included FAODs in their newborn screening programs, thereby enabling early diagnosis and treatment. Fatty acid oxidation disorders (FAOD), are included in the Swedish Newborn Screening Program since 2010. One of the most severe disorders is long-chain 3-hydroxyacyl-CoA dehydrogenase deficiency (LCHAD) (OMIM 609016), which impairs the breakdown of long-chain fatty acids. Despite treatment, patients with LCHAD develop a specific chorioretinopathy not seen in other fatty oxidation disorders. From a clinical perspective, treatment and management of patients with LCHAD is challenging, since symptoms and complications arise regardless of the complicated dietary treatment. Thus, the focus of this thesis has been on studying the metabolism in patients with LCHAD and the related complications, with the foremost intention to improve the care and treatment of these patients.

## 1.4 FATTY ACID OXIDATION

### 1.4.1 Fatty acid mobilization

The final degradation of fatty acids up to 20 carbon chain-lengths (C20) predominantly occurs in the mitochondrial-matrix by beta-oxidation, while very long-chain fatty acids (> C20) have to be chain shortened in the peroxisomes before they can be completely oxidized to acetylCoA in the mitochondria (13). Fasting and exercise release adrenaline/epinephrine and noradrenaline/norepinephrine, which induce lipolysis by the action of hormone-sensitive lipase. Hormone-sensitive lipase in the adipose tissues facilitates the hydrolysis of TAGs, producing one molecule of glycerol and three molecules of fatty acids (14). The free fatty acids are released into the circulation and transported to the target cell, while the glycerol backbone is either recycled in the liver for TAG synthesis or used as a substrate for gluconeogenesis (15). The most potent inhibitor of lipolysis is insulin, which binds to the surface of the adipocytes and inactivates hormone-sensitive lipase (16).

### 1.4.2 Fatty acid transportation into the cells

Several different membrane-bound proteins mediate the transportation of long-chain fatty acids (LCFAs) across the plasma membrane into the cytoplasm. These proteins include fatty acid translocase (FAT)/CD36, plasma membrane fatty acid binding protein (FABPpm), caveolins, and fatty acid transport proteins (FATP) (17, 18). The FATPs are encoded by the SLC27 gene and are crucial for maintaining lipid homeostasis. In humans, six FATP have been identified, with different chain length-specificity and different tissue distribution (17). The fatty acids are activated to acyl-CoA-esters in the cytosol by a family of ATP-dependent acyl-CoA synthases (ACSs) (19, 20).

### 1.4.3 Fatty acid transport into the mitochondria

The mitochondrial membrane is impermeable to long-chain acyl-CoAs. The carnitine palmitoyl transferase system is essential for LCFA passage into the mitochondria, although

fatty acids shorter than 12 carbon atoms enter the mitochondrial matrix without the carnitine shuttle (21) (Figure 2). Carnitine levels are maintained by dietary intake of dairy and meat products, as well as by endogenous synthesis from lysine and methionine, or by renal absorption (18, 22). Its cellular uptake is by the OCTN2 carnitine transporter which is essential for sufficient carnitine supply (18).

Carnitine palmitoyl transferase 1 (CPT-1), on the outer mitochondrial membrane transfers fatty acids from Coenzyme-A to carnitine to form acyl-carnitine. Acyl-carnitine is translocated across the inner mitochondrial membrane by carnitine acylcarnitine translocase (CACT) in exchange for free carnitine. CPT-2 on the inside of the inner membrane removes carnitine and the long-chain fatty acid acyl-CoA can undergo beta-oxidation (18, 21, 23). CPT-1 is present in different isoforms CPT-1A (liver CPT-1), and CPT-1B (muscle CPT-1) (24). In addition, CPT-1 is the rate-limiting step of fat oxidation. Postprandially, levels of ATP and citrate rise, and thereby stimulate the synthesis of malonyl-CoA, which binds to the inner mitochondrial membrane and prevents entry of the fatty acid into the mitochondria. The fatty acid is thus directed toward lipogenesis instead of oxidation (3, 25).

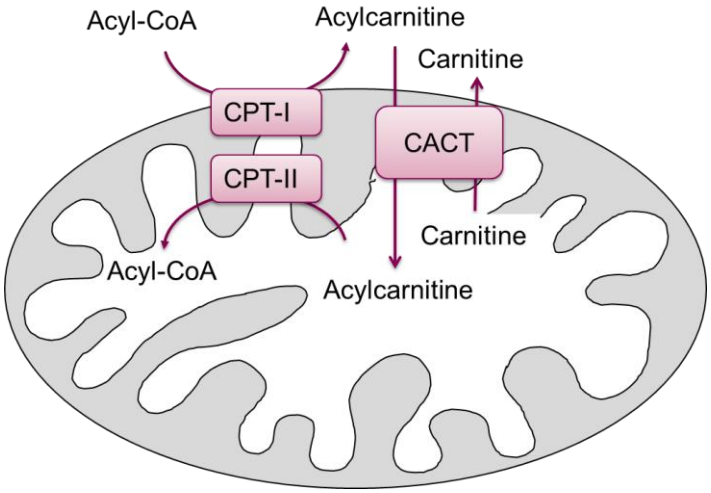



**Figure 2. The Carnitine shuttle**

CPT-1 on the outer mitochondrial membrane forms fatty acid acyl-carnitine which is translocated across the inner mitochondrial membrane by carnitine acylcarnitine translocase (CACT) in exchange for free carnitine. CPT-2 on the inside of the inner membrane removes carnitine and re-esterifies the acylcarnitine into a long-chain fatty acyl-CoA that undergoes beta-oxidation (18, 21, 23).

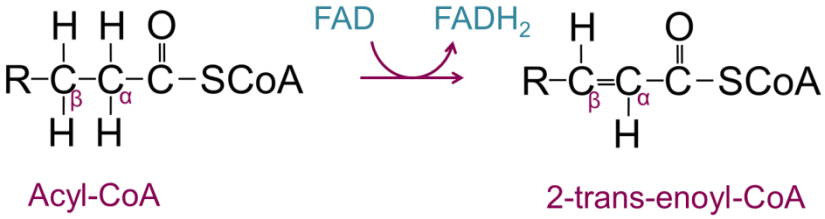
### 1.4.4 Mitochondrial beta-oxidation of even-numbered unsaturated fatty acids

Once inside the mitochondria the long-chain fatty acyl-CoA undergoes beta-oxidation, a series of four reactions affecting the third carbon of the fatty acid, the β-carbon. Beta-oxidation results in a fatty acid shortened by two carbons, and the production of acetyl-CoA, NADH, and $FADH_2$. The initial rounds of beta-oxidation are catalyzed by enzymes bound to the mitochondrial membrane. As the fatty acid becomes shorter and more hydrophilic, further degradation takes place in the mitochondrial matrix by different enzymes. The beta-oxidation process is also described as a spiral since the shortened fatty acyl-CoA cycles through beta-oxidation until all acetyl groups are cleaved off. The faith of acetyl-CoA is further oxidation in the citric acid cycle to $CO_2$ and water, or ketone body synthesis in the liver (21, 23, 24, 26).

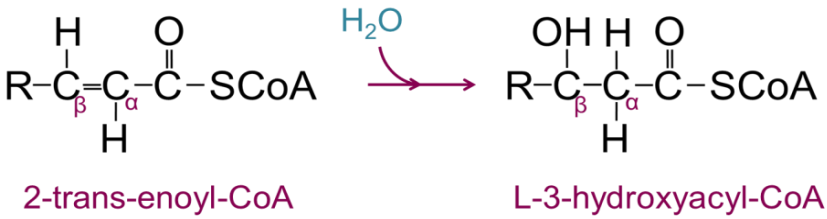
The beta-oxidation enzymes are chain-length specific, with overlapping activities (Figure 3). The first step in the spiral is catalyzed by a family of acyl-CoA dehydrogenases (ACADs): very long-chain acyl-CoA dehydrogenase (VLCAD) with specificity for fatty acids C14–C18, long-chain acyl-CoA dehydrogenase (LCAD) C10–C12, medium-chain acyl-CoA dehydrogenase (MCAD) C6–C12, and short-chain acyl-CoA dehydrogenase (SCAD) C4–C6. VLCAD is membrane-bound, while LCAD, MCAD and SCAD are located in the mitochondrial matrix (21, 23, 26). The role of LCAD in humans is uncertain; however, it is important in mouse models (Section 1.5.9) (27). ACAD enzymes produce $FADH_2$ thereby transferring electrons to the electron transport chain. The subsequent three beta-oxidation reactions are catalyzed by the membrane-bound mitochondrial trifunctional protein enzyme complex (TFP), with activity toward longer chain substrates. Substrates with shorter chain lengths are oxidized by homologous enzymes in the matrix. The TFP is a hetero-octamer with four α- and four β-subunits, encoded by the *HADHA* and *HADHB* genes (28-31). The α-subunits contain the hydratase and long-chain hydroxyacyl- CoA dehydrogenase activities, while the β-subunits comprise the thiolase activity. The second beta-oxidation step is catalyzed by long-chain 2, 3-enoyl-CoA hydratase (LCEH) in the TFP and short-chain 2,3-enoyl-CoA hydratase (SCEH or crotonase) for shorter substrates in the matrix. The third step, yielding $NADH_2$, is mediated by long-chain 3-hydroxyacyl-CoA dehydrogenase (LCHAD) in the TFP with activity toward C12–C16, or short-chain 3-hydroxyacyl-CoA dehydrogenase (SCHAD) in the matrix with an optimal activity toward C6. The fourth step is catalyzed by long-chain 3-ketoacyl-CoA thiolase (LKAT) in the TFP. The matrix enzymes are medium (MKAT), and short-chain 3-ketoacyl-CoA thiolase (SKAT), and the SKAT reaction remains the final step in mitochondrial beta-oxidation (21, 26, 32).

**Figure3. Mitochondrial beta-oxidation reactions**

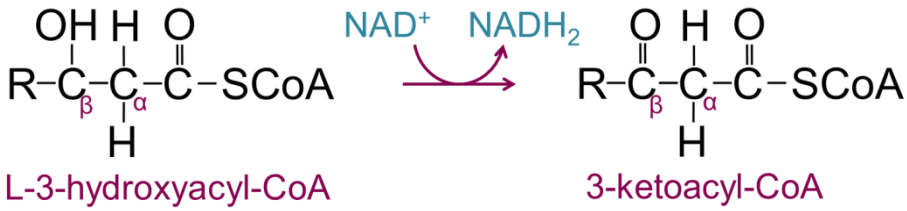
Mitochondrial beta-oxidation involves four different reactions affecting the β-carbon.



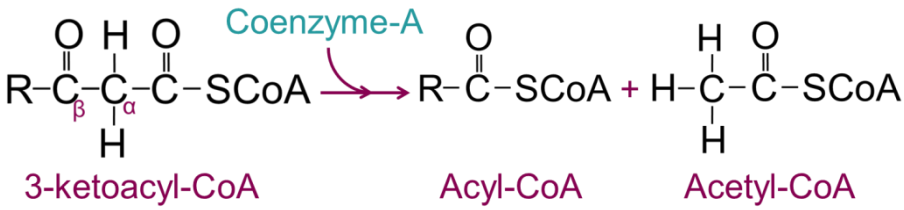

1. The first step of beta-oxidation is an oxidation reaction. A family of acyl-CoA dehydrogenases mediate the reaction, creating a trans-double bond between the α- and β-carbons, forming 2-trans-enoyl-CoA and $FADH_2$.




2. The second reaction is hydration of the 2-trans-enoyl-CoA by 2-enoyl-CoA hydratase, forming L-3-hydroxyacyl-CoA. The double bond between the α- and β-carbon is converted to a hydroxyl group on the β-carbon.




**3.** L-3-hydroxyacyl CoA dehydrogenase catalyzes the oxidation reaction from L-3-hydroxyacyl-CoA, producing 3-ketoacyl-CoA and $NADH_2$. The hydroxyl group on the β carbon is converted to a keto group.




4. The fourth and final reaction of beta-oxidation is a thiolytic cleavage of the bond between the α- and β-carbons. The reaction is facilitated by 3-ketoacyl-CoA thiolase in the presence of coenzyme-A and results in a fatty acyl-CoA shortened by two carbons and acetyl-CoA.

### 1.4.5 Oxidation of mono- or poly-unsaturated fatty acids

Oleic acid (18:1), an unsaturated fatty acid with one double bond, is oxidized by the same set of reactions as saturated even-numbered fatty acids, until the double bond is reached. The enzyme Cis-$\Delta^3$ enoyl-CoA-isomerase converts the double bond in the cis-configuration into a trans-configuration. The compound bypasses the hydratase reaction and reenters β-oxidation. Polyunsaturated fatty acids, linoleic acid (18:2) and linolenic acid (C18:3), requires an additional NADPH dependent enzyme, 2, 4 dienoyl-CoA reductase, for further oxidation(23, 26).

### 1.4.6 Odd chain fatty acid oxidation

Fatty acids with odd numbers of carbons are oxidized in a similar manner similar to that of even- numbered ones, until three carbons remain. This molecule, propionyl-CoA, is further degraded by the ATP dependent enzyme propionyl CoA carboxylase (using biotin co-factor), methylmalonyl-CoA racemase and methylmalonyl-CoA mutase (using vitamin B12 co-factor) to form succinyl-CoA which enters the citric acid cycle (23, 26).

### 1.4.7 Additional fatty acid oxidation pathways

In addition to the mitochondrial β-oxidation, fatty acids are degraded by pathways that may be complementary when mitochondrial β-oxidation is overloaded or impaired (33, 34).

*Peroxisomal β-oxidation* is of minor importance for energy generation, and is directed toward very long-chain fatty acids, particularly C24:0, 26:0 (6, 13) and pristanic acid (6, 35). The enzymatic steps, also involving oxidation, hydration, oxidation and thiolytic cleavage, are equivalent to mitochondrial β-oxidation; however, the first reaction is catalyzed by acyl-CoA oxidase (and not by ACADs) (13), which transfers electrons via $FADH_2$ to create $H_2O_2$. The peroxisomal pathway is also different from the mitochondrial one, since the trans-membrane import does not require carnitine. The oxidation proceeds to the medium chain level, when the fatty acyl-CoA is transported to the mitochondria for further oxidation.

*Omega oxidation* for medium chain fatty acids occurs in the endoplasmic reticulum. The oxidation does not involve the β-carbon, but it begins from the ω-carbon, i.e., on the opposite end of the carbon chain, and yields dicarboxylic acids which are fatty acids with a carboxyl group at each end. The dicarboxylic acids can be transported to the mitochondrial matrix or to peroxisomes for β-oxidation. When β-oxidation is compromised, high levels of dicarboxylic acids are excreted in urine as markers of fatty acid oxidation defects (18, 36).

### 1.4.8 Ketone body production

The ketone bodies acetoacetate and 3-hydroxybutyrate, are formed in the liver from acetyl-CoA during fasting, by HMG CoA synthase and HMG CoA lyase. The ketone bodies provide supplementary energy for the brain and cardiac muscle.

## 1.5 LONG-CHAIN 3-HYDROXYACYL-COA DEHYDROGENASE DEFICIENCY (LCHAD)

### 1.5.1 Symptoms and prevalence

LCHAD deficiency was first described in the 1980s (36-38) in patients presenting with liver involvement, and concurrent hypoglycemia, free carnitine deficiency and long-chain acylcarnitine excess. Today, most patients with FAO defects are identified through newborn screening programs before any symptoms occur. However, not all countries perform newborn screening and mortality and morbidity rates are still high (10, 39, 40).

The symptoms of LCHAD are usually initiated by fasting, infection or other catabolic situations when the energy demand is high, and affect organs with a high energy demand or turnover such as the liver, heart and skeletal muscles. The presentation may involve acute symptoms with hypoketotic hypoglycemia, hepatic failure, hypertrophic cardiomyopathy, coma, cardiac arrest, and sudden death. Chronic symptoms such as muscle hypotonia, failure to thrive, and episodes of rhabdomyolysis, are also common presentations at diagnosis (9-11) (12-14). Despite treatment, symptoms may still occur as recurrent episodes of rhabdomyolysis or development of cardiomyopathy and liver affection. A common complication in LCHAD is rhabdomyolysis in conjunction with exercise. The etiology is unknown, but it may include an insufficient energy supply to the muscles or toxic effects of acylcarnitines or free fatty acids which cause membrane destabilization and leakage of intracellular creatine kinase and transaminases(41, 42). Peripheral neuropathy and chorioretinopathy are specific features of LCHAD, not present in other FAO defects. In addition, mothers carrying a fetus with LCHAD are at risk for severe pregnancy complications such as preeclampsia, HELLP syndrome (hemolysis, elevated liver enzymes, low platelets) and acute fatty liver of pregnancy (AFLP) (Section 1.5.4.1).

### 1.5.2 Genetics

Patients with mutations in one of the enzymes active in the TFP, are diagnosed with either LCHAD or TFP deficiency, and may exhibit slightly different phenotypes (21, 43). However, the disorders cannot easily be distinguished clinically or with acylcarnitine profiles and are managed in similar manners (44).

LCHAD is inherited in an autosomal recessive pattern. Patients who are homozygous or compound heterozygous for the common G1528C mutation, are diagnosed with LCHAD and trifunctional protein deficiency (TFPD) if any other α- or β-mutations are found (21) (43, 45). The α and β subunits are encoded by separate nuclear genes, *HADHA* and *HADHB* (30), located within the same chromosomal region, 2p23 (46). The G1528C mutation in the α-subunit of the TFP (47, 48) is the most common mutation in isolated LCHAD and is present in 60% to 87% of alleles in Caucasian patients with LCHAD (47, 49). The carrier frequency is 1:680 (Dutch population) (50). The mutation severely reduces the LCHAD activity by altering glutamic acid into glutamine acid at position 474 (E474Q) (51, 52), which inactivates the catalytic domain, but preserves the other TFP activities at >60% of normal activity (32)

(53). Hence, general TFP deficiency is characterized by reduced enzyme activities of all three TFP enzymes (31, 43). The frequency of the combined deficiency is unknown because most reports on LCHAD lack data on the activities of enoyl-CoA hydratase (LCEH) and long-chain 3-ketoacyl-CoA thiolase (LKAT). Patients with mutations that preserve the hydratase activity accumulate more acylcarnitines than patients with loss of all three enzymes (54). Mutations in TFP cause instability of the TFP complex, thereby affecting all enzymatic reactions, and thus leading to less production of acylcarnitines (45, 55).

### 1.5.3 Diagnostic procedures

The LCHAD diagnosis is based on the analysis of accumulated specific metabolites, and confirmatory testing with measurements of enzyme activity and/or identification of mutations in the *HADHA* gene (51, 52, 56, 57). In LCHAD, levels of long-chain metabolites of 3-hydroxy fatty acids, 3-hydroxy acylcarnitines, 3-hydroxy acyl-CoAs and 3-hydroxy-dicarboxylic acids are increased in serum and urine (56, 58). Some centers also conduct FAO flux studies in cultured skin fibroblasts or lymphocytes, to measure the end products of FAO. Enzyme assays and flux studies are time-consuming, and may take several months to complete (12).

The diagnostic procedure also involves plasma measurements of glucose, lactate, ammonia, liver transaminases, creatine kinase, blood gases, lactate, ketones, free and total carnitine, electrolytes, blood count, platelets, and also amino acids and organic acids in urine. Analysis of dicarboxylic acids in urine is no longer part of the diagnostic procedure at our laboratory.

### 1.5.4 Pathogenesis

The pathogenic mechanism in LCHAD is not fully understood. The impaired fatty acid oxidation and subsequent build-up of fatty acid intermediary metabolites result in insufficient acetyl-CoA and ketone body production during catabolic states, and thereby energy deficiency. Accumulated fatty acids bind to Coenzyme A and thereby reduce levels of free Coenzyme A, needed for the tricarboxylic acid cycle and production of ATP. Histopathological investigations have demonstrated fat accumulation in skeletal muscle, liver, kidney and heart (56, 59-61), and a potential toxic effect of the accumulated long-chain fatty acids or acylcarnitines is currently debated (52, 62-64). It has been suggested that the intermediates may disrupt mitochondria and disturb cellular calcium homoeostasis, thereby inducing oxidative stress and increasing the formation of reactive oxygen species (ROS) (64, 65). In addition, is has been shown that fatty acid derivatives may inhibit the respiratory chain directly (66). Many symptoms in LCHAD/FAOD can be linked with the current hypotheses. Energy deficiency may alter calcium flux and increase membrane permeability and hence rhabdomyolysis and necrosis of myocytes (41, 67). Arrhythmias and impaired cardiac function has been linked to accumulated lipids and possible intracellular calcium overload (63, 68, 69). It has also been suggested that leakage of tricarboxylic acid cycle intermediates from the cells would cause energy deprivation (70). Hence, cellular calcium overload and increased cell membrane permeability seem to be involved in the pathogenesis in

LCHAD/FAOD. Possible toxicity of acylcarnitines has also been discussed in connection with maternal liver disease and the specific chorioretinopathy observed in patients with mutations in the trifunctional protein.

#### *1.5.4.1 Pregnancy complications*

There is an increased risk that the pregnancy of mothers carrying a fetus with LCHAD or TFP deficiency will be complicated by maternal preeclampsia, maternal HELLP syndrome (hemolysis, elevated transaminases, low platelets) and/or acute fatty liver of pregnancy (AFLP) (36, 40, 43, 45, 48, 71, 72). Moreover placental maternal floor infarction has been reported in mothers to fetuses with LCHAD (73, 74). HELLP and AFPL may coexist and are responsible for high maternal and fetal mortality (75). AFLP is characterized by fatty infiltration of the hepatocytes in the third trimester, and the HELLP syndrome involves endothelial dysfunction, complement system activation, fibrin deposition and platelet aggregation, leading to thrombocytopenia and hepatocellular necrosis.

The frequency of AFLP or HELLP syndrome in mothers of patients with LCHAD has been reported to be between 20–79% (10, 40, 72, 76, 77), and this seems to be unrelated to the severity of the fetal phenotype (43). In Asia, where the G1528 C mutation is uncommon, reports of AFPL/HELLP in mothers of patients with LCHAD/TFPD are scarce (78), proposing a possible link between the mutation and the development of maternal liver disease. It has also been suggested that women with AFLP should undergo molecular testing for LCHAD/TFPD, since maternal liver disease is more likely to occur in pregnancies with an affected fetus (79, 80). LCHAD does not, however, appear to be a major cause of the HELLP syndrome (50).

The mechanism for the high incidence of maternal liver disease is unknown. The heterozygous mother has reduced fatty acid oxidation capacity and is asymptomatic until she becomes pregnant with a homozygous fetus. It has been speculated that the placenta, mainly of fetal origin, relies on fatty acid oxidation for energy supply (81-83). In a pregnancy with a fetus with defect beta-oxidation potentially toxic intermediates accumulate. The intermediates may injure cell membranes as well as the maternal endothelium and trigger an inflammatory response (52).The combination of accumulated fatty acid intermediates, reduced maternal fatty acid oxidation capacity and increased metabolic stress during late gestation, eventually leads to AFLP (21, 80). The maternal liver disease and placental energy depletion may cause intrauterine growth retardation and premature birth (21).

#### *1.5.4.2 Chorioretinopathy in LCHAD*

Patients with LCHAD/ TFP deficiencies develop a specific and progressive chorioretinopathy. It is believed that the initial changes affect the retinal pigment epithelial cell layer (RPE) (84, 85), which constitutes a barrier between the photoreceptors and choroid vessels. The pigmented RPE cells are essential for visual function by absorbing light, reducing oxidative stress, and transporting/secreting nutrients and metabolic intermediates, and are involved in phagocytosis and immuno-modulating functions (86).

The chorioretinal changes are progressive with deteriorating retinal function as evaluated by electroretinography (ERG), and lead ultimately to loss of vision (85, 87). At birth, the fundus is normal/pale, and visual acuity is normal (Stage 1). The first signs of chorioretinopathy may already be visible after a few months, initially as hypopigmentation, pigment clumping and a declined ERG response with intact vision (Stage 2), progressing to atrophy with sparing of the central macula and affected color and night vision (Stage 3). Finally, the photoreceptors and choroidal vessels are eradicated, and a bare sclera is discerned, leading to blindness (Stage 4) (87, 88).

Mitochondrial beta-oxidation is involved in the metabolism of the (RPE) (89); however, the pathogenesis of the unique chorioretinopathy is unknown (55, 88). A possible mechanism may be toxic effects of acylcarnitine and hydroxy fatty acid accumulation, since high levels of these intermediates are associated with decreased retinal function (90). Histological examinations have demonstrated RPE cell death (91), and recent research has revealed lipid accumulation and apoptosis in an *in vitro* RPE cell model (84).

Chorioretinopathy has been described in patients with LCHAD or TFP deficiency, but not in patients with other FAODs (92). In addition, patients lacking the common G1528C mutation appear to have milder progression of the chorioretinopathy (90). Chorioretinopathy has not been reported in patients of Asian descent, in whom the common mutation is rare (78, 93, 94), although it is unclear whether ocular examinations and/or ERG were commenced. Altogether, the mutant protein produced in normal levels in LCHAD may lead to high levels of accumulated intermediates that are potentially toxic for the RPE (54).

To date, there is no treatment for the chorioretinopathy; however, supplementation with docosahexaenoic acid (DHA), abundant in the retinal cells as well as a low-fat diet supplemented with medium-chain triglycerides (MCTs), may prevent or delay progression of visual impairment(90, 95).

### 1.5.5 Management of patients with LCHAD

The management of patients with fatty acid oxidation defects involves regular monitoring by a multidisciplinary team consisting of metabolic specialists, dieticians, and psychologists. The treatment in LCAHD is initiated as soon as the diagnosis is established and consists of an individualized, rigorous and lifelong dietary intervention. The goal of the treatment is to provide sufficient energy and nutrients to maintain health and normal growth, and at the same time limit fatty acid oxidation from exogenous and endogenous lipids, and thus the accumulation of metabolites from defective fatty acid oxidation.

Treatment guidelines are based on expert recommendations and few clinical trials, and they differ between centers, especially regarding the length of fasting periods and the need for nocturnal feeding, but also concerning levels of carnitine and essential fatty acid supplementation (96-101).

The cornerstones of the specific LCHAD treatment are fasting avoidance and a low-fat diet with 15–20% of the calories derived from fat, and restriction of long-chain fatty acid intake accompanied by supplementation of medium chain triglycerides (MCTs), essential fatty acids, vitamins, and minerals (95). During any catabolic situations such as febrile illness, fasting or vigorous physical activity the patients are liberally treated with a carbohydrate-rich supplements or intravenous 10% glucose infusions (10–12 mg/kg/min) to avoid metabolic derangement and deleterious consequences. To normalize plasma acylcarnitine levels, it is recommended that the LCFA intake should be less than 10% of the total energy intake. The remaining fat-intake, up to 20% of total energy, is replaced with MCT, containing mainly C8 and C10 fatty acids, which bypass the metabolic block (95, 98, 102). The MCT constitutes an important energy substrate for heart and skeletal muscle that utilizes fatty acids under well-fed conditions. In addition, MCT is thought to yield adequate levels of acetyl-CoA to produce ketone bodies and also malonyl-CoA which inhibits CPT-1 and thus further accumulation of long-chain intermediates (58). The MCT is administered as an MCT- containing infant formula for toddlers and infants, and for older children as a liquid supplement or is used in cooking. Adverse effects may involve gastrointestinal symptoms as nausea and stomach ache, loose stools, and steatorrhea.

The low LCFA intake poses a risk for essential fatty acid deficiency (95) and the diet is therefore supplemented with α-linolenic and linoleic acids, calculated as part of the LCFA intake (101) and given as flax/walnut oil (103). In addition, patients with LCHAD may suffer from docosahexaenoic acid (22:6 n-3) (DHA) deficiency (104). The mechanism is not known, but it has been postulated that the conversion from precursor α-linolenic acid to DHA is impaired in the presence of fatty acid oxidation defects, making it an essential compound in LCHAD.

Short fasting intervals and lipid restriction in combination with sustained age-appropriate protein intake, result in a relatively high-carbohydrate diet compared to normal dietary guidelines (105). The desired effect is increased insulin secretion which act as a potent inhibitor of lipolysis (106, 107). A higher protein intake may have positive effects on energy balance and metabolic control, although long-term effects require further evaluation (108). Cornstarch, that slowly releases glucose for many hours, is only recommended as emergency treatment and not before bedtime (101), as it is difficult to individualize the correct dose that prevents lipolysis.

Patients with LCHAD and other long-chain FAODs are at risk of secondary carnitine deficiency, since carnitine conjugates with accumulated long-chain acyl-groups from Coenzyme-A (18). Acylcarnitines cross the plasma membrane and are excreted in the urine or bile (24). Supplementation with carnitine is, however, controversial. Studies on mice with VLCAD have shown that carnitine biosynthesis is induced by increased carnitine demand and that levels in plasma do not reflect tissue levels (109), thus, the endogenous synthesis is very effective and need not be substituted. Intravenous carnitine administration during acute metabolic derangements is discouraged due to the arrhythmogenic effect of increased intra-

mitochondrial long-chain acylcarnitines (68, 110). Others argue that carnitine supplementation is essential for export of accumulated long-chain fatty acid intermediates and to release free Coenzyme-A, and that the large amounts of energy required for creatine synthesis may actually be a trigger for rhabdomyolysis or cardiomyopathy during illness when energy levels are low (111).

Supplementation with carbohydrates and MCT is recommended prior to physical exercise (112). Carbohydrates constrain FAO through the inhibiting effect of insulin, and MCT generates ketone body synthesis, which have beneficial effects on the cardiac energy supply (112). Thus, regular exercise is recommended for patients with LCHAD, if a "MCT/ carbohydrate sport drink" is consumed before exercise in addition to adequate rest and rehydration post-exercise (112, 113).

Despite treatment, patients with LCHAD may still develop cardiomyopathy, recurrent episodes of rhabdomyolysis, and even sudden death, especially during periods of higher energy demand. Energy deprivation may arise from defective fatty acid oxidation and decreased levels of acetyl-CoA and decreased conversion of NAD to NADH. Accumulating fatty acid intermediates limits the availability of free Coenzyme-A, thus compromising important pathways, including the citric acid cycle. In addition, acylcarnitines may have toxic effects, with destabilized membranes and increased cellular leakage (64). It has therefore been hypothesized that the acetyl-CoA-yield from MCT oxidation may not be adequate to replenish the leakage of citric acid cycle intermediates (114, 115).

One possible supplement for replenishing the citric acid cycle is triheptanoin. Triheptanoin is a triacylglyceride with three molecules of heptanoate which is an odd-numbered fatty acid with 7 carbon atoms. Mitochondrial beta-oxidation of heptanoate yields two acetyl-CoA molecules and propionyl-CoA, which is converted to the citric acid cycle intermediate succinyl-CoA. As a result, triheptanoin increases the energy supply by replenishing the citric acid cycle and thereby supplying substrates for gluconeogenesis (111). Moreover, triheptanoin is converted to C5 ketone bodies, which are efficient substrates for the brain and other tissues (114). Replacing MCT with triheptanoin, equivalent to 30–35% of total caloric intake, relieved cardiomyopathy in patients with VLCAD (70) and a retrospective chart review suggested that triheptanoin decreases the number of hospital days and hypoglycemic events, but not the number of episodes with rhabdomyolysis (99). Gillingham et al. conducted a randomized double-blind study comparing supplementation with 20 E% MCT vs. 20 E% triheptanoin in patients with long-chain FAOD (unpublished data, Gillingham et al, INFORM Meeting, Lyon, 2015) and found that triheptanoin decreased the heart rate during exercise and improved left ventricular function. Patients supplemented with either MCT or triheptanoin had complains about upset stomach and gastric cramps, but there was no difference between MCT and triheptanoin regarding gastrointestinal symptoms or other adverse effects. More studies are needed to evaluate dose-response relationships and compliance, especially when a larger amount than 20 E% of triheptanoin is prescribed. At the

present time, a pharmaceutical company is investigating the effect of triheptanoin in a phase II study (http://www.ultragenyx.com/pipeline/triheptanoin-faod/).

Another therapeutic approach for mild muscular forms of long-chain FAOD with residual enzyme activity may be treatment with compounds of the bezafibrate group (116). Bezafibrates stimulate the nuclear peroxisome proliferator activated receptor (PPAR) and enhance gene transcription expression in both fibroblasts and patients with muscular forms of CPT-2 and VLCAD {Djouadi, 2008 #524;Bonnefont, 2010 #470}. The activation of PPAR restores levels of mutated proteins, and accordingly the residual enzyme activity and FAO capacity. Recent research has shown that bezafibrate treatment improved FAO capacity in 23% of TFP-deficient cell lines, including those heterozygous for the G1528C mutation (119). Although increases in mutant proteins were seen, FAO flux was not restored and accumulated acylcarnitines were not cleared. This is expected as the G1528C mutation target the catalytic site and increased levels of the protein would not improve FAO capacity.

The Swedish dietary instructions for children with LCHAD deficiency follow the general guidelines, with restricted intake of long-chain fatty acids of 10 E% and MCT supplementation up to 20 E %. To reduce the total daily energy intake, the fat restrictions are emphasized as grams of long-chain fatty acid intake (120) instead of a percentage of the total energy intake. Fasting periods are limited to 3–4 hours, and all LCHAD patients are recommended to have nocturnal feedings through a gastrostomy tube (with a low-fat formula containing whey protein, carbohydrates, MCT fat, vitamins, minerals, and trace elements). The intake of carbohydrates and protein is not adjusted in detail, but is altered if the patients show significant deviations in weight, height or BMI. Essential fatty acids are predominantly given as walnut oil, and DHA is supplemented to maintain plasma levels just above or within the upper reference range. Carnitine is supplemented if the patients have low carnitine levels. Presently no patients at our centers receive treatment with triheptanoin or bezafibrates.

### 1.5.6 Follow-up

Regular clinical check-ups and informative briefings for patients with LCHAD and their families are important to prevent morbidity and mortality (96) and are take place at least annually at the metabolic center. Follow-ups include physical examinations, such as regular ocular examinations, including ERG, and echocardiography. Hepatic ultrasonography is performed if serum transaminases are increased or the liver is enlarged. Biochemical follow-ups include plasma measurements of long-chain acylcarnitines, total and free carnitine, CK, and transaminases. Essential fatty acids and docosahexaenoic acid are analyzed in the phospholipid fraction of plasma samples and expressed as a relative percentage of total fatty acids.

### 1.5.7 Animal models for studying FAOD

Cultured skin fibroblasts are often the only specimen provided for clinical studies, since there are no viable animal models for LCHAD or TFP deficiencies (121), although several mouse models have been developed to study other FAODs. While the LCAD enzyme expression is

very low in humans, it plays an important role in mouse fatty acid oxidation, and has overlapping enzymatic activities with VLCAD (27). There are two different VLCAD mouse models, both with stress-induced phenotypes. The LCAD -/- model resembles human VLCAD with fasting intolerance, cardiomyopathy, hypoketotic hypoglycemia, and sudden death (122, 123). The VLCAD -/- mouse model displays a milder clinical disease than the LCAD -/- mouse, with mild hepatic steatosis and cardiac fatty acid accumulation. In addition, the LCAD -/- mice and the LCAD +/- mice have an increased loss of pups in utero (121), which indicates that impaired fatty acid oxidation may play an important role in intrauterine life. Studies on these mouse models have also demonstrated the relationship between energy deficiency and development of cardiomyopathy, and that supplementation with carnitine does not prevent low tissue carnitine levels, but induces acylcarnitine production (124).

## 1.6 NORMAL GROWTH IN INFANCY, CHILDHOOD AND PUBERTY

A healthy child follows an individual growth curve, and regular measurements of height and weight are cornerstones in pediatric healthcare. Any deviations from the individual curve may be an indicator of physical disease or psychosocial distress. The regulation of linear growth is complex and multifactorial, and occurs in three different phases: infancy, childhood, and puberty (125). Nutritional, as well as hormonal factors control the infancy component from birth to 3 years of age. Insulin growth Factor I, IGF-I, plays a major role in fetal and post-neonatal growth (126), as well as insulin and thyroid hormones(127). The infancy growth velocity is rapid and decelerating, the growth rate is being about 2.5 cm/month from birth to 6 months and 1.3 cm/month between 6 and 12 months. From about 1 year of age, the childhood component of growth becomes significant as the influence of growth hormone (GH) becomes gradually important, although infancy and childhood growth overlap until 3 years of age (128). At birth, the child's size is influenced by prenatal growth, but as childhood growth is initiated, genetic components become increasingly important. From 2 years of age, the child will begin to grow according to its genetic potential and thus follow an individual curve, so-called canalization (129). Thus, whereas growth in infancy may tend to oscillate, growth from the second year of life is stable. A change of more than 0.75 SDS between 2 and 3 years of age indicates an abnormal development, and the attained annual height seldom changes more than 0.3–0.4 SDS during childhood growth (125). Boys and girls show little difference in growth rate during childhood, and growth velocity slowly decelerates until the beginning of puberty. The onset of puberty varies between individuals and populations, with a slightly different pubertal growth spurt in boys (28 ± 8 cm) and girls (25 ± 8 cm) (130). Pubertal growth is regulated by GH and the sex hormones testosterone and estrogen (125).

An uncomplicated method to determine if the child is growing to his/her genetic potential, or target height, is to compare the predicted final height with the midparental height, calculated by adding or subtracting 6.5 cm for boys and girls, respectively, to or from the mean parental height (131). The child is growing according to its genetic potential if it is growing within the

TH reference range and the final height is predicted to be within 1 SDS of the target height. However, this method may underestimate height for children with short parents (132).

The different oscillations in height and weight are mirrored in the BMI curve (133). The mean BMI (weight in kg/height in meters$^2$) is 14 kg/m$^2$ at birth and increases to 18 kg/m$^2$ around the age of nine months, when GH begins to stimulate growth. This characteristic point is defined as the BMI peak (134). The influence of GH results in an increased growth rate and a decreased percentage of body fat, thus lowering the BMI scores. At age 6 the decline in fat mass has stabilized, and BMI has decreased to a minimum of 16 kg/m$^2$ defined as the adiposity or BMI rebound. After this age the lean body mass increases and the BMI rises to a mean value of 22 kg/m$^2$ at age 20 years (135). Reports on growth in patients with LCHAD are scare, although some reports indicate that the frequency of overweight or obesity is increased (108, 113).

## 1.7 COGNITIVE OUTCOME

Psychological testing is part of the routine checkup at our clinics, since it is well known that metabolic diseases affect neuropsychological outcomes (136-138). Concerning fatty acid oxidation defects developmental delays in speech, language and motor function have been reported for medium- and very long-chain defects (MCAD and VLCAD) (139-142), although reports on cognitive outcomes in LCHAD are scarce (141).

The highly metabolically active neurons in the central nervous system are vulnerable to metabolic derangements (143). Consequently, toxic intermediates and a suboptimal energy and/or substrate supply, as well as seizures and coma, may cause cognitive and behavioral impairments. In addition, the metabolism of omega-3 fatty acids, essential for brain and retinal development (144), may be affected in fatty acid oxidation disorders.

### 1.7.1 Intellectual Disability

Intelligence involves an individual's ability to reason, plan, solve problems, do abstract thinking and to understand complex ideas. It also involves a person's ability to adapt to the environment and to learn from experience (145). A person's adaptive skills are vital to be able to live and function independently, and include life skills such as taking care of personal hygiene and health, handle money, social skills etc. Intellectual disability is defined as "deficits in intellectual and adaptive functioning presenting before 18 years of age", in the American Psychiatric Association's Diagnostic and Statistical Manual of Mental Disorders (DSM-V) (146).

### 1.7.2 Executive Function

Executive functioning is an important component of behavioral control, is essential for learning new information, recovering old information, and using the information to solve problems of everyday life. In general, executive functions are needed in complex cognitive processes like problem solving, planning, and decision-making. The principal executive components are cognitive flexibility, inhibitory control, and working memory (147).

Cognitive flexibility reflects the ability to shift actions and attention in response to changing situations, and inhibitory control is the mind's ability to suppress interfering stimuli. The working memory system is responsible for maintenance and manipulation of information over short periods of time(148) and is necessary for language comprehension, learning, and reasoning, thus forming the basis for intelligence (149). The psychological models describing the relationship between executive functioning and working memory differ. One of the most accepted models (150, 151) describes working memory as a multi-compartment model, with a storage component that holds visual or auditive information, while the executive component controls and processes the information.

# 2 AIMS

## 2.1 GENERAL OBJECTIVE

The aim of this thesis was to describe the clinical outcome and complications in patients with LCHAD, and, in addition, investigate the energy metabolism, with particular emphasis on the dynamics of fasting. The ultimate ambition was to improve the care and treatment of these patients.

## 2.2 SPECIFIC AIMS

1. How is height, weight, and BMI affected in LCHAD?
2. How long is the fasting tolerance in patients with LCHAD?
3. What energy substrates do children with LCHAD utilize during fasting?
4. How does age at diagnosis, dietary treatment, and number of metabolic decompensations affect visual function and retinal pathology?
5. How is cognition affected in LCHAD?

# 3 MATERIALS AND METHODS

## 3.1 ETHICAL CONSIDERATIONS

The studies included in this thesis have been carried out on children. The families were informed orally and received written information. The children received age appropriate oral and written information, and all procedures and interviews were performed by experienced pediatric examiners. Before participation all patients and families gave their informed consent. Our aim was to ensure that all invasive procedures were pain-free by using EMLA® and/or nitrous oxide, and that the research procedures were coordinated with the patients' regular check-ups.

All procedures followed were in accord with the ethical standards of the responsible committee on human experimentation (Ethics Committee of Uppsala University, Sweden, Decision Number 2006/005, 2009-09-30) and with the Helsinki Declaration of 1975. One major ethical concern was the low number of participants which may have affected anonymity. In the study investigating cognitive outcome, the results were reported on a group level to avoid identification of single patients.

## 3.2 PATIENTS

This thesis is based on investigations on ten patients with LCHAD followed at the Karolinska University Hospital and Uppsala Akademiska Hospital, Sweden. They were diagnosed with LCHAD between 1990 and 2002, before newborn screening for FAODs was introduced in Sweden. The patients lived in different parts of Sweden, but they had at least annual checkups at our centers. During the same time period, there were three additional patients with LCHAD in Sweden, under control at other centers. They were not included in this study.

## 3.3 MEDICAL CHART REVIEW

In order to compare retinal outcomes with clinical parameters and to study growth, medical records and charts were reviewed systematically for major clinical events. Age and symptoms at diagnosis, signs of intrauterine and perinatal stress (maternal hypertension, maternal preeclampsia, maternal HELLP syndrome and/or AFLP), gestational age, birth weight, neonatal hypoglycemia, cardiomyopathy, episodes of metabolic decompensations, and/or number of hospitalizations were included. Information on height, weight and biochemical parameters (plasma acylcarnitines, creatine kinase, transaminases) was assembled. Furthermore, information on gross psychomotor development, epilepsy incidence, and dietary regimen including information on length of fasting periods and night feeds, was collected. Food in-take was monitored with diaries.

## 3.4 HEIGHT AND WEIGHT MEASUREMENTS

Bodyweight was measured to the nearest 0.1 kg, and height to the nearest centimeter. The measurements were compared with Swedish reference data (152) and plotted as height standard deviation scores (SDS) and BMI SDS. Parental heights were recorded and the target heights (THs) were calculated using the Tanner method (131), by adding 6.5 cm to the mean of the parental heights for male TH and subtracting 6.5 cm for female TH. In order to assess whether the children achieved their final height (FH) according to their genetic potential, TH SDS was compared with FH SDS. In addition, we aimed to estimate growth velocity by analyzing mean annual changes in height, weight and BMI SDS.

## 3.5 METHODS FOR STUDYING LIPOLYSIS AND GLUCOSE HOMEOSTASIS

### 3.5.1 Study protocol

The patients were admitted to our hospital units for two days. The first day corresponded to a regular annual follow-up with microdialysis, a standard FAOD diet, as well as night feeds, while studies on fasting metabolism took place on day 2. Nitrous oxide anesthesia and EMLA® cream were used to insert two peripheral intravenous lines for the tracer infusions and blood sampling, and also for insertion of a microdialysis probe in the abdominal subcutaneous adipose tissue.

On the second day, the patients were fed an evening meal and had regular night feeds until just before 2 a.m. when the night feeds were omitted and a standardized meal consisting of 233 mL Monogen (80% MCT, 20% LCT)/$m^2$ body surface was given through the gastrostomy tube. The patients were fasted for 6 hours until 8 a.m. when the fast was interrupted by a standardized morning meal consisting of 233 mL Monogen/$m^2$ body surface. The study ended 2 hours postprandially. Subcutaneous adipose tissue metabolism was monitored by microdialysis of glucose, glycerol, lactate, and pyruvate on days 1 and 2. Lipolysis during fasting was studied by measurements of stable isotope enrichments of glucose and glycerol, accumulation of fatty acid intermediates (from dried blood spots), glycerol, and non-esterified fatty acids (NEFA). In addition, plasma glucose, insulin,

glucagon, cortisol, growth hormone, lactate, pyruvate, and biochemical parameters (plasma Hb, ASAT, ALAT, CK, 3-hydroxybuturate) were followed. Resting energy expenditure and substrate utilization were evaluated by indirect calorimetry.

### 3.5.2 Dietary treatment

All patients continued with their regular dietary intervention during the study with the exception of the 6 hour fast. The diet involved a low-fat diet, with the fat-intake adjusted as the maximum intake of grams of fat/day, and restriction of long-chain fatty acids and supplementation with essential fatty acids, docosahexaenoic acid (DHA) and MCT fat. All children had continuous night feeds, and fasting periods were limited to 3–4 hours. One patient received carnitine supplementation.

### 3.5.3 Microdialysis

Microdialysis is a minimally invasive sampling technique that facilitates continuous sampling and analysis of extracellular metabolites without repeated blood sampling (153). The basic principle is that a thin double-lumen catheter (microdialysis probe) is placed in the tissue of interest and perfused with a physiological solution (Figure 4). Small molecules cross the outer semipermeable membrane by passive diffusion and the outgoing dialysate is collected at regular intervals for analysis, thereby reflecting the concentrations in the interstitial fluid over that interval. In this study, the dialysates were collected for analysis every 30 minutes. When the probe is implanted there is initial vasoconstriction and tissue damage, and it takes minutes to hours before baseline levels are reached. In addition it is important that the composition of the perfusate is close to being physiological.

To study adipose tissue metabolism, the probe was inserted in the abdominal subcutaneous tissue and the dialysates were analyzed for glucose, glycerol, lactate, and pyruvate. The steady-state concentration of glucose is similar to that in venous blood (154). The level of glycerol in adipose tissue is higher than that in blood, since glycerol is produced by fat cells and the levels in blood only partly reflect adipose tissue lipolysis (154).

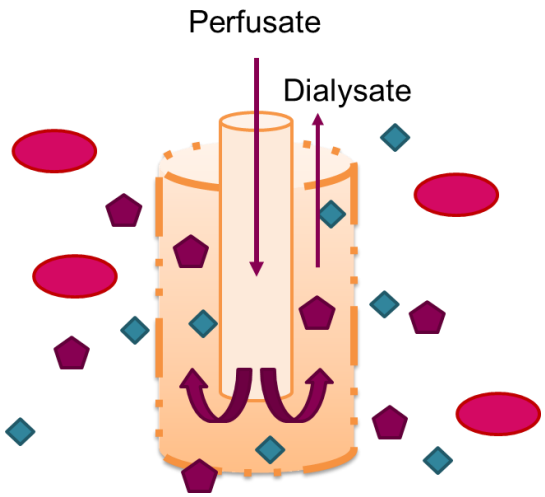



**Figure 4. Basic principles of the microdialysis technique**

The microdialysis probe simulates a capillary blood vessel. Small molecules cross the outer semipermeable membrane by passive diffusion and the dialysate is collected for analysis.

### 3.5.4 Stable isotopes

The stable isotope technique is a method for studying flows of metabolites and metabolic processes. Stable isotopes are naturally occurring variants of an atom with different numbers of neutrons and thus different molecular weights, compared to the naturally dominant isotope. Isotopes can therefore be detected by mass spectrometry. As an example, the dominant isotope of carbon has 6 protons and 6 neutrons (12C), but a small amount of carbon isotopes have 6 protons and 7 neutrons (13C), creating a stable carbon isotope. Stable isotopes do not decay over time, but persist in their same elemental form, contrary to radioactive isotopes.

In metabolic studies, stable isotopes are administered intravenously or orally to record, or trace, a specific substance, the tracee. The tracer has to be chemically identical to the tracee, but differ in some ways to be detected. The tracer is infused in the bloodstream at a constant rate, resulting in an increase in concentration, until it reaches a metabolic plateau or steady state. At that point, the relation of tracer and tracee leaving the bloodstream is identical to the measurable levels within the bloodstream, and also identical to that entering the bloodstream (rate of appearance, Ra). It may take several hours to reach steady state, so the constant tracer infusion is usually combined with an initial bolus injection (priming dose). The tracee is determined by measuring the amount of tracer relative to the tracee, referred to as the enrichment (Figure 5).

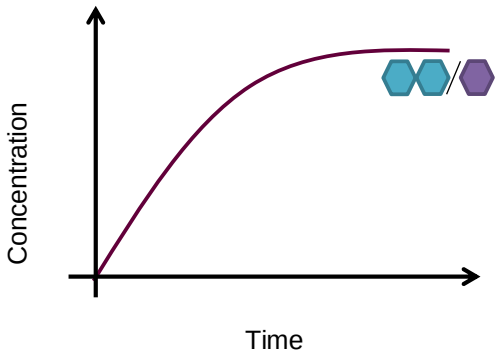



**Figure 5. Principle of the stable isotope technique**

At metabolic steady state, the concentration of the enrichment is identical to the concentration in plasma and the rate of appearance. The different molecular weights of the tracer/tracee enable detection of the substance of interest.

In the course of lipolysis, 1 mole of triglyceride is hydrolyzed to 1 mole of glycerol and 3 moles of fatty acids. Glycerol is fully released into the bloodstream, while some of the fatty acids are recycled to triglycerides. Hence, the rate of appearance of glycerol is a better lipolytic marker than fatty acids (14). The assumptions are that whole-body lipolysis reflects adipose tissue lipolysis, and that glycerol is not produced from any other metabolic pathway (155). Tracers for studying gluconeogenesis do not differentiate between hepatic and renal gluconeogenesis (156).

To study lipolysis in patients with LCHAD, we measured the enrichment of glycerol in the bloodstream after a priming dose (0.6 mg/kg) and a constant rate of infusion of [1.1.2.3.3-2H5]-glycerol (0.015 mg/kg/min) (Cambridge Isotope Laboratories, Woburn, MA, USA). The rate of appearance of unlabeled glycerol corresponded to glycerol released by lipolysis in steady state. Glucose production was studied using a [U-13C2]-glucose tracer. A priming

dose was given (5 mg/kg) prior to the constant rate infusion (0.1 mg/kg/min), and unlabeled glucose was equivalent to glucose production at metabolic steady state. The glycerol and glucose tracers were infused during the fasting period of 6 hours, and for 2 additional hours postprandially. Blood, analyzed for plasma glucose, glycerol, and isotopic enrichments was sampled at the beginning of the infusions and every 10 minutes during the last 60 minutes of the fasting period and also during the last 60 minutes of the study period. The postprandial measurements were not analyzed. The isotopic enrichments were analyzed by gas chromatography- mass spectrometry (GC-MS).

### 3.5.5 Indirect calorimetry

Substrate utilization and resting energy expenditure (REE) were estimated after 5 hours of fasting, by indirect calorimetry with the use of a ventilated hood system (Sensor Medics, Vmax29n), pre-and post-prandially. During the procedure the children were resting in bed. Measurements of REE were done every minute for 30 minutes and the data showing steady state measurements were used to calculate REE expressed as kcal/day. The respiratory quotient (RQ) was assessed by measuring $CO_2$ production and $O_2$ consumption in the breathing air. A $CO_2/O_2$ ratio close to 1 would indicate carbohydrate oxidation, while an RQ near 0.7 is an indicator of mainly fat oxidation. The Schofield reference was used to estimate predicted resting energy expenditure (REE) (157).

### 3.5.6 Fatty acid metabolites

Disease-specific acylcarnitines C16-OH, C16-OH/C16, C18-OH, C18-OH/C18 were analyzed by tandem mass spectrometry from dried blood spots (NeoBaseTM Non-derivatized MSMS Kit, Perkin Elmer, analyzed by LC-MS/MS Micromass Quattro microTM, Waters). Tandem mass spectrometry (MS/MS) is a method used to identify and measure carnitine esters and many other metabolites in blood and urine, and allows quick and accurate measurements with minimal sample preparation. The mass spectrometer ionizes and separates the molecules according to their mass (m)-to-charge (z) ratios (m/z), followed by detection and data processing, and resulting in a graph illustrating, molecular mass and the relative quantity of the different molecules (158). In addition to acylcarnitines, analysis of hydroxy-fatty acids, 3OHC16:0 and 3OHC18:1, were measured in plasma and analyzed by GC-MS (36).

### 3.5.7 Biochemical analyses

Capillary blood glucose was analyzed at bedside (HemoCue Glucose 201, Hemocue AB, Ängelholm, Sweden). Levels of glycerol (159), non-esterified fatty acids NEFA (Wako Chemicals GmbH, Neuss, Germany), triacylglycerides (enzymatic, colorimetric method) and levels of 3-hydroxybutyrate (160) were analyzed in plasma. Levels and outlines of hormones involved in fat metabolism were investigated; Insulin (radioimmunoassay, Pharmacia Insulin RIA, Pharmacia Uppsala, Sweden), glucagon (radioimmunoassay), cortisol (Electro Chemi Luminescence Immuno Assay), and growth hormone (monoclonal antibodies). Catecholamines, crucial for the regulation of lipolysis, require large blood-sampling volumes,

which is not realistic considering the total blood volume required for all analyses. Instead heart rate was monitored as an indirect indication of catecholamine release. Heart rate was registered by Actiheart, a small light-weight accelerometer worn on the chest; Figure 6.

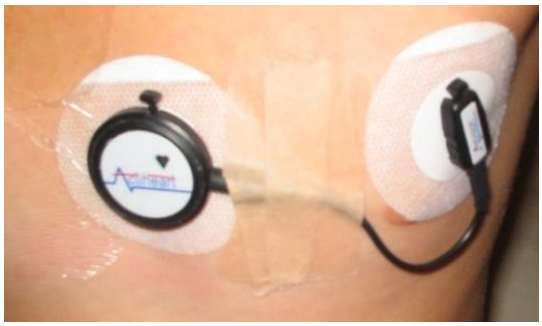


**Figure 6. Actiheart**

The Actiheart is a compact and waterproof device that records heart rate and physical activity, intended for measuring activity energy expenditure.

## 3.6 OCULAR EXAMINATIONS

All examinations were performed by the same orthoptist and pediatric ophthalmologist and included best corrected visual acuity, stereopsis (three-dimensional vision) (Lang, Forch, Switzerland), ocular alignment, color vision testing, slit-lamp investigation (a magnified assessment of the eye structures), ophthalmoscopy, fundus photography and refraction with dilated pupils. Best visual acuity was classified according to the WHO reference (http://www.who.int/blindness/Change%20the%20Definition%20of%20Blindness.pdf); blindness 0–<0.05, severe visual impairment 0.05–<0.1, moderate visual impairment 0.1–0.3. Retinal function was investigated under general anesthesia by electroretinography (ERG), measuring the electrical responses to light of the different retinal cell types. After dark adaptation, the eye was stimulated with bright light provoking a biphasic waveform with a negative a-wave and a positive b-wave recordable at the cornea. The amplitudes of the a- and b-waves were measured, as well as the time from the flash to the peak of the b-wave. Retinal pathology is detected by deteriorating amplitudes. Tissue morphology was examined by optical coherence tomography (OCT) in three patients. OCT is a non-invasive imaging technique that provides a high-resolution- image of the ocular tissues. The technique is comparable to an ultrasound procedure, but measure the reflection of light rather than sound, resulting in a cross-sectional view of the retinal structures.

## 3.7 COGNITIVE ASSESSMENTS

### 3.7.1 Intelligence

The level of cognitive function was measured using the Wechsler intelligence scales. These scales are considered to be the “gold standard” for evaluating cognitive outcome in Sweden and have been translated into Swedish and adapted to Swedish norms. Children aged 3−7 years were tested with the Wechsler Preschool and Primary Scale of Intelligence-III (WPPSI-III) and patients aged 6−16 were tested with the Wechsler Intelligence Scale for Children-IV (WISC-IV). Patients 16 years old and older were evaluated with the Wechsler Adult Intelligence Scale-III (WAIS-III). The test generates a Full-Scale IQ score, representing the individual’s general intellectual ability and intellectual function in different cognitive

domains (Figure 7). The Intelligence quotient (IQ) was defined as being within the normal range (IQ 70–130) or below normal (IQ ≤70).

The *Verbal Comprehension Index* evaluates the children's verbal communication, cover questions on how words are similar, word definitions, comprehension of common concepts and general knowledge. The *Perceptual Reasoning Index* measures non-verbal abstract problem solving and is tested by letting the children organize pictures and puzzles according to predefined models. It also contains logic matrix reasoning. The *Working Memory Index* is based on timed subtests assessing attention, concentration, and ability to memorize information by repetition of number/letter sequences, and *The Processing Speed Index* assesses the ability to process information under time pressure, and requires visual-motor coordination and persistence. For example the patients may be asked to search for or draw symbols below numbers according to key. The number of items completed in a specific period of time equals processing speed.

The Wechsler scales are mostly based on auditive tests. The Spatial Span Board Subtest (161) was added to assess the visuospatial processes. During the test the examiner points to a sequence of symbols, and the person performing the test has to repeat the pattern.

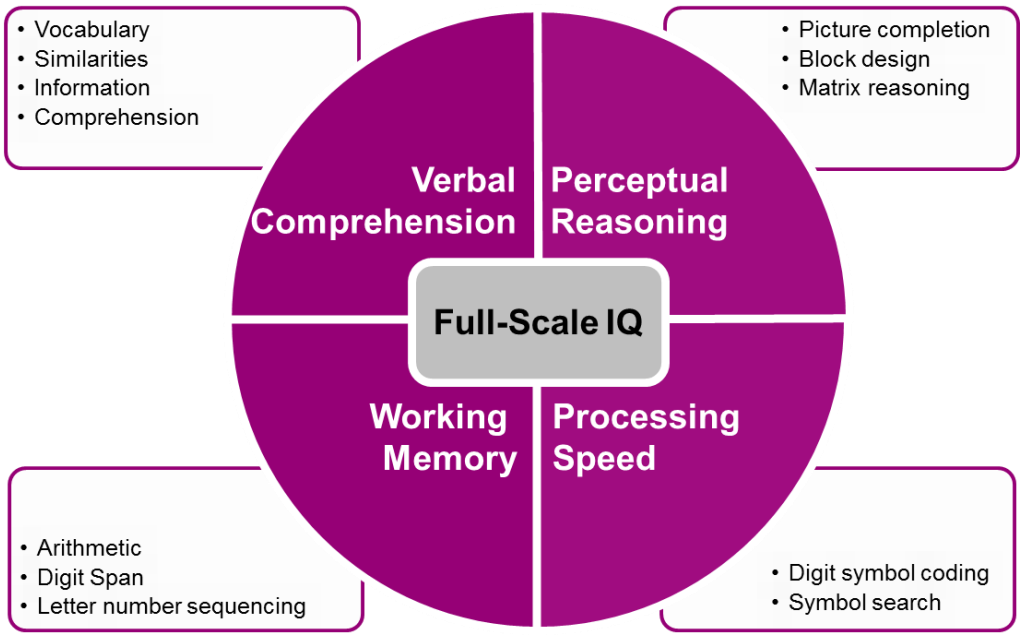



**Figure 7. Schematic illustration of the Wechsler scales**

The Wechsler tests assess Full-Scale IQ and intellectual function in different cognitive domains, represented by perceptual reasoning, verbal comprehension, working memory and processing speed. The IQ scores were compared to Swedish norms (mean 100, SD ± 15).

### 3.7.2 Adaptive and Executive Functions

To evaluate areas of personal independence and daily functioning, we used ABAS-II (162), which has been translated into Swedish and adapted to Swedish norms and takes 20 minutes to administer. The results are reported as an overall score, the General Adaptive Composite (GAC), including conceptual, social and practical domains. The conceptual domain consists of communication skills (language, speech), functional academic skills (basic reading, writing, and math skills) and self-direction (following directions, independence). The social domain represents skills important for social interaction, and the practical domain comprises skills important for personal care and hygiene, home living, health and safety skills and getting around the community. The ABAS scale has a mean of 100, with an SD of 15. A higher GAC score indicates better adaptive functioning.

Executive functions can be measured by different methods, depending on executive component of interest and explanatory psychological model. In addition to the Wechsler tests, we measured executive functioning with the Behavior Rating Inventory of Executive Function (BRIEF) (163), which is a standardized method of asking parents/caregivers/teachers of children, and adolescents aged 5–18 for executive functions in daily life. The BRIEF is not diagnosis-specific and is used to evaluate executive function in children and adolescents with various disabilities, ranging from learning, attention or developmental disorders to different medical conditions, including fatty acid oxidation defects (136, 141). The questionnaire is completed in 15 minutes and has been translated into Swedish. The BRIEF consists of different scales that produce two different indexes, the Behavioral Regulation Index (ability to control impulses, alternate between activities and regulate emotional responses) and the Metacognition Index (ability to generate ideas, hold information in mind to complete a task, set goals, check own work), as well as a Global Executive Composite (GEC) that represents the overall executive function. A high score indicates a dysfunction in a specific domain of executive function. The scale consists of T-scores, with 50 being the mean and 10 being one SD. Scores above 65 are considered to be clinically significant.

## 3.8 STATISTICAL ANALYSES

All studies included basic descriptive statistics expressed as the mean, median, and standard deviation.

# 4 RESULTS

## 4.1 PATIENTS

The clinical characteristics of all patients are listed in table 1. Three pregnancies (30%) were complicated by maternal liver disease, and three patients (30%) were born small for gestational age, however they were not born to the mothers with preeclampsia. Four children were born preterm (40%), two of which being very preterm (gestational weeks, GWs, 29 and 31), and two preterm (GW 35). The girl born in GW 31 suffered a complicated neonatal period along with stage III cerebral hemorrhage. Hypoglycemia was a common symptom in the neonatal period (70%), and three patients (30%) have developed epilepsy. The mean age for diagnosis was 6.4 months (median 7.5 months). No patients have died after the diagnosis. Five patients (50%) had acute symptoms at diagnosis with hypoketotic hypoglycemia, enlarged liver, coma, seizures and/or cardiomyopathy, while three patients had less dramatic symptoms with recurrent episodes of hypoglycemia, hypotonia, and failure to thrive. Two patients (5 and 6) were treated for suspected FAOD because of a family history of diseased siblings. Patient 5 had treatment with a MCT containing formula from birth until diagnosis at 8 months. Seven patients were homozygous for the common G1528C mutation, while three were compound heterozygous for G1528C with three novel *HADHA* mutations on the second allele. There was no difference in acylcarnitine profiles or phenotypes, although preeclampsia was only found in mothers of homozygous patients. Enzyme assays have not been performed and the hydratase and thiolase activities are thus unknown. Since one copy of the common mutation was found, LCHAD and not trifunctional protein (TFP) deficiency is assumed to occur also in the heterozygous patients.

Our ambition was to determine the number of episodes of metabolic decompensation and/or the number of hospitalizations. However, the chart review revealed that different families seek medical attention to different extents. Some families seek medical attention often, and for symptoms that may not be related to the FAOD, while others tend to manage the decompensations at home with “emergency treatments”. Therefore the episode may not always be documented in the medical charts. Instead of counting the number of strict metabolic decompensations, we recorded the occasions when the patients contacted the hospital due to infections or myopathy. Information from food diaries showed that the patients did adhere to the recommended LCHAD diet. The fat intake constituted 13–24% of the total calorie intake, with 8–19% of total fat as MCT and 4–5% as LCT.

## Table 1. Clinical characteristics

| Patient's | Age at diagnosis (months) | Symptoms at diagnosis | Preeclampsia /AFLP | Caesarian section | Gestational age (weeks) | Birth weight (g) | Birth weight SDS | Neonatal hypoglycemia | Epilepsy | Mutations |
|---|---|---|---|---|---|---|---|---|---|---|
| 1 ♀ | 5 | Vomiting, metabolic acidosis, seizures, lethargy, liver enlargement, hypotonia. | | Yes | 37 | 2165 | -2.3 | Yes | | Homozygous c.1528G>C |
| 2 ♂ | 8 | Hypoglycemia, elevated liver enzymes, seizures, cardiomyopathy. | NA | | 38 | 3070 | -0.5 | Yes | Yes | Homozygous c.1528G>C |
| 3 ♂ | 4.5 | Hypoglycemia, elevated liver enzymes, metabolic acidosis, seizures, cerebral edema, cardiomyopathy. | Yes | Yes | 38 | 2865 | -1.0 | | Yes | Homozygous c.1528G>C |
| 4 ♀ | 13 | Vomiting, failure to thrive, elevated liver enzymes, anemia, seizures, metabolic acidosis, coma, heart arrest, cardiomyopathy. | Yes | | 35 | 2016 | -1.8 | Yes | | Homozygous c.1528G>C |
| 5 ♀ | 8 | Neonatal hypothermia elevated liver enzymes and 3OH-FA.Treated from birth due to suspected metabolic disease. | | | 35 | 1910 | -2.1 | Yes | | Compound heterozygous c.1528G>C |
| 6 ♀ | 0.25 | No symptoms. Treated from birth due to family history. | NA | | 38 | 3510 | 1.0 | | | Homozygous c.1528G>C |
| 7 ♀ | 0.25 | Vomiting, diarrhea, apnea, lethargy, hypoglycemia, renal, liver and heartfailure, cardiomyopathy, seizures. | | | 40 | 3260 | 0.3 | Yes | | Homozygous c.1528G>C |
| 8 ♀ | 8 | Lethargy, hypotonia. | Yes | Yes | 29 | 1101 | -1.5 | | | Homozygous c.1528G>C |
| 9 ♀ | 10 | Neonatal hypoglycemia, intraventricular/intraparen-chymal hemorrhage grade III-IV, periventricular leukomalacia. Failure to thrive, liver enlargement. | | Yes | 31 | 1309 | -2.0 | Yes | Yes | Compound heterozygous c.1528G>C |
| 10 ♂ | 7 | Hypoglycemia, hypotonia. | | | 40 | 3165 | 0.2 | Yes | | Compound heterozygous c.1528G>C |

## 4.2 HEIGHT, WEIGHT AND BMI

### 4.2.1 Height

We found accelerated linear growth up to 5 years of age with an increasing annual height SDS (mean SDS 0.2 at age 2 years to mean SDS 0.7 at age 5 years) (Figure 8). Annual growth velocity, measured as annual change in height SDS, was difficult to obtain from the retrospective data as measurements were made with irregular intervals, especially when the child became older. The measurements were more systematic in early childhood, and height SDS increased by 0.2–0.3 SDS between the second and fourth year.

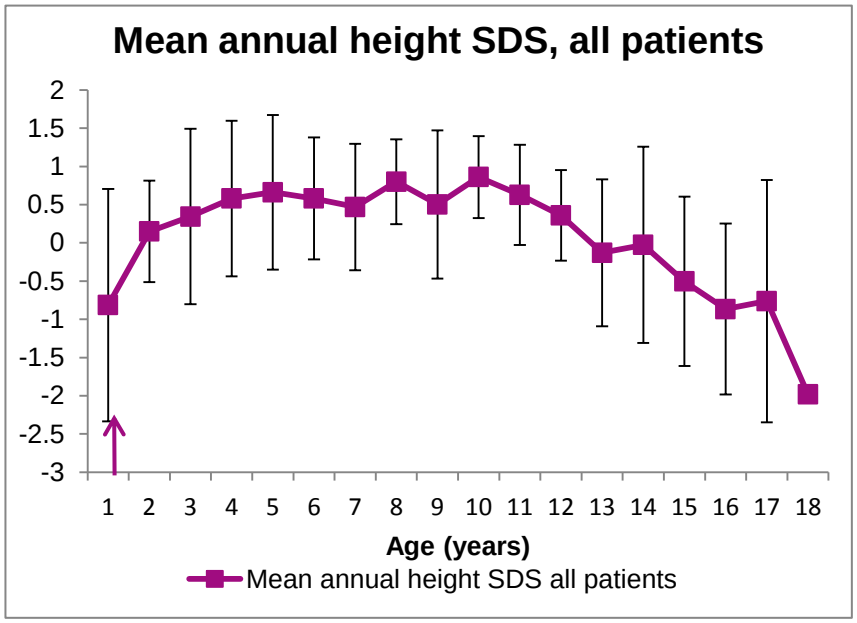



**Figure 8. Mean height SDS all patients**

Height SDS increased up to the fourth year. The arrow indicates mean age for diagnosis, error bars ± 1 SD.

**Table 2. Anthropometric data on final height and target height.**

Pubertal growth was compared with the Tanner reference (28 ± 8 cm for males, 25 ± 8 cm for females) (130).One boy had subnormal pubertal growth (patient 3). Three patients had final height (FH) SDS within target height (TH) range (±1 SDS), representing growth according to genetic potential.

| Patient | Pubertal growth (cm) | FH (cm) | FH SDS | Mother´s height (cm) | Father´s height (cm) | TH (cm) | TH SDS | FH SDS –TH SDS |
|---|---|---|---|---|---|---|---|---|
| 1 | 24 | 156 | -2 | 162 | 187 | 168 | 0.1 | -2.1 |
| 2 | 26 | 185 | 0.7 | 159 | 187 | 180 | 0.9 | -0.2 |
| 3 | 18 | 180 | -0.1 | 168 | 196 | 189 | 1.3 | -1.4 |
| 4 | 18 | 169 | 0.2 | 167 | 189 | 172 | 0.7 | -0.5 |
| 5 | 20 | 166 | -0.2 | 162 | 187 | 168 | 0.1 | -0.3 |

During the study period, five patients (patients 1–5) reached their final height (FH). Patients 2, 4, and 5 had a final height that was within ±1.3 SDS of target height, and two patients' final heights were slightly below their target; Table 2. Patient 1 had an early puberty and was treated with a GnRH-agonist between ages 8 and 10 years. Her pubertal growth spurt was normal (24 cm). Patient 3 developed severe epilepsy, accompanied by neurological and cognitive disabilities and poor pubertal growth; Table 2. The mean final height SDS was 0.3

### 4.2.2 Weight and BMI

The greatest annual change in weight SDS occurred during the second year of life and, to a lesser extent during years 3 and 4 (Figure 9).

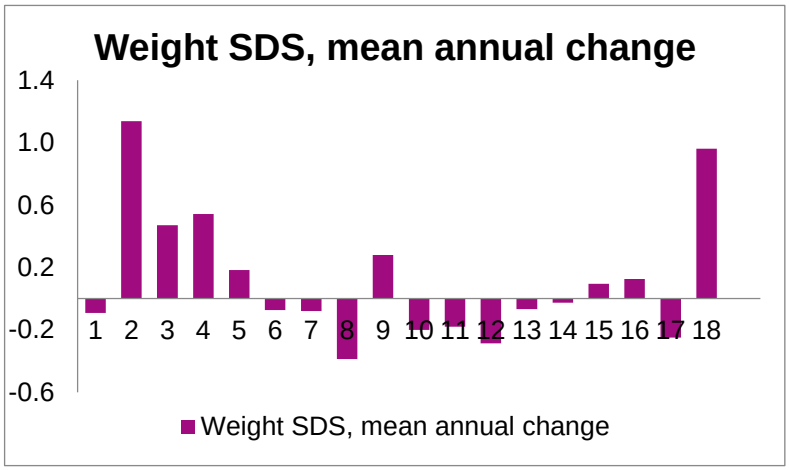



**Figure 9. Weight SDS annual change.**

The greatest annual change in weight SDS occurred during the second year of life and to a lesser extent during year 3 and 4.

Peak BMI values before 1 year of age, were recognizable for 9 out of 10 patients (data missing for one patient). The majority of patients with diagnosis after the neonatal period had a sudden interruption in the BMI trajectory around the time of diagnosis, with declining BMI values. After the diagnosis, BMI increased for all but one patient. On average, the BMI peak occurred at age 8.4 months (± 2.4 months) in the girls, with a mean BMI of 16.8 kg/m$^2$ (± 1.2 kg/m$^2$) (n = 6). In boys, the corresponding BMI peak occurred at age 8.2 months (± 2.0 months) with a slightly higher mean BMI of 17.1 kg/m$^2$ (± 0.3 kg/m$^2$) (n = 3).

Four patients (40 %) had a declining BMI after the peak, but only for a short period of time, while the other patients either maintained their BMI or had an increase in it. The highest BMI SDS were observed between ages 5 and 7 years, hence a BMI rebound with a nadir around age 6, was not seen (Figure 10). At age 6 years, six patients were overweight and 1 patient obese.

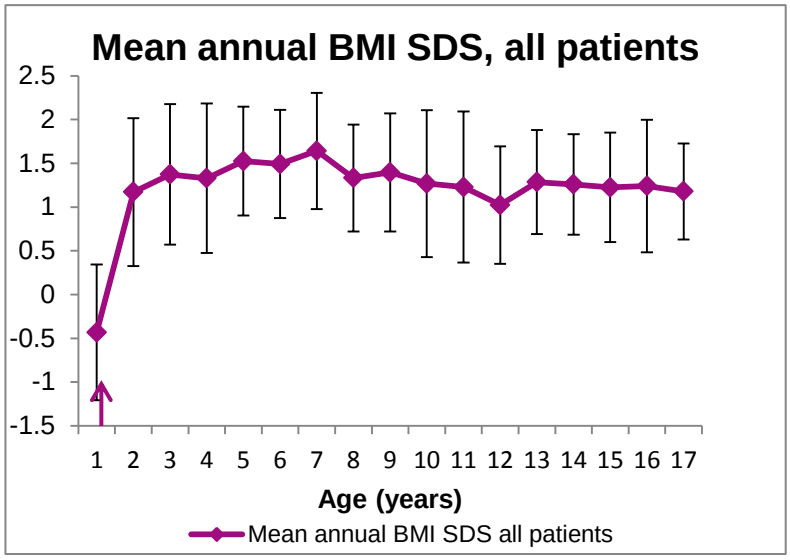



**Figure 10. Mean annual BMI SDS**

The highest BMI SDS were observed between ages 5 and 7 years. The arrow indicates mean age of diagnosis, error bars ± 1 SD. For individual BMI scores please see paper III.

## 4.3 ENERGY HOMEOSTASIS

Lipolysis was investigated in five patients (patients 6–10), aged between 5.5 and 9.5 years at the time of the study (mean age 7.5 years). The results of the studied parameters are depicted in Figure 11.

No patients developed hypoglycemia or had clinical or laboratory signs of rhabdomyolysis. Mean plasma glucose was 5.7 ± 0.7 mM (min 3.9 mM, max 9.4 mM) with the lowest concentration recorded after 2 hours of fasting. Glucose levels in subcutaneous adipose tissue were slightly lower than in plasma, with a mean concentration of 4.4 ± 0.5 mM (min 3.0 mM, max 6.8mM) and the lowest levels observed between 1 and 3.5 hours of fasting. Glucose production was normal, with a rate of 19.6 ± 3.4 µmol/kg/min (3.5 ± 0.6 mg/kg/min) at fasting hour 5–6.

Increasing plasma and dialysate levels of glycerol were detected after 3–4 hours of fasting, without any concurrent changes in levels of NEFA and TAG. The peak level of plasma glycerol was 143 µM (Figure 11), detected after 5 hours of fasting. The peak level of glycerol in subcutaneous adipose tissue, i.e. the microdialysate, was 530 µM detected after 6.5 hours of fasting. In addition, the levels of microdialysate glycerol were 59% higher during the night when the child was fasting, compared to the night with regular LCHAD diet and night feeds (mean 304 ± 96 µM compared to 191 ± 64 µM). The mean endogenous rate of glycerol production was 7.7 ± 1.6 µmol/kg/min, which is a higher rate than normal for age (Table 3). Levels of long-chain acylcarnitines increased after 4 –5 hours of fasting, particularly levels of C16-OH (Figure 11e).

| Study population | Number of subjects | Hours of fasting | Glycerol production rate µmol/kg/min | Reference |
|---|---|---|---|---|
| LCHAD | 5 | 6 | 7.7 | Current study |
| Healthy | 9 | 12 | 2.0 | (164) |
| Healthy | 4 | 12 | 4.3 | (165) |
| Afro-American | 20 | 12 | 2.4 | (166) |
| White American | 20 | 12 | 3.8 | (166) |

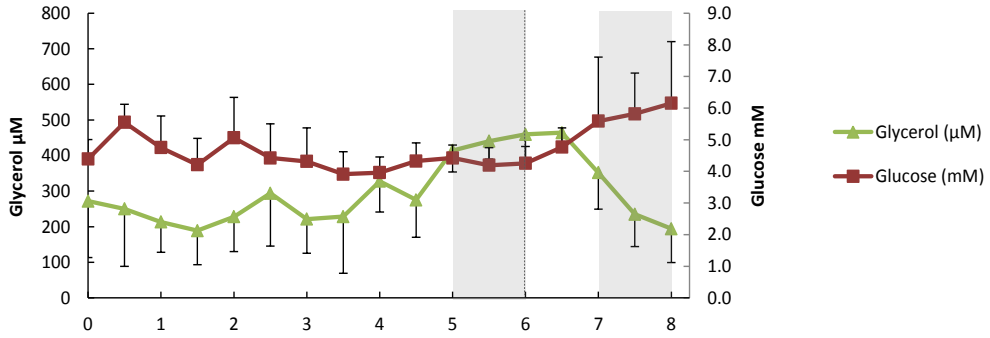
**Table 3. Glycerol production in different populations**

Glycerol production was increased in patients with LCHAD with a rate of 7.7 ± 1.6 µmol/kg/min.

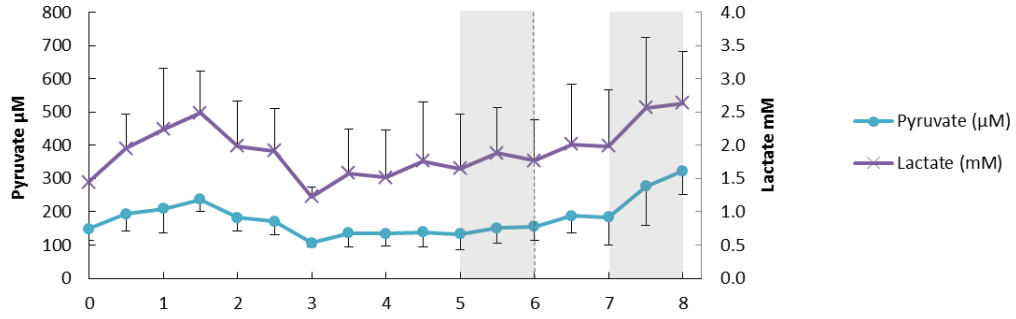
Profiles of hormones of central importance for lipolysis were followed. Insulin decreased after the standardized MCT intake that preceded the fast, and remained low (Figure 11d). Mean glucagon levels were also low with the highest measurements recorded at time 0 hours. Levels of glucagon at the time of the lowest recorded glucose levels were not available. Cortisol-secretion followed the regular endogenous circadian rhythm showing normal concentrations. Interestingly, four patients displayed a growth hormone peak after 3 hours of fasting while sleeping (mean peak value 13 ± 11.2 mU/L, min 6.9 mU/L, max 30 mU/L) (Figure 11d).

Plasma 3-hydroxybutyrate remained low (<0.2 mM) throughout the fast. Pyruvate (mean 162 ± 35 µM, min 87 µM, max 290 µM) and lactate (mean 1.8 ± 0.3 mM, min 1.0 mM, max 3.6 mM) in the microdialysates peaked at hour 1.5 after the initial glucose peak, but declined and remained low during the continuing fast (Figure 11b).

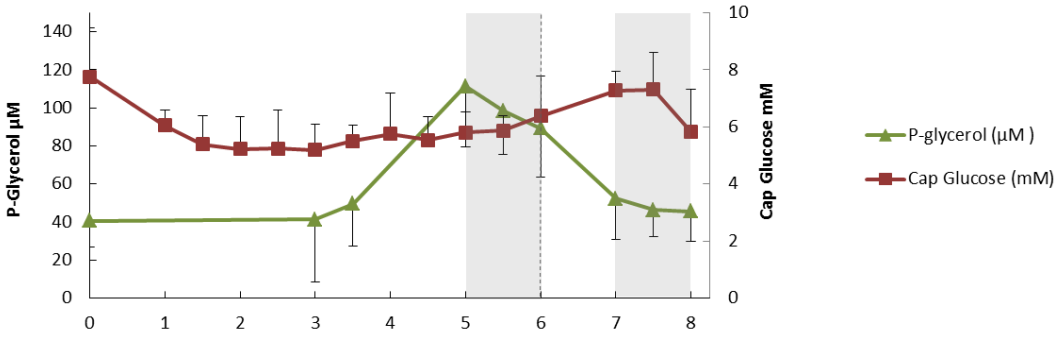
a. Microdialysis Glycerol and Glucose




b. Microdialysis Pyruvate and Lactate




c. Capillary Glucose and Plasma Glycerol



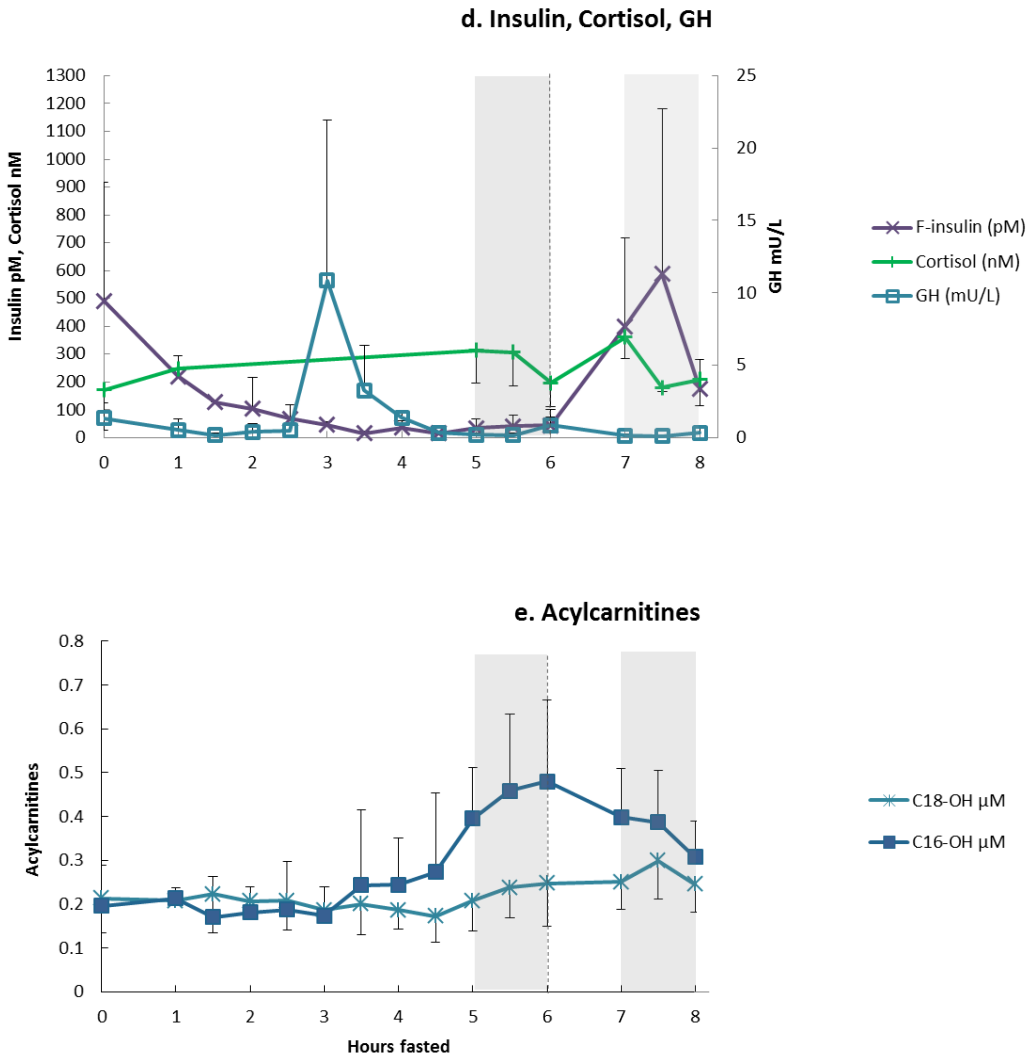



**Figure 11. Results of lipolytic parameters and hormones during fasting**

The lowest recorded glucose, pyruvate, and lactate levels in the capillary and dialysate measurements (a–c) were followed by a growth hormone peak (d) and increased heart rate (Figure 12). Levels of long-chain acylcarnitines and levels of plasma and microdialysate glycerol increased after 3–4 hours of fasting (e and c). Levels of insulin were low and the cortisol secretion followed the endogenous circadian rhythm. The grey fields indicate times for stable isotope measurements and the dotted line when the fasting was interrupted.

An increased mean heart rate was recorded after 2 hours of fasting (Figure 12).

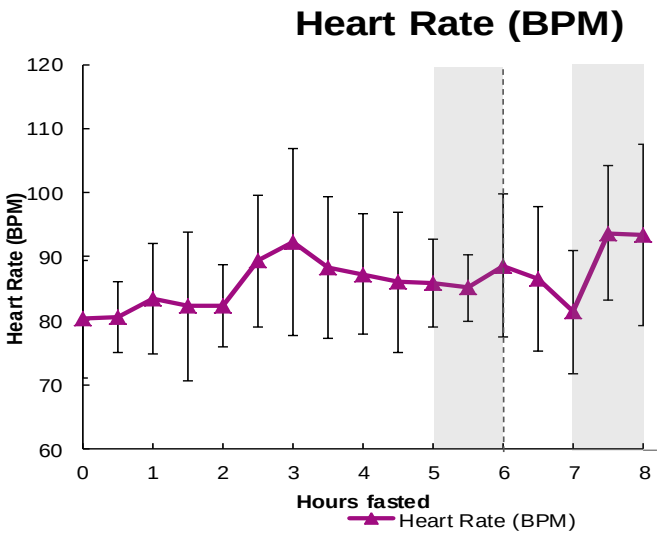



**Figure 12. Heart rate**

The mean heart rate increased after 2 hours of fasting. The grey fields indicate times for stable isotope measurements and the dotted line when the fasting was interrupted, error bars ± 1 SD.

The fast was discontinued at 8 a.m., followed by a decreased rate of appearance and levels of plasma glycerol, acylcarnitines, 3-hydroxy fatty acids, and mean heart rate. As expected plasma glucose and glucose production, as well as insulin secretion, increased as the fast was interrupted.

The energy production at rest was 1074 ± 80 kcal/day, which is normal or slightly lower than that reported for healthy peers (157). Mean RQ was 0.9, indicating carbohydrate oxidation rather than fat oxidation. A similar RQ has been reported in other children with FAOD (108); however, the fat oxidation is lower in children with LCHAD than in corresponding prepubertal children without an FAO disorder (164, 167).

## 4.4 OCULAR CHARACTERISTICS

Retinal pathology of different severities was found in all 10 children (Figure 13). The initial changes were subtle pigmentations in the posterior retinal poles, detected at a median age of 3.6 years (range 14 months to 6 years), progressing to granular pigmentations and atrophies around the optic disc and the macula. Six patients had no or only slight vision loss, while two patients (1 and 4) developed central chorioretinal atrophy and severe myopia. Development of chorioretinopathy was also monitored by ERG, which was pathological in seven patients, and subnormal in three patients, suggesting that ERG changes developed after the fundus changes were visible.

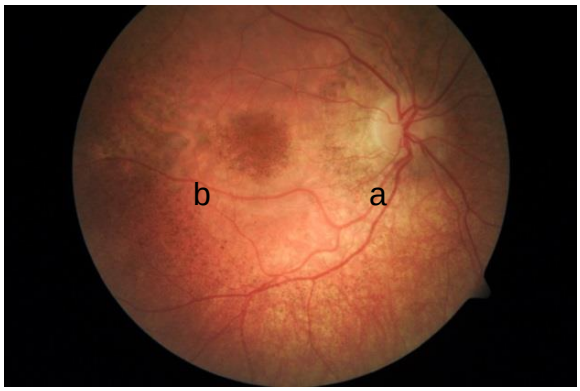



**Figure 10. Fundus photograph**

A fundus photograph from a 4 year old patient with LCHAD, illustrating scattered granular pigmentations, peripapillary atrophy (a) and hyper-pigmentation in the macula (b). Visual acuity was normal for age.

In order to compare retinal function with the patients' clinical condition, major clinical events like age and symptoms at diagnosis, neonatal hypoglycemia, gestational age, pregnancy complications, psychomotor development, number of metabolic decompensations, epilepsy, night feeds and present age, were scored depending on severity, and the summed scores were compared with the ocular outcome. Children with higher summarized scores for clinical parameters also had the most pronounced chorioretinopathy. It was not apparent, however, which clinical events were most harmful to the retina. There was an association between age at diagnosis and fundus stage; hence, chorioretinopathy was less pronounced in children with an early diagnosis and more severe in children with a late diagnosis. One of the patients with severe myopia was diagnosed with LCHAD at five months and was hospitalized for LCHAD-related symptoms on numerous occasions while the other patient with severe myopia was diagnosed at 13 months, but was hospitalized only a few times. The children with best ocular outcome and subnormal ERG results (patients 5, 7 and 10) were diagnosed at 8, 0.25, and 7 months, respectively, although patient 5 was treated for a suspected metabolic/FAO disorder from birth. Patients 5 and 10 were hospitalized on numerous occasions, and were compound heterozygous for the common mutation. Patient 7 was diagnosed within her first week of life due to hypoglycemia, lethargy, liver enlargement, cardiomyopathy and seizures, but has had few hospitalizations once a dietary regimen was initiated. Moreover, a child diagnosed and treated presymptomatically developed granular pigmentations at age 5.5 years and declining ERG responses from 7 years.

## 4.5 COGNITION

Eight patients were evaluated for cognitive outcome (patients 1–2, 5–10). The tests took approximately 45 to 90 minutes to complete. The mean Full-Scale IQ Scores was 82, but the results had a wide distribution ranging from 42 to 112, and two subgroups were identified (Figure 14).

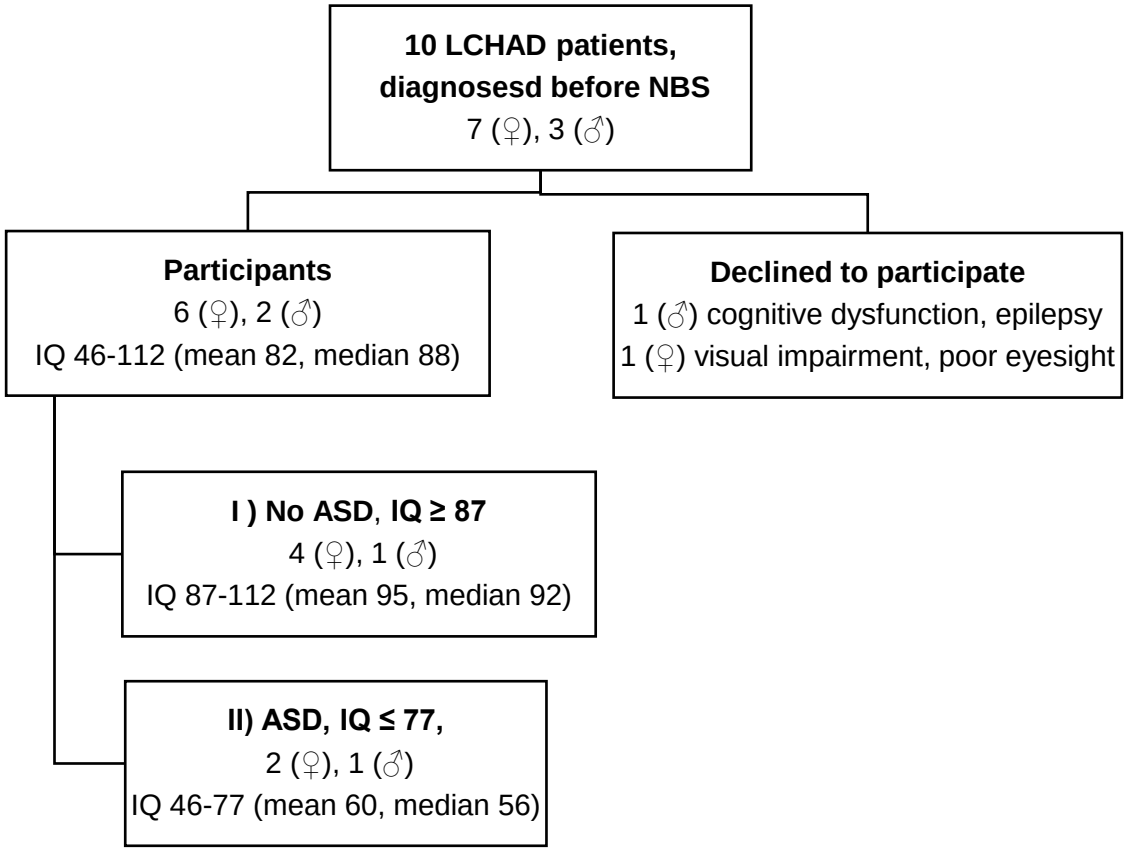



**Figure 14**. **Patients evaluated for cognitive outcome**

Eight patients participated in cognitive evaluations, and two subgroups were identified.

The scores on the different scales and subgroups are presented in Table 4. Group I had a mean IQ score of 95, range 87–112 (n = 5), with average scores for verbal (mean 101), perceptual (mean 97), and processing speed (mean 102) skills. However, the mean scores for working memory were lower (mean 81). The results from the visuospatial span board subtest were normal; hence, the lower scores for working memory were derived primarily from the auditory component of the working memory. Parents reported deficiencies in adaptive functioning with a mean GAC score of 83. They also reported dysfunctions in executive functioning, particularly in the domains of shifting, flexibility, and planning. There were vast inter-individual differences with a GEF score ranging from 50 to 69, but the mean GEF score for all five patients was 60, which is considered to be within the normal range of variation. All the patients in Group I attended regular schools.

In Group II the IQ scores ranged from 46 to 77 (n = 3). The patients displayed autistic-like behavior and two children were diagnosed with mental retardation and epilepsy. They all had low scores on verbal (mean 65), perceptual (mean 67), processing speed (mean 57) and working memory (mean 67) skills and had special educational needs. As expected, the parents of patients with autism spectrum disorder (ASD) and intellectual disabilities, reported lower scores for adaptive skills than parents of children in Group I, with a mean GAC of 34

(range 28 to 42). Evaluation by the BRIEF was not carried out. The patients in Group II were diagnosed at a somewhat older age than the patients in Group I (mean age for diagnosis, 8 and 5 months, respectively).

| | All patients, latest assesment (n=8) | | | Patients without ASD (n=5) | | | Patients with ASD (n=3) | | |
|---|---|---|---|---|---|---|---|---|---|
| | Mean | Median | Range | Mean | Median | Range | Mean | Median | Range |
| *Intelligence (WPPSI-III /WISC-IV/ WAIS-III/ WNV)* | | | | | | | | | |
| Full-scale IQ | 82 | 88 | 46-112 | 95 | 92 | 87-112 | 60 | 56 | 46-77 |
| Verbal | 87 | 92 | 57-112 | 101 | 102 | 87-112 | 65 | 68 | 57-70 |
| Perceptual | 85 | 93 | 55-106 | 97 | 98 | 80-106 | 67 | 56 | 55-90 |
| Working memory | 77 (n=7) | 77 | 65-91 | 81 | 80 | 73-91 | 67 (n=2) | | 65-68 |
| Processing Speed | 85 | 90 | 49-128 | 102 | 101 | 85-128 | 57 | 60 | 49-63 |
| Visual working memory | | | | 53 | 56 | 43-68 | | | |
| *Adaptive functioning (ABAS)* | | | | | | | | | |
| GAC | 69 (n=7) | 80 | 28-102 | 83 | 89 | 52-102 | 34 (n=2) | | 28-42 |
| Cognitive | 68 (n=7) | 78 | 12-97 | 85 | 92 | 59-97 | 26 (n=2) | | 12-40 |
| Social | 70 (n=7) | 75 | 4-109 | 87 | 83 | 72-109 | 27 (n=2) | | 4-50 |
| Practical | 77 (n=7) | 92 | 12-114 | 97 | 94 | 83-114 | 28 (n=2) | | 12-44 |
| *Executive Functioning (BRIEF)* | | | | | | | | | |
| Inhibit | | | | 48 | 50 | 39-55 | | | |
| Shift | | | | 66 | 67 | 57-77 | | | |
| Emotional Control | | | | 53 | 51 | 47-61 | | | |
| Behavioral Regulatory Index | | | | 55 | 58 | 47-63 | | | |
| Initiate | | | | 57 | 56 | 50-68 | | | |
| Working Memory | | | | 64 | 64 | 43-79 | | | |
| Plan/Organize | | | | 65 | 61 | 51-84 | | | |
| Organization of materials | | | | 55 | 59 | 46-62 | | | |
| Monitor | | | | 57 | 57 | 47-67 | | | |
| Metacognition Index | | | | 62 | 60 | 51-75 | | | |
| Global Executive Function | | | | 60 | 61 | 50-69 | | | |

**Table 4. Scores for cognitive outcomes**

Five patients had mean full-scale IQ above 87, with a specific deficit in working memory and individual deficiencies in executive functions. Three patients had mean full-scale IQ below 77 and were diagnosed with autism spectrum disorder (ASD). Adaptive function was evaluated by Adaptive Behavior Assessment System® (ABAS) (mean 100, SD 15). Executive function was evaluated using the Behavior Rating Inventory of Executive Function® (BRIEF) (mean 50, SD 10). Lower scores on BRIEF indicated a better outcome, and scores above 65 were considered clinically significant.

# 5 DISCUSSION

The first cases of LCHAD were reported in the early 1990s. Since then the general knowledge about the pathophysiology and complications has increased, and different treatment protocols have been developed. Despite dietary therapy there is still marked morbidity and mortality associated with the disorder (10, 39, 111). Serious clinical complications remain a problem, including the development of chorioretinopathy and peripheral neuropathy, recurrent episodes of myopathy and rhabdomyolysis, along with cardiomyopathy, and liver manifestations (21, 23, 57, 111).

This thesis is based on clinical investigations of ten patients with LCHAD, with the aim to increase our understanding of the energy metabolism in LCHAD, especially during fasting, and to give an overall description of the clinical outcomes as well as complications. The older patients have been followed from infancy to adulthood. This cohort is comparable to other cohorts of patients with LCHAD diagnosed by symptoms, since age and symptoms at presentation and frequency of maternal liver disease is similar (10, 12, 39, 40, 72, 79, 168). None of the patients have died after the diagnosis.

## 5.1 GROWTH

Assessment of growth is an important indicator of health and nutritional status. The LCHAD diet with frequent meals and low fat content differs substantially from the traditional nutritional recommendations for normal children. As part of the study, data on height, weight, and BMI were collected and analyzed. The majority of the children had a rapid weight gain between 2 and 4 years of age, and the characteristic decline in BMI after the BMI peak was not observed. We found that the BMI peaked at age 8.4 months in females and at 8.2 months in males, occurring slightly earlier than in normal children (9.6 and 8.8 months respectively) (134). Also, the mean peak values were lower with a peak BMI of 16.8 $kg/m^2$ and 17.1 $kg/m^2$ compared to 17.7 $kg/m^2$ and 18.1 $kg/m^2$ in a large study on Finnish children (134). Several patients had an abrupt disruption of the BMI trajectory with declining BMIs around time for diagnosis (mean and median ages for diagnosis 6.4 and 7.5 months respectively). This may have affected the timing and the BMI peak value in patients with LCHAD, since the time for diagnosis occurred in close proximity to when the BMI peak normally occurs.

The mean BMI at 6 years of age, when the BMI normally decreases to a minimum, was 18.1 $kg/m^2$ (17.3 $kg/m^2$ for males and 18.4 $kg/m^2$ for females). Five of the girls and one boy were overweight (60%), and one girl was obese at age 6 years (10%). Two patients had a normal BMI (20%), and one boy did not have any measurements at that point in time. This shows that the adiposity rebound was early or even non-existent, and that overweight was overrepresented. In comparison, only 11% of the girls and 15% of the boys were overweight in a study of normal Swedish school children (169). Three patients were born SGA, but did not show catch-up growth.

Height velocity was also affected after the start of treatment, with the greatest increases in height SDS observed up to 4 years of age, followed by a period of stable or decelerated

growth. The increase in weight occurred before acceleration in height. It is likely that the dietary intervention resulted in a nutritional surplus, and thus increased the secretion of IGF-I and insulin resulting in growth acceleration in early childhood (170). Furthermore, three out of five patients reached their final height within their target height, and it appears that neither the disorder itself nor the dietary treatment affects final height negatively.

In patients with LCHAD several factors constitute risks for development of insulin resistance. Rapid weight gain and early adiposity rebound are associated with adult adverse metabolic profiles and obesity, and it has also been suggested that early rebound is part of a developmental pathway to the metabolic syndrome (135, 171). In addition, regular feeds and night feeds subsequently results in constant hyperinsulinemia, which also constitutes a risk-factor for development of insulin resistance (172, 173). In addition, high insulin levels and inhibited lipolysis result in "fat trapping", with further enlargement of the adipose tissue (14, 174). Moreover the capacity to oxidize fatty acids seem to play an important role in the development of insulin resistance, although it is unclear whether intramyocellular lipid accumulation causes decreased insulin sensitivity or not (175, 176).

It has been suggested that FAOD may be protective against the development of insulin resistance since patients with LCHAD seem to have normal glucose tolerance (177), and mice with VLCAD did not develop insulin resistance (178). In contrast, ageing mice heterozygous for mutations in the mitochondrial trifunctional protein developed insulin resistance and liver steatosis (65).

The majority of the patients studied in this thesis were overweight and some had very high insulin levels, but they have not developed signs of impaired glucose metabolism or type 2 diabetes mellitus. The associations between insulin resistance and defect fatty acid oxidation, overweight, hyperinsulinemia, and a low fat diet with MCT are interesting and need further evaluation.

Overweight and obesity have also been observed in other patients with LCHAD (108, 177). In addition to the diet, patients with FAOD may be physically less active due to muscular weakness and/or fear of rhabdomyolysis or they may have an altered total energy expenditure, which contributes to the weight increase. The risks associated with childhood obesity are well known, and will not be further discussed here. However, obesity in FAOD constitutes an extra challenge since planned weight reduction involves endogenous fatty acid oxidation and therefore is not endorsed. It is therefore crucial to follow weight development and adjust the diet to avoid over-feeding at an early age. A higher dietary protein content may contribute to an improved weight and energy balance, although long-term effects are not known (108). Both weight and metabolic control may be facilitated by fat recommendations in grams instead of percentage of total calories, since a higher caloric intake allows for a higher fat intake. Recommendations for exercise and exercise intensity levels should also be considered. Theoretically, fatty acid oxidation is more pronounced during prolonged intervals of moderate exercise, in comparison to short bursts of high-intensity activities in which glycogen is the preferred substrate. With supplementation of MCT (0.3–0.4 g/kg body

weight) prior to physical activity, exercise at a moderate intensity (60–70% of maximal heart rate) for up to an hour is considered safe and does not increase levels of acylcarnitines or cause rhabdomyolysis(112, 113, 179). Hence, exercise in LCHAD patients should be encouraged if the patients undertake precautions with diet, rehydration and recovery in conjunction with the physical activity.

## 5.2 ENERGY TURNOVER AND SUBSTRATE UTILIZATION IN PATIENTS WITH LCHAD

Fasting tolerance has previously mainly been determined in terms of the risk of developing hypoglycemia (101). Hence, many centers do not generally recommend nocturnal intragastric feedings, but consider nightly fasting intervals of 10–12 hours safe, since the glycogen stores built up during the day would maintain blood glucose levels during the night (101). However, in this disorder there is also a concern regarding the risk of increasing levels of toxic metabolic intermediates coming from the defective breakdown of long-chain acylcarnitines. Emerging evidence highlights an indirect association between accumulation of fatty acid metabolites and lipotoxicity. Gillingham et al. found reduced progression of retinopathy when levels of acylcarnitines and hydroxyacyl fatty acids declined (90), and Polinati and co-workers demonstrated lipid accumulation and possible toxicity to the retinal pigment epithelium (RPE) cells (84). Furthermore, the association with LCHAD and acute fatty liver of pregnancy, preeclampsia and the HELLP syndrome is believed to be caused by accumulation of toxic fatty acids (80). Likewise, elevated levels of myocardial triglyceride content in LCAD mice may be responsible for the impairment of cardiac function (63, 180). Others have suggested structural and functional mitochondrial abnormalities, secondary to the accumulation of toxic intermediates (62). It has also been suggested that high levels of hydroxylated fatty acids accumulating in LCHAD deficiency may disturb mitochondrial energy and redox homoeostasis (64).

We were interested in studying glucose homeostasis, as well as the degree and timing of lipolysis, levels of acylcarnitines, and the main regulating hormonal balance in the fasting situation in patients with LCHAD. Lipolysis and glucose production were studied using stable isotopes of glucose and glycerol, microdialysis, and analysis of acylcarnitines. Heart rate, hormones associated with fasting, and biochemical parameters were also followed. We found considerably shorter fasting tolerance than in healthy children, since fatty acid metabolites had already increased after 3–4 hours, despite normal blood glucose and glucose production rates. The lipolysis was preceded by slightly lower microdialysis glucose levels, elevated heart rate, and a growth hormone peek, most likely representing a hormonal counterregulation necessary to supply substrates and sufficient energy to maintain glucose homeostasis. Declining levels of substrate availability (glucose, pyruvate, and lactate) and insufficient acetyl-CoA supply to the citric acid cycle may contribute to reduced ATP availability, possibly with initiation of catecholamine release and increased lipolysis. Blood levels of long-chain acylcarnitines and 3-hydroxyfatty acids were also elevated after 4 hours of fasting. The inability to utilize lipids as energy substrates was demonstrated by an

increased respiratory quotient, indicating higher carbohydrate oxidation than fatty acid oxidation compared with peers, but not with other patients with LCHAD (108, 167, 177).

Increased concentrations of fatty acid intermediates occurred much earlier than the development of hypoglycemia. Hence, hypoglycemia is a late indicator of metabolic derangement. Increased lipolysis in LCHAD has also been reported previously (181). With the increasing evidence of deleterious effects of acylcarnitines, it is essential to evaluate the fasting intervals not only from a glycemic perspective, but from the risk for accumulation of fatty acids. The levels of acylcarnitines that may be harmful require further assessment.

The antilipolytic effect of insulin is used in clinical practice, as the patients are recommended a low fat, high carbohydrate diet, and fasting avoidance, which triggers insulin secretion. Insulin binds to the adipocytes and inhibits lipolysis by inactivation of hormone-sensitive lipase, thus preventing the breakdown of triacylglycerols to free fatty acids and glycerol (182). To avoid lipolysis, fasting intervals that do not exceed 4 hours are essential, – even during the night. Hence, continuous nocturnal intragastric feedings are required to avoid lipolysis and fatty acid intermediate build-up, and should be weighed against the associated risks and influence on the quality of life in a longer perspective (183, 184).

## 5.3 OCULAR CHARACTERISTICS AND COGNITION

Chorioretinopathy is a well-studied and debilitating complication in patients with LCHAD, which is not seen in other forms of FAODs. We found retinal pigmentations in all patients with LCHAD, which is a higher frequency than previously reported (10, 12, 40, 59, 76). The higher occurrence of chorioretinopathy in this cohort is probably due to a longer follow-up interval and no mortality after the diagnosis. The pathogenesis of this specific type of chorioretinopathy is largely unknown (55), and most likely multifactorial. It was not possible to identify any single clinical factor that was directly related to the progression of the chorioretinopathy in the current study, but the patients with more pronounced ocular changes and decreased retinal function had a combination of more serious clinical events such as older age at diagnosis, severe symptoms at diagnosis and epilepsy. The patients with the most pathological fundus and ERG findings were also the ones with IQ scores in the range of mental retardation and with ASD, suggesting a possible common underlying pathophysiology.

Patients diagnosed at a younger age had, as a group, better ocular and cognitive outcomes, assessed as fundus stage, ERG response, and IQ scores. This may be counterintuitive since it may be assumed that patients with lower residual enzymatic activity would develop energy deficiency and high levels of long-chain acylcarnitines with dramatic symptoms at an earlier age and therefor have a more severe clinical situation. On the other hand, they subsequently received an earlier diagnosis and treatment, which may be a more important positive factor. However severe initial symptoms such as hypoglycemia, metabolic acidosis, coma, heart arrest, and seizures may also cause irreversible harm to the retina and CNS. Therefore, both

the severity of symptoms and the age at diagnosis may be associated with ocular and cognitive disability.

The numbers of metabolic decompensations as a measure of metabolic control are interesting, but challenging to evaluate. Intermittent episodes of metabolic derangements cause overload of acylcarnitines, energy deficiency, and/or hypoglycemia and may be harmful to the retina and CNS. In addition, any event of metabolic decompensation treated with either glucose infusions or "emergency treatment" typically involves extra caloric intakes, and may thus affect weight gain. To arrive at a strict definition of metabolic decompensation is, however, challenging. The tendency to seek healthcare differed between families and was affected by many factors such as age of the child, distance to the nearest hospital, magnitude of pain and symptoms, and knowledge of and attitude to the disease. The numbers of metabolic decompensations were therefore either under- or over-reported, and associations with outcome results were not credible.

Seven of the patients were homozygous for the common G1528C mutation, and three were compound heterozygous, resulting in production of a mutant LCHAD protein and high levels of fatty acid intermediates. Both the specific chorioretinopathy and the cognitive profiles are unique for patients with LCHAD, and are not seen in other forms of FAO defects. This suggests that the defective protein and accumulated intermediates are involved in the development of the retinal changes and cognitive symptoms. Acylcarnitines and fatty acids are important for the CNS. While glucose is the dominant cerebral fuel, fatty acids may be used as well, predominantly by the astrocytes (185). Fatty acids, as well as acylcarnitines, are involved in neuroprotection, gene modification, and neurotransmission (185, 186). Accumulated acylcarnitines easily cross the blood-brain barrier, and hence lipotoxicity may also affect the brain. However, neuropathological light microscopy examinations using specimens from deceased patients with LCHAD, have shown unspecific changes but not fat accumulation (59).

Three patients had IQ scores below normal and autistic symptoms and epilepsy. Furthermore, a third patient, not participating in the neuropsychological testing, had recurrent seizures and delayed psychomotor development. Some of the patients have undergone brain MRI investigations, which have been inconclusive. Autism is a developmental disorder of unknown origin, characterized by persistent deficits in social communication and social interaction, as well as restricted, repetitive patterns of behavior, interests, or activities (146). Intellectual disability and autism spectrum disorder frequently co-occur. Information on neuropsychological function and autism in patients with LCHAD is very limited, and prior to our publication only two patients have been described (141); a boy homozygous for G1528C with developmental delay and IQ< 85, and a girl heterozygous for G1528C with speech delay. Both were diagnosed by newborn screening and had mild retinal pigmentary changes. Several other metabolic defects have been associated with autistic symptoms (187-189) . There are a number of possible interacting factors in LCHAD that may cause epilepsy and affect the development of the CNS and the brain. The medical histories of the patients are

complex, and it is likely that the epileptic seizures have contributed to the lower IQ scores, but it is also possible that the disease has caused cerebral lesions that result in epilepsy. It may not only be the hypoglycemic episodes *per se*, or energy deficiency, but toxic effects of metabolites or deficiencies of certain fatty acids may cause the described deficiencies in cognitive outcome, or a combination of all of them.

A specific cognitive pattern was also noticed in patients with IQ scores in the normal range with a noticeable deficit in verbal auditory working memory. This may influence vocabulary, speech development and reading comprehension, all tasks when phonics or sounds and oral instructions are important (151). Moreover, parental questionnaires stressed the patients' difficulties in executive functions. Deficient executive functions result in disability when skills such as planning, monitoring, multitasking and being flexible become increasingly important with age. These skills are crucial when the patients need to manage the disease and dietary therapy independently. Larger studies with more sophisticated neuroimaging are needed to investigate causalities and draw general conclusions from these findings. Nevertheless, the results are important to be able to identify special educational needs early on.

Another factor essential for ocular and cognitive outcomes in LCHAD may be DHA. Increasing levels of DHA are present in the prefrontal cortex and in the retina during late gestation and during the first years of life (190). DHA has numerous roles in neurocognitive development and affect memory formation by stimulating neuron growth in the hippocampus (7, 191). DHA also plays a major role in visual development and prevents damage of photoreceptors (192-194). It has been suggested that patients with LCHAD may develop DHA deficiency (95, 104), either due to defective synthesis or to low levels of the precursor, linolenic acid. Low DHA levels, especially during the first years of life when neuronal development is particularly vulnerable, could have critical effects on the retina and the developing brain. In our studies, DHA deficiency was not detected. The patients were substituted with DHA and no correlation with DHA levels was seen over the relatively short study period. Hence, we cannot express an opinion on how DHA might have affected the ocular outcome in the patients studied.

## 5.4 METHODOLOGICAL CONSIDERATIONS

The patients in this cohort were investigated for ocular complications and important clinical parameters. Data on growth and diet were collected by reviewing medical charts and lipolysis during fasting was studied by means of microdialysis, infusion of stable isotopes of glucose and glycerol, and biochemical and hormonal blood samples. In addition, cognition and executive functions were studied by means of cognitive tests and parental questionnaires. Some methodological concerns have been identified, beyond the small size of the cohort. Chart reviews are retrospective by nature and data on follow-up parameters, dietary regimen, and height and weight measurements were at times missing or not frequently documented or documented at irregular intervals. In any type of dietary intervention, it is important to monitor compliance. The only way to check whether the patients adhered to the

recommended diet was by checking food diaries and results from blood samples. It is well known that misreporting of food intake is common and that food diaries should be considered to be an approximate guide to food intake rather than a comprehensive research tool (195). The difficulties with estimating the number of metabolic decompensations have already been discussed. The study of lipolysis involved intravenous and subcutaneous catheterization, as well as repeated blood sampling. Due to the limitation of the amount on blood that it was possible to draw, some of the analyses had to be restricted or omitted. Furthermore, we did not include a healthy control group for comparisons, but used the patients as their own controls by analyzing microdialysates during intragastric feeding versus fasting. Moreover, the patients had been on the diet for several years, and it is not known how the lipolytic indicators would be affected with another diet or without night feeds. Two patients with severe phenotypes did not participate in the cognitive tests, which may have caused a selection bias toward patients with better cognitive outcomes in this study.

## 5.5 SUMMARY

In summary, fasting tolerance, assessed by timing of lipolysis, is shorter than was expected and fasting intervals should be determined by taking lipolysis into consideration. Since lipolysis occurs before hypoglycemia it is essential to establish thorough metabolic control around the clock, hence nocturnal intra-gastric feedings with a low-fat formula supplemented with MCT, protein, and carbohydrates should indeed be considered. LCHAD deficiency and the treatment present a challenge, since it is difficult to achieve balanced optimized metabolic control without a corresponding weight gain and overweight. In addition, despite dietary intervention and strict metabolic control, the evolution of chorioretinopathy seems inevitably. The neuropsychological outcome is affected and differs from that in patients with other FAODs. We have speculated that the same factors involved in the development of retinal changes may also impact cognitive outcomes.

The results in this thesis emphasize the importance of frequent clinical follow-ups in order to improve metabolic control and monitor the development of ocular and cognitive complications as well as overweight. Clinical examinations should involve assessments of height, weight, and BMI, with extra attention to cardiac, hepatic, and neurological examinations. It may be possible to counteract a further increase in weight and BMI, by means of dietary alterations with less carbohydrates and increased protein if it is recognized early on. The intake of fat quantities may also be facilitated by recommendations in grams instead of percentages of total calories. Cardiac and hepatic complications require individualized treatment and handling. Ocular examinations are recommended annually, with repeated fundus photography and ERG examinations. Systematic evaluations of developmental and neuropsychological outcomes beyond regular developmental milestones are important. Parental or self-report screening questionnaires are easy to administer, but patients with LCHAD should also participate in formal neuropsychological testing in order to identify specific disabilities and need for special education.

With expanded newborn screening and early treatment, the disease may have a less dramatic development and, hopefully, lead to improved health in patients with LCHAD. Future studies will determine whether the described complications will also occur in asymptomatic patients.

# 6 CONCLUSIONS

- The majority of the patients develop overweight in early childhood, presumably due to excessive caloric intake.
- The disease and the treatment do not seem to affect final height negatively.
- Lipolysis and accumulation of acylcarnitines occur after 3–4 hours of fasting, before hypoglycemia, and despite a normal glucose production rate.
- The recommendation for an acceptable length of fasting periods should be limited and night feeds considered.
- Microdialysis and the stable isotope techniques are suitable methods for studying the dynamics of metabolic processes. Microdialysis is convenient for clinical use.
- The fasting periods in patients with LCHAD should be limited to 4 hours, and night feeds should be considered.
- All patients develop retinal changes to different degrees.
- The current management does not prevent, but possibly delays the development of ocular symptoms.
- Early diagnosis and treatment results in better ocular and cognitive outcomes.
- LCHAD patients demonstrate a specific cognitive pattern. Patients with normal IQ scores have a particular deficit in auditive verbal memory and deficiencies in executive functions, which may affect their learning and ability to independently manage the dietary treatment.
- The neuropsychiatric dissabilities in patients with LCHAD present as autistic spectrum disorders.
- The development of chorioretinopathy and the cognitive outcome may have a common underlying pathophysiology, since the patients with low IQs and autism also have the most pathological ocular examinations.
- Screening for neuropsychological deficits should be included in the routine follow-up, with the purpose of identifying special educational needs early on.

# 7 FUTURE PERSPECTIVES

The major future challenges are to further investigate the causes of the morbidity in LCHAD and thereby improve management and treatment. However, LCHAD is difficult to study due to the lack of viable animal models, few clinical cases, and a lack of common guidelines and treatment protocols. Furthermore, not all countries perform newborn screening for FAOD.

Systematic investigations and comparisons of screened and unscreened cohorts will increase our understanding of the pathogenesis and effectiveness of treatments. This emphasizes the need for multicenter collaborations to facilitate recruitment of a larger selection of study participants. It will be important to evaluate clinical, developmental and neuropsychological outcomes in patients identified by newborn screening and those diagnosed by symptoms in lager numbers of patients.

As discussed in this thesis, fatty acid accumulation and lipotoxicity have been suggested to be part of the pathogenesis in many of the LCHAD-related complications. Going forward it will be vital to further investigate the toxic mechanisms and to explore the levels of acylcarnitines that may be considered harmful. For this, additional and appropriate research methods will be required. The pluripotent stem-cell technology (84), generating LCHAD-deficient retinal pigment epithelial cells, may constitute an interesting model for experimental research. The method could be used to further study the development and background to the chorioretinopathy and also to perform *in vitro* experiments of possible treatment approaches. Viral and non-viral gene replacement therapies have been proposed as future treatment possibilities to lower toxic byproducts (32), and may possibly be explored by the pluripotent stem-cell method.

As also discussed in this thesis, symptoms and complications occur despite treatment. Thus, treatment-alternatives that more effectively inhibits fatty acid intermediate accumulation and provide sufficient energy is warranted. Triheptanoin and anaplerotic substrates, which replenish the citric acid cycle intermediates, are interesting and require further investigation to assess the possible treatment effects.

The earliest discovered patients with LCHAD are now in their 20s and it is not clear what symptoms and complications might arise with increasing age. The dietary intervention with frequent feeds and constant hyperinsulinemia comprises a possible risk for the development of insulin resistance, and it will be important to follow and further evaluate metabolic profiles and insulin sensitivity.

# 8 SVENSK SAMMANFATTNING

## 8.1 KROPPENS ENERGIBALANS

Kroppens celler behöver energi för att fungera. Energin kommer från kolhydrater, protein och fett i maten, men även från kroppsegna reserver. Insulin frisätts i samband med matintag och ökar upptaget av socker (glukos) i cellerna, och hämmar samtidigt fettnedbryningen. Stress och fasta ger utsöndring av adrenalin som stimulerar fettnedbrytningen. Det mest näringstäta ämnet utgörs av fettsyror som består av långa kedjor av kolatomer. Fettsyrorna lagras i fettväven som triglycerider vilka består av tre fettsyror kopplade till glycerol. Vid fettnedbrytning ökar nivåerna av glycerol, som alltså är ett indirekt mått på hur mycket fett som bryts ner. Fettsyrorna omvandlas till energi i cellens kraftverk, mitokondrien, genom *beta-oxidation*. De olika biokemiska reaktionerna i beta-oxidationen underlättas med hjälp av olika proteiner, eller enzymer.

Olika organ föredrar olika sorters energi-bränsle. Hjärnan föredrar glukos, medan hjärtmuskeln föredrar fettsyror. Muskler använder fett vid låg intensivt arbete, men använder glukos lagrat som glykogen vid högintensivt arbete.

## 8.2 FETTSYRA OXIDATIONS DEFEKTER

### 8.2.1 Bakgrund

Fettsyrorna transporteras in i mitokondrien för beta-oxidation. Ett varv av beta-oxidation kortar fettsyran med två kolatomer, varefter fettsyran genomgår ett nytt varv av beta-oxidation, tills hela kedjan är förkortad. För varje varv utvinns energi De sammankopplade sekvenserna gör att beta-oxidationen brukar beskrivas som en spiral. Enzymerna i beta-oxidationen är anpassade efter längden på fettsyran, och det finns enzymer specifika för långa, medellånga respektive korta fettsyror. Vid brist på något av beta-oxidationens enzym drabbas patienten av en fettsyraoxidationsdefekt, vilket medför att fettsyrorna bryts ner ofullständigt och cellen får brist på energi. De ofullständigt nedbrutna fettsyrorna är skadliga och kan inlagras i olika organ. Det finns defekter beskrivna i nästan alla enzymsteg. I den här avhandlingen har vi fokuserat på en av de allvarligaste fettsyra-oxidations defekterna, som beror på en skada i enzymet *Long-chain 3-hydroxyacyl-CoA-dehydrogenas* och orsakar *Long-chain 3-hydroxyacyl-CoA-dehydrogenas-brist*, förkortat LCHAD.

LCHAD är en recessivt ärftlig sjukdom som drabbar ca 1 per 60 000 födslar, vilket innebär att det i genomsnitt föds 1-2 barn per år med LCHAD i Sverige. De första fallen upptäcktes i början av 1980 talet, och de äldsta patienterna är i dag i 20-30 årsåldern. I Sverige ingår sedan 2010 utredning av fettsyraoxidationsdefekter inklusive LCHAD i det blodprov som tas i samband med födseln (nyföddhetsscreening). Det innebär att vi sedan 2010 kan diagnostisera patienter med LCHAD och andra fettsyraoxidationsdefekter innan symptom uppkommer.

### 8.2.2 Symptom

Symptomen uppkommer under de första levnadsåren, ofta i samband med tillstånd då extra energi behövs t.ex. vid en infektion, men även då barnet har långa intervaller mellan måltiderna som vid amningsavvänjning. De första symptomen kan vara dramatiska med lågt blodsocker, hjärtmuskelförstoring, leverpåverkan och plötslig död. En del patienter har mer ospecifika symptom som muskelsvaghet och återkommande episoder av muskelvärk och muskelsönderfall och otillräcklig viktuppgång. På sikt utvecklar patienter med LCHAD specifika ögonbottenförändringar som kan orsaka, synnedsättning och i vissa fall även blindhet. En del patienter utvecklar nedsatt känsel. Kvinnor som väntar ett barn med LCHAD kan drabbas av leversjukdom i samband med graviditeten. Mekanismen för ögonbottenförändringarna och graviditets-komplikationerna är okänd, men tros höra ihop med ansamling av ofullständigt nedbrutna fettsyror. Ögon och graviditets-komplikationerna förekommer inte vid några andra beta-oxidationsdefekter, och det verkar därför finnas en speciell sjukdomsmekanism vid LCHAD.

### 8.2.3 Diagnostik

Misstanke om LCHAD bör utredas snabbt eftersom symptomen kan vara livshotande och förbättras med insatt behandling. För att ställa diagnosen krävs rutin-blodprover som blodvärde, blodsocker, leverprover, kreatin-kinas, men även specifika blodprover av ofullständigt metaboliserade fettsyror samt mutationsanalys. En specifik mutation (G1528C) i genen för LCHAD enzymet är särskilt vanlig och resulterar i att enzymet blir felaktigtt.

### 8.2.4 Behandling

Behandlingen skiljer sig i olika länder i världen. I Sverige behandlas patienter med LCHAD av ett team med läkare, dietister, sjuksköterskor och psykologer som har specialistkunskap om ärftliga ämnesomsättningssjukdomar. Behandlingen är livslång och syftar till att bromsa fettnedbrytningen och samtidigt tillgodose näringstillförseln. Insulins hämmande effekt på fettnedbryningen utnyttjas i behandlingen av patienter med LCHAD, genom att rekommendera täta måltider. I Sverige rekommenderar vi också att patienterna erhåller ett näringsdropp nattetid. Fettintaget minskas kraftigt så att mindre än 20 % av det totala kaloriintaget utgörs av fett. Familjerna får noggranna instruktioner om hur fettinnehåll beräknas och hur många gram fett som max tillåts i kosten. Det fett som ges ska framför allt bestå av medel-långa fetter, så kallat MCT fett, som förbränns utan LCHAD-enzymet. Kosten vid LCHAD skiljer sig väsentligt från den som rekommenderas för växande barn.

För att undvika allvarliga symptom bör infektioner behandlas med extra näringstillförsel, vilket ofta innebär att barnet måste vårdas på sjukhus med glukosdropp. Trots behandling, får patienter med LCHAD återkommande episoder av muskelpåverkan.

## 8.3 SYFTE

Syftet med den här avhandlingen har varit att beskriva det kliniska förloppet hos patienter med LCHAD. Särskilt har vi velat studera hur kosten och sjukdomen påverkar längd och

vikt, samt hur energiomsättningen påverkas vid fasta. Vi har också undersökt om det finns något samband mellan ålder vid diagnos, sjukdomssymtom och ögonbottenförändringar och kognitiv utveckling. Studierna är gjorda på tio patienter med LCHAD som följts vid Karolinska Universitetssjukhuset och Uppsala Universitetssjukhus, innan nyföddhetsscreening för fettsyraoxidations defekter infördes i Sverige.

## 8.4 METODER

Medicinska data från tillväxtkurvor och patientjournaler registrerades. Kliniska symtom graderades och relaterades till resultatet av ögon och kognitiva undersökningar.

Mikrodialys innebär att en tunn slang sätts in i underhudsfettet på magen under bedövning. Slangen, som har två hålrum, spolas med en lösning som liknar vätskan mellan cellerna. Små molekyler som socker och glycerol passerar fritt från fettväven in i slangen och analyseras med regelbundna intervaller. Metoden är ett okomplicarat sätt att få en uppfattning om ämnesomsättningen i fettväven och uppfattas inte som besvärlig av de flesta barn.

Stabil isotop teknik möjliggör att ämnen och olika ämnesomsättningsvägar kan spåras i kroppen. Samma grundämne kan ha olika atomvikter, eller olika isotoper. En stabil isotop förändras inte över tid till skillnad från en radioaktiv isotop som faller sönder. De stabila isotoperna ges som dropp intravenöst under flera timmar, varvid isotopkoncentrationen ökar. Slutligen uppnås en platå där inte koncentrationen ändras något mer. Med hjälp av blodprover går det att skilja på tillsatt isotop och det ämne som kroppen tillverkat.

Indirekt kalorimetri ger ett mått på energiomsättningen och vilka ämnen(substrat) som kroppen använder för att tillverka energi. Syre- och koldioxidmängden mäts i in- och utandningsluften. Energiomsättningen beräknas utifrån syreförbrukningen, och den så kallade respiratoriska kvoten (RQ) ger uppgift om substratutnyttjande. En respiratorisk kvot på 1.0 visar att det är kolhydrater som är den huvudsakliga energikällan, medan en respiratorisk kvot runt 0.7 motsvarar fettförbränning.

Ögonundersökningar syftar till att undersöka synskärpan och funktionen av näthinnan, samt för att registrera eventuell pigmentering av ögats näthinna. Med elektroretinografi (ERG). Registreras näthinnas reaktion på ljusstimulans. Vid ERG måste små barn sövas.

En psykolog genomförde åldersanpassade kognitiva tester med hjälp av Wechsler skalorna och undersökte exekutiva funktioner med specifika frågeformulär som ifylldes av föräldrarna (ABAS och BRIEF).

## 8.5 RESULTAT

Vi fann att patienterna hade en snabb viktökning efter påbörjad kostbehandling i samband med att de fått LCHAD-diagnosen. De täta måltiderna och näringstillförseln nattetid gav upphov till övervikt hos majoriteten av barnen. Vi såg också att längdtillväxten ökade efter viktuppgången, däremot verkade inte slutlängden påverkas.

Undersökningarna av energiomsättningen vid fasta gjordes med barnet inneliggande på sjukhus under två dygn. Första dygnet motsvarade en ordinarie årskontroll inklusive mikrodialysundersökning. Under andra dygnet fastade barnet sex timmar nattetid. Fett- och kolhydratomsättningen undersöktes med stabil isotop teknik (glukos och glycerol), mikrodialys, indirekt kalorimetri samt blodprover för hormoner viktiga för ämnesomsättningen. Trots normala blodglukosvärden och normal glukosproduktions hastighet (19.6 ± 3.4 umol/kg/min), började patienterna bryta ner fett redan efter 3-4 timmar, vilket illustrerades av ökad glycerol produktionshastighet (7.7 ± 1.6 umol/kg/min). Dessutom steg nivåerna av ofullständigt nedbrutna fettsyror, samt glycerol i plasma och i vätskan från mikrodialysen.

Ämnesomsättningen i vila var normal för åldern och den respiratoriska kvoten var hög (0.9), vilket tyder på att kolhydrater utgjorde den huvudsakliga energikällan. Resultaten visar att fettförbränningen inte fungerade optimalt, eftersom barnen borde förbränna fett efter fasta.

Alla patienter utvecklade pigmentförändringar på ögats näthinna. Ögonförändringarna orsakade kraftig synnedsättning hos två av de äldre patienterna. Vi såg att patienter med tidig diagnos och behandling utvecklade mildare förändringar som uppkom vid en högre ålder.

Kognitiv funktionsnedsättning var vanligare än väntat, och uppvisade ett specifikt mönster. Majoriteten av patienterna hade normalt IQ med en svaghet i auditivt verbalt arbetsminne, vilket kan ha betydelse för ordförråd, talutveckling och läsförståelse. Patienterna hade nedsatta exekutiva funktioner vilket kan påverka förmågan att självständigt följa diet och behandlingsrekommendationer. Tre patienter (38 %) hade en försenad utveckling och autistiska beteenden.

## 8.6 SLUTSATSER

Sammanfattningsvis visar denna avhandling att patienter med LCHAD börjar bryta ner kroppens fettreserver avsevärt tidigare än friska barn. Det innebär att täta måltider och näringsdropp nattetid är viktiga för att minska ansamlingen av ofullständigt nedbrutna fettsyror. Den defekta fettförbränningen gör att patienterna använder kolhydrater som bränsle under fasta. Dieten gör att patienterna har en risk för viktökning och övervikt. Alla patienter utvecklade ögonförändringar, men både ögon- och kognitiva komplikationer var mindre utpräglade vid tidig diagnos och strikt tidig behandling. Specifik kognitiv påverkan förekommer, och det är därför viktigt att testa kognitiva och exekutiva funktioner hos patienter med LCHAD, så att särskilda stöd- och utbildningsbehov identifieras tidigt.

# 9 ACKNOWLEDGMENTS

This thesis reflects an almost ten-year journey from concept to publication, which would certainly not have been possible without the assistance, advice and encouragement from several respected individuals. Above all I am greatly indebted to all the families who participated in the studies, and admire your efforts, diligence and daily challenges that living with a complicated disease entails.

In particular I would like to express my genuine gratitude to

*Anna Nordenström*, my supervisor, for ensnaring me into the fascinating world of research, and beta-oxidation defects in particular. You are a strong-willed, unpretentious and dedicated clinician and researcher, and I admire your ability to fearlessly manage the role as national expert in numerous medical areas. Also, thanks for being a great friend.

*Maria Halldin Stenlid*, my co-supervisor, for introducing me to the stable isotope technique, and to Uppsala University. It has been very rewarding to meet and learn from you and your knowledgeable colleagues. I treasure your careful, thoughtful and balanced opinion which has nurtured this research and thesis.

*Ulrika von Döbeln*, my co-supervisor, for welcoming me to the Center for Inherited Metabolic Diseases and for cooperation with your talented team. Your energetic and passionate approach to metabolism is truly contagious. I appreciate your ability to always listen, think, and come up with a wise response, as well as your capability to add the finishing touch.

*Sara Ask*, co-author, for sharing your vast knowledge on nutrition, and for daring to be different and follow your dreams.

*Anna Bengtsson Strandqvist*, co-author, for educational discussions on neuropsychology and executive functions, which have been extra rewarding on account of your subtle sarcasm and intellectual approach.

*Jan Gustafsson*, co-author, Uppsala University, for expert assistance with analyses and interpretations of the stable isotope data.

*Kristina Teär Fahnehjelm*, *Gerd Holmström*, and *Liu Ying*, co-authors, for performing and sharing the results of the ocular investigations and ERG examinations.

*Jan Alm*, *Antal Nemeth* and *Rolf H Zetterström*, co-authors, for valuable and insightful discussions on the contents of the studies.

Collaborators *Siw Siljerud*, *Sindra Isetun*, *Martin Engvall*, *Sten Salomonsson*, *Roger Olsson* and *Elisabeth Söderberg* for skillful laboratory and technical expertise, nurses *Jenny Gårdman*, *Jaana Nejla*, *Kerstin Ekbom*, *Karin Johansson*, and *Gunilla Wallin* for excellent

assistance and patient care at the Center for Inherited Metabolic Diseases, Karolinska University Hospital and Uppsala University.

*Melanie Gillingham* and colleagues at the Oregon Health & Science University, Portland, USA, for outstanding and clinically applicable research. I am very grateful for the opportunity to visit you, – it was one of the absolute highlights of my graduate studies.

*Mona-Lisa Engman*, Head of the General Pediatrics and Emergency Medicine, Karolinska University Hospital Huddinge, for being a clever person, great boss, and for bearing with me despite my very long absence of leave.

All the wonderful *doctors and nurses* at the departments of General Pediatrics and Emergency Medicine, as well as Diabetes Endocrinology Metabolism and Obesity (DEMO), for professional patient care and for being great colleagues.

*Hans Lindblad*, late Head of the General Pediatrics and Emergency Medicine, Karolinska University Hospital Huddinge, for always being generous and accepting time away for research. I will always remember you as a wise man with a big heart.

*Ann-Britt Bohlin*, former Head of Children's Hospital, Karolinska University Hospital, Huddinge, for accepting me for pediatric residency training, and creating a great atmosphere for both clinical work and research.

*Barbro Friden*, former Division Head of Children's Hospital, Karolinska University Hospital, for demonstrating and sharing great leadership skills in a complex organization. You encouraged me to take on new challenges beyond my regular responsibilities which have been very rewarding.

*Isaac Austin*, for efficient, correct and much appreciated comments on the English language in this thesis.

*Veronica Strandell*, for designing the cover illustration and teaching me precious computer tricks. You are a wonderful friend as well as SWEA Chicago teammate.

*Folke Flam*, my mentor, and your wife Nomi Flam for giving me my first stethoscope and for encouraging me to become a pediatrician. You always make things seem so easy, no matter if it is completing a thesis or being a successful entrepreneur in health care.

*Ove and Siv Haglind*, my incredible and very helpful in-laws. Without your passionate support it would not have been possible to attend research conferences and graduate courses. Thanks for being a frequent visitor to the North Shore – we have enjoyed your company.

*Lena and Wolf Bieneck*, my beloved parents, for always being considerate and for your never-ending support and encouragement. Your positive attitude and great sense of humor is truly inspiring. Spending time with you is always very enjoyable and comforting, and we are grateful to share so many joint memories from our years overseas.

*Ella and Carl*, my funny, wise and wonderful children, you are my greatest joy and inspiration.

*Magnus*, my dear husband and life companion. Thank you for encouraging and inspiring me, and for bringing so much happiness to my life.

Last but not least, I would like to thank *friends and family* for friendship and all your motivating support.

The studies in this thesis have been supported by grants from the regional agreement on medical training and clinical research (ALF) between Stockholm County Council and the Karolinska Institutet and Uppsala County and Uppsala University, The Swedish Order of Freemasons (Frimurare Barnhuset), HRH Crown Princess Lovisa's Association for Child Medical Care (HKH Kronprinsessan Lovisas förening för barnasjukvård), The Sven Jerring Foundation, The Samariten Foundation, The Child Care Society, The Gillberg foundation, Vera Ekström Foundation and the Swedish Neuropsychological Society.

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