Mitochondrial trifunctional protein in disease and development

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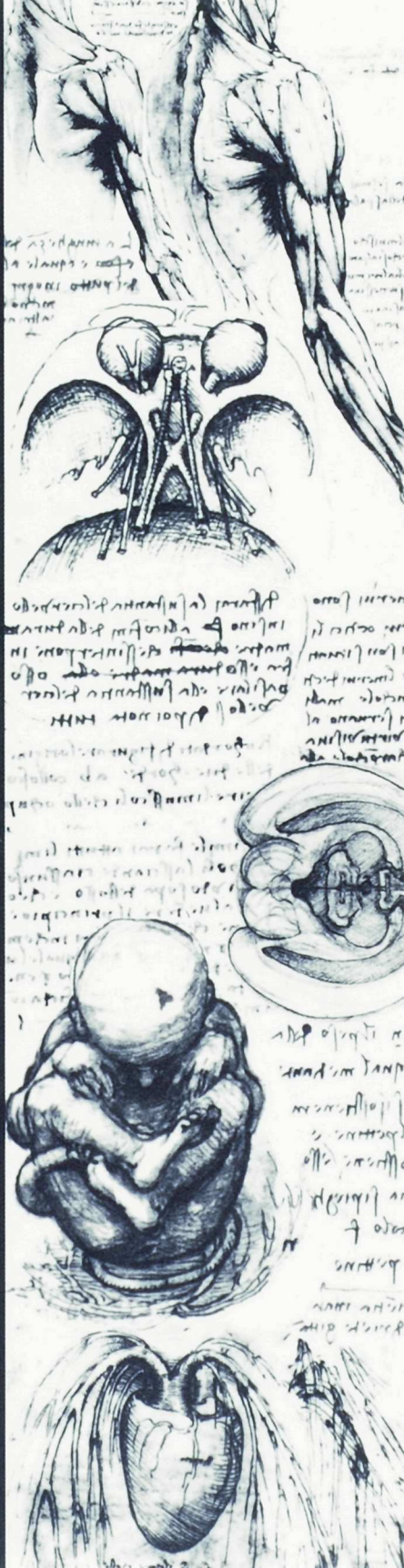
Lisette den Boer



Stellingen

behorende bij het proefschrift

Mitochondrial Trifunctional Protein in disease and development

1. MTP- en LCHAD-deficiëntie horen thuis in de differentiaal diagnose van cardiomyopathie, hypotonie, perifere neuropathie, failure to thrive en cholestase (dit proefschrift).
2. Hydrops foetalis en intra-uteriene groeiretardatie kunnen symptomen zijn van een lang-keten vetzuuroxidatiestoornis (dit proefschrift).
3. De hoge incidentie van HELLP-syndroom en AFLP in moeders die zwanger zijn van een LCHAD- of MTP-deficiënt kind wordt vooral veroorzaakt door een verstoorde placentaire vetzuuroxidatie (dit proefschrift).
4. De pasgeboren kinderen van vrouwen die tijdens hun zwangerschap een ernstige vorm van HELLP-syndroom of een AFLP doormaakten, behoren onderzocht te worden op de aanwezigheid van een aangeboren deficiëntie van de lang-keten vetzuuroxidatie (dit proefschrift).
5. Niet alleen MCAD-deficiëntie, maar ook de lang-keten vetzuuroxidatiestoornissen moeten worden opgenomen in het neonatale hielprik-screeningsprogramma, omdat hierdoor zowel de mortaliteit als de morbiditeit kan worden verminderd (dit proefschrift).
6. Lang-keten vetzuuroxidatie is van belang tijdens de vroege ontwikkeling van het humane embryo (dit proefschrift).
7. Het verrichten van promotie onderzoek tijdens de opleiding tot medisch specialist leidt ertoe dat men van beiden niet optimaal kan genieten.
8. Kennis is nog geen wijsheid (A. Schopenhauer).
9. Als Nederland een kenniseconomie wil blijven, zal zij er zorg voor moeten dragen dat haar kennisdragers van voldoende financiële middelen worden voorzien.
10. Wetenschap is de titanische poging van het menselijk intellect zich uit zijn kosmische element te verlossen door te begrijpen (W.F. Hermans).
11. Als artsen meer tijd aan een gedegen anamnese zouden besteden, zou dit leiden tot een aanzienlijke besparing van ziektekosten.
12. Elk afscheid is de geboorte van een mooie herinnering (Michelangelo).

Lisette den Boer, 18 december 2003

Mitochondrial Trifunctional Protein in disease and development

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Mitochondrial Trifunctional Protein in disease and development

ACADEMISCH PROEFSCHRIFT

ter verkrijging van de graad van doctor
aan de Universiteit van Amsterdam
op gezag van de Rector Magnificus

prof. mr. P.F. van der Heijden

ten overstaan van een door het college
voor promoties ingestelde commissie,
in het openbaar te verdedigen in de Aula der Universiteit

op donderdag 18 december 2003, te 12.00 uur

door

Margarethe Elizabeth Joyce den Boer

geboren te Gouda

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List of Abbreviations

AcAc	acetoacetate
ADP	adenosine diphosphate
AFLP	acute fatty liver of pregnancy
ATP	adenosine triphosphate
CACT	carnitine acylcarnitine translocase
CK	creatine kinase
CPT1	carnitine palmitoyl transferase 1
CPT2	carnitine palmitoyl transferase 2
DHA	docosahexaenoic acid
ETF	electron-transferring flavoprotein
FAD	flavine adenine dinucleotide
FAO	fatty acid oxidation
FFA	free fatty acid
3HB	ß-hydroxybutyrate
HELLP	hemolysis elevated liver enzymes and low platelets
HMG-CoA	3-hydroxy-2-methylglutaryl-CoA
IUGR	intra-uterine growth retardation
LCAD	long-chain acyl-CoA dehydrogenase
LCEH	long-chain enoyl-CoA hydrogenase
LCHAD	long-chain 3-hydroxyacyl-CoA dehydrogenase
LCHADD	long-chain 3-hydroxyacyl-CoA dehydrogenase deficiency
LCKAT	long-chain ketoacyl-CoA thiolase
LCT	long-chain triglycerides
MCAD	medium-chain acyl-CoA dehydrogenase
MCADD	medium-chain acyl-CoA dehydrogenase deficiency
MCKAT	medium-chain ketoacyl-CoA thiolase (general thiolase)
MCT	medium-chain triglycerides
MTP	mitochondrial trifunctional protein
MTPD	mitochondrial trifunctional protein deficiency
NAD	nicotinamide adenine dinucleotide
OCTN2	carnitine transporter (organic cation transporter 2)
SBCAD	short-branched-chain acyl-CoA dehydrogenase
SCAD	short-chain acyl-CoA dehydrogenase
SCEH	short-chain enoyl-CoA hydrogenase (crotonase)
SCHAD	short-chain 3-hydroxyacyl-CoA dehydrogenase
SCOT	succinyl-CoA oxoacid transferase
T2	acetoacetyl-CoA thiolase
VLCAD	very long-chain acyl-CoA dehydrogenase
VLCFA	very long-chain fatty acids

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Introduction

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Fatty acid oxidation

Introduction

The fatty acid oxidation (FAO) pathway degrades fatty acids. Fatty acids, obtained from our diet, synthesis by the liver or release from adipose tissue, are the most important energy storage depot in man. Whereas the complete oxidation of fatty acids yields 37 kJ per gram or even more, proteins and carbohydrates yield only about 17 kJ per gram. Additionally, fatty acids are non-polar and will be stored anhydrous, in contrast to proteins and carbohydrates. Consequently, the intracellular storage of fatty acids is by far the most economic energy storage depot in the human body. The oxidation of fatty acids is stimulated during catabolic, aerobic circumstances, like fasting and prolonged mild exercise. In some tissues, like myocardial tissue and renal cortex, fatty acids are preferred as the main energy source under all circumstances. In the liver, acetyl-CoA produced by FAO can also be used for the synthesis of ketone bodies. Ketone bodies can be used as fuel by several tissues, especially by the brain, which is not capable to oxidize fatty acids. FAO takes place in both mitochondria and peroxisomes. Mitochondrial and peroxisomal FAO show important similarities, but they differ in regulation, energy production and especially substrate specificity. The mitochondria are responsible for the oxidation of the bulk of the fatty acids derived from the diet and from fatty acids stored in adipocytes, mainly represented by long-chain fatty acids. The peroxisomes are responsible for the oxidation of those fatty acids that cannot be oxidized in the mitochondria, like for example very long-chain fatty acids (VLCFA), bile acid intermediates, pristanic acid and long-chain dicarboxylic fatty acids. Major differences in the clinical presentation of defects in the mitochondrial and peroxisomal ß-oxidation indicate that these ß-oxidation systems have to be considered as two separate systems with completely different physiological functions.

Mobilization and activation of fatty acids

In catabolic situations, the hormones glucagon, adrenalin and cortisol will activate hydrolysis of triacylglycerols, releasing glycerol and free fatty acids. Via the circulation most of the glycerol will be taken up by the liver to serve as a substrate for gluconeogenesis. The fatty acids, in the circulation bound to albumin, will be taken up by different tissues. Inside the cell fatty acids are transported to the mitochondria both by passive diffusion and by protein-mediated binding mechanisms by fatty acid transport proteins (FATPs). Intracellular, fatty acids are bound by fatty acid binding proteins (FABPs), which are considered to be important carriers for intracellular fatty acids[1]. Cytosolic acyl-

CoA synthetases subsequently activate the fatty acids to form acyl-CoA esters, which may undergo different fates, including incorporation into triglycerides, phospholipids and cholesteryl esters. Under catabolic circumstances, the acyl-CoA esters will primarily be channeled into the mitochondrial matrix for ß-oxidation via the carnitine cycle.

The carnitine cycle

The carnitine cycle (figure 1) transfers the long-chain acyl-CoA esters from the cytosol into the mitochondrial matrix, by binding carnitine to the acyl-CoA ester. Carnitine palmitoyl transferase (CPT) 1, located in the outer mitochondrial membrane, converts the acyl-CoAs to their acylcarnitine derivatives, which cross the outer mitochondrial membrane.

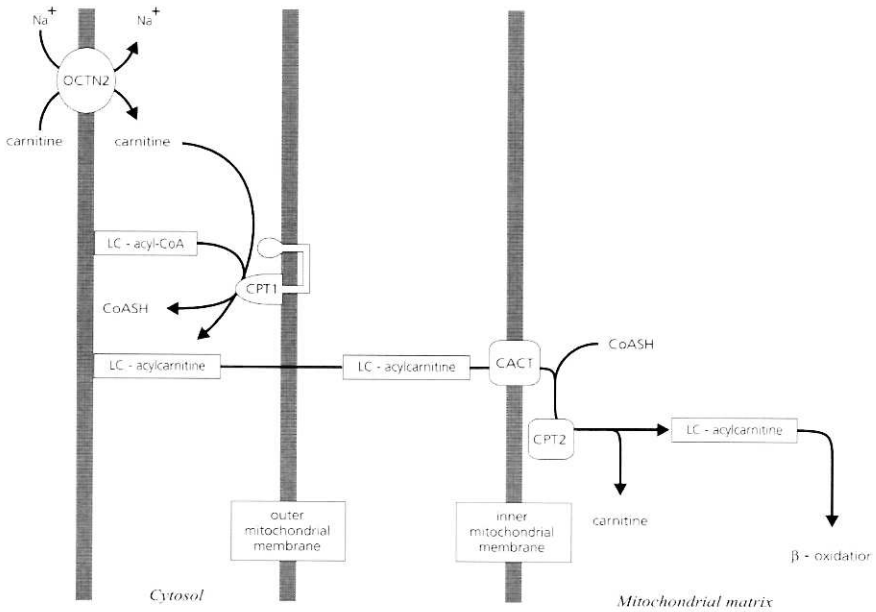


Figure 1- Carnitine cycle

The long-chain acylcarnitine esters easily can be transported across the mitochondrial membranes by a specific carrier, carnitine-acylcarnitine-translocase (CACT). CPT2, located on the matrix side of the inner mitochondrial membrane, reconverts the acylcarnitines to their acyl-CoA esters. The acyl-CoA esters can now enter the β-oxidation pathway (figure 2). The released carnitine is transported to the cytosol for reentering the carnitine cycle.

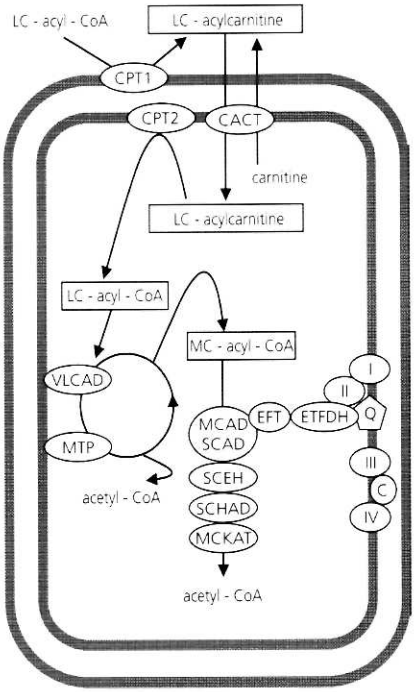


Figure 2 – Enzymatic organisation β-oxidation

Medium-chain fatty acids can enter the mitochondrial matrix without the involvement of the carnitine cycle. They will be activated to their acyl-CoA esters in the mitochondrial matrix by a medium-chain acyl-CoA synthetase. Liver CPT1 is very important in the regulation of FAO, because it is inhibited by malonyl- CoA[2], the first intermediate in fatty acid synthesis. Via this mechanism, a high concentration of malonyl-CoA, synthesized from glucose in the fatty acid synthesis process, prevents fatty acids from being transported to into the mitochondrial matrix for FAO.

The ß-oxidation pathway

Inside the mitochondrial matrix the acyl-CoA enters the ß-oxidation pathway (figure 3). The ß-oxidation pathway is cyclic and shortens the long-chain acyl-CoA with 2 carbons by the formation of an acetyl-CoA unit each time a cycle is fully completed. The ß-oxidation pathway involves four reactions: dehydrogenation, hydration, a second dehydrogenation and thiolytic cleavage. For each reaction of the ß-oxidation spiral several chain-length specific iso-enzymes are known (figure 3). The reactions and the involved iso-enzymes are discussed below.

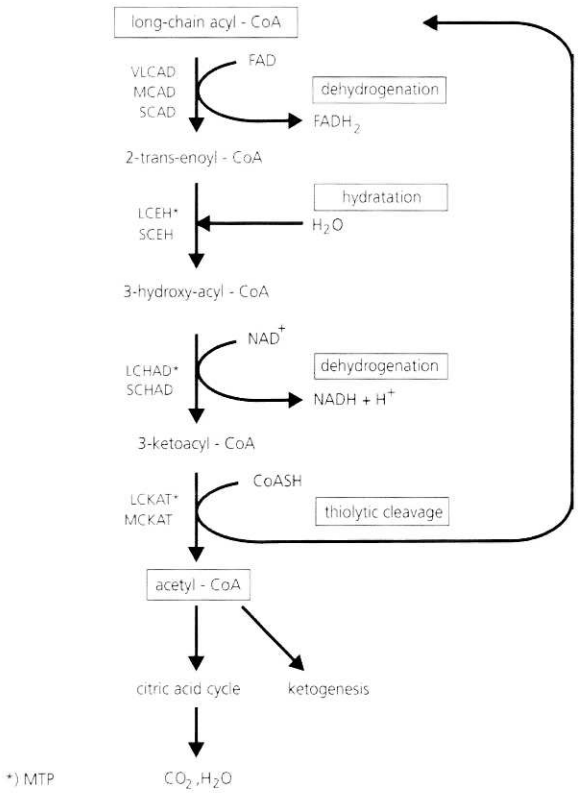


Figure 3 – β-oxidation pathway

Fatty acyl-CoA dehydrogenases

The first dehydrogenation of the ß-oxidation is catalyzed by fatty acyl-CoA dehydrogenases, located in the mitochondrial matrix. Most dehydrogenases are composed of four identical subunits and are noncovalently carrying a flavine adenine dinucleotide (FAD). The dehydrogenases insert a double bond between the second and third carbon atom, resulting in reduction of FAD into $FADH_2$ and the formation of a 2-trans-enoyl-CoA. The enzyme bound $FADH_2$ is reoxidized to FAD via transfer of a pair of electrons to electron-transferring flavoprotein (ETF). The reduced ETF produced in this way is than reoxidized via the enzyme ETF dehydrogenase (ETFDH) finally resulting in the reduction of co-enzyme Q of the respiratory chain (figure 2). A wide range of substrate specific dehydrogenases is known: very long-chain acyl-CoA dehydrogenase (VLCAD), long-chain acyl-CoA dehydrogenase (LCAD), medium-chain acyl-CoA dehydrogenase (MCAD), short-chain acyl-CoA dehydrogenase (SCAD), short branched-chain acyl-CoA dehydrogenase (SBCAD), isovaleryl-CoA dehydrogenase (IVD) and glutaryl-CoA dehydrogenase.

Although the different dehydrogenases prefer substrates of different chain lengths, their substrate specificity shows some overlap. The membrane-bound VLCAD accepts C12-CoA to C24-CoA, preferring C16-CoA as a substrate[3]. MCAD accepts acyl-CoAs ranging from C4 to C16, but is most active with C6- and C8-CoA. The short-chain enzymes, accept both C4- and C6-CoAs as substrate. For a long time the LCAD enzyme was thought to play an important role in the degradation of long-chain fatty acids, but only recently it became clear that the role initially attributed to LCAD, is in fact performed by VLCAD. LCAD in contrast, mainly reacts with 2-methyl branched-chain acyl-CoAs[4]. SBCAD, preferentially reacting with methylbutyryl-CoA and isobutyryl-CoA, appears to be the isoform of LCAD, sequentially involved in the degradation of short-chain 2-methylbrainched-chain acyl-CoAs[5].

Enoyl-CoA hydratases

The hydratation of 2-trans-enoyl-CoAs to their 3-hydroxyacyl-CoAs is catalyzed by 2-enoyl-CoA hydratases (figure 3). A short-chain and a long-chain hydratase are known, both localized in the mitochondrial matrix. The short-chain enzyme, a homohexamer called crotonase (SCEH), has a substrate specificity ranging from crotonyl-CoA (C4), which it prefers as a substrate, to 2-trans-hexadecenoyl-CoA (C16), on which it acts less efficient. The long-chain enoyl-CoA hydratase (LCEH), which has appeared to be part of the mitochondrial trifunctional protein (MTP)[6,7], shows some overlap in substrate specificity with crotonase and reacts with all 2-trans-enoyl-CoAs except for crotonyl-CoA.

3-Hydroxyacyl-CoA dehydrogenases

The third step of the ß-oxidation cycle, a second dehydrogenation, is catalyzed by 3-hydroxyacyl-CoA dehydrogenases. The dehydrogenation of 3-hydroxyacyl-CoA results in the formation of a 3-ketoacyl-CoA and the reduction of nicotinamide adenine dinucleotide (NAD) into NAD reduced form (NADH), which is subsequently oxidized by the respiratory chain at the level of complex I.

In mammals, two 3-hydroxyacyl-CoA dehydrogenases are known to be involved in the oxidation of fatty acids: a short-chain enzyme (SCHAD), a dimer of two identical subunits formed in the cytosol and subsequently transported into the mitochondrial matrix, and a long-chain enzyme (LCHAD), associated with the inner mitochondrial membrane. The LCHAD enzyme is one of the three components of the MTP[6,8], which will be discussed later.

Although SCHAD can react with 3-hydroxyacyl-CoA esters ranging from C4 to C16, it is most active with C4-, C6-, C8- and C10-substrates[9]. LCHAD is active with medium- and long-chain substrates from C6 and more carbons, but shows a rapidly increasing activity with substrates containing more than 10 carbons[10].

3-Ketoacyl-CoA thiolases

The fourth and final reaction is the thiolytic cleavage of the bond between the second and third carbon atom, catalyzed by a 3-ketoacyl-CoA thiolase in the presence of a CoA-unit.

The result is the production of an acetyl-CoA and the fatty acyl-CoA ester, shortened with two carbons.

In mammalian two thiolases are known to be active in the oxidation of fatty acids. The medium-chain enzyme, MCKAT or general thiolase, reacts with ketoacyl-CoA esters ranging from C4 to C106. Its activity is rapidly decreasing with substrates longer than C10. The long-chain thiolase (LCKAT), part of the MTP[6,8], prefers substrates ranging from C8 to C16.

Mitochondrial Trifunctional Protein (MTP)

MTP harbors the activity of three out of the four enzymes required for the oxidation of long-chain fatty acids: the long-chain enoyl-CoA hydratase, the long-chain 3-hydroxyacyl-CoA dehydrogenase (LCHAD) and the long-chain thiolase (figure 3). MTP is a heterooctamer of 4 α- and 4 ß-subunits and is, like VLCAD, associated with the inner mitochondrial membrane (figure 2). The α-subunit carries the LCEH and the LCHAD

activities; the ß-subunit harbors the LCKAT activity. The α- and ß-subunits are encoded by different nuclear genes, containing 20 and 16 exons respectively, which lay head-to-head, adjacent to each other on chromosome 2p23[11,12]. Probably they are transcribed from the same bi-directional promoter region[13], resulting in the possibility to coordinate expression of the two subunits[14].

Ketone body metabolism

Introduction

The main ketone bodies, acetoacetate (AcAc) and ß-hydroxybutyrate (3HB) are energy-rich compounds that transport energy from the liver to other tissues, especially the brain. Unlike other tissues, the brain is not capable to oxidize fatty acids, and is therefore completely dependent on glycolysis for all energy requirements under normal circumstances. During prolonged fasting, the brain can utilize ketone bodies as a substrate.

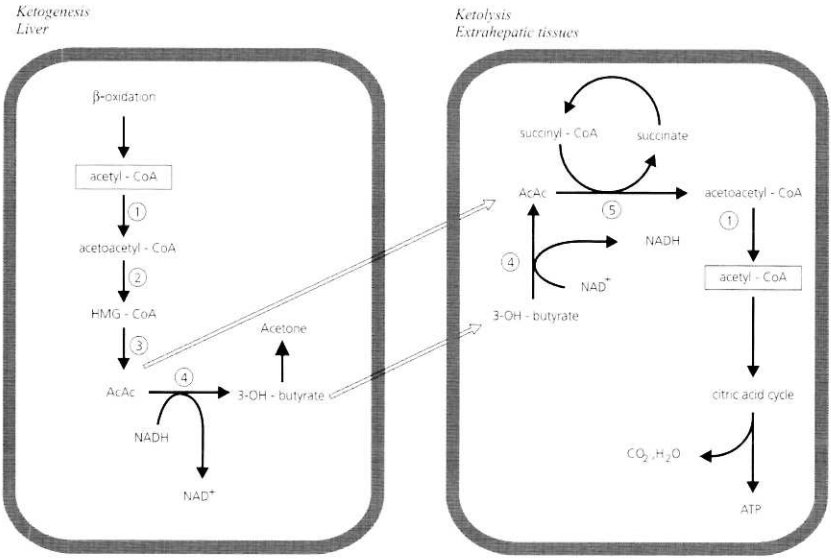


Figure 4 – Ketogenesis and ketolysis

Ketogenesis

Ketogenesis is the mitochondrial process that converts acetyl-CoA, derived from the FAO pathway, into the ketone bodies AcAc, 3HB and acetone (figure 4). The pathway is mainly hepatic, but to a lesser extent it is also active in kidney[15], the intestines of suckling mammals[16] and in the cortical astrocytes of neonates[17,18].

The rate of ketogenesis is most likely determined by the concentration of acetyl-CoA in the mitochondria and the availability of oxaloacetate. Acetyl-CoA can be derived from

both glycolysis and FAO. To enter the citric acid cycle acetyl-CoA will condense with oxaloacetate. Oxaloacetate can be formed from pyruvate, the product of glycolysis, via the enzyme pyruvate carboxylase. During fasting conditions oxaloacetate is, in addition to the production from pyruvate now also produced from amino acids and lactate, used mainly for gluconeogenesis. Under these circumstances, the acetyl-CoA produced from intensified FAO, can not condense with oxaloacetate, resulting in an increased acetyl-CoA concentration in the mitochondrial matrix. This condition results in an acceleration of ketogenesis.

Ketogenesis involves four enzymatic steps and starts two acetyl-CoA units. Firstly, an acetoacetyl-CoA is formed from two acetyl-CoA units by a reversible thiolase reaction. If the inner mitochondrial acetyl-CoA concentration is high, the involved enzyme mitochondrial AcAc-CoA thiolase (T2) shifts the equilibrium to AcAc-CoA synthesis. Secondly, a third acetyl-CoA unit is added to acetoacetyl-CoA by the highly regulated hepatic mitochondrial 3-hydroxy-2-methylglutaryl-CoA (HMG-CoA) synthase (mHS), which results in the formation of 3-hydroxy-3-mehylglutaryl-CoA (HMG-CoA). HMG-CoA can also be produced by leucine catabolism, using the same set of enzymes. Thirdly, HMG-CoA is converted into AcAc and an acetyl-CoA unit by 3HMG-CoA lyase. Fourthly, a NADH dependent reduction of AcAc, catalyzed by ß-hydroxybutyrate dehydrogenase, results in the formation of 3HB. Small amounts of AcAc will spontaneous decarboxylize to acetone, a volatile ketone body.

AcAc and 3HB, both short-chain organic acids enter the circulation and are transported to the tissues (figure 4). They pass the blood-brain barrier by the monocarboxylate carrier. Catabolic states accompanied by low concentrations of insulin and subsequent reduced levels of malonyl-CoA, will stimulate lipolysis, FAO and ketogenesis. In this, malonyl-CoA controls ketogenesis as it controls FAO, by inhibiting substrate availability by regulation of CPT1. Another site of regulation during prolonged fasting is at the level of the expression of genes encoding for CPT1[19] and hepatic mitochondrial HMG-CoA synthase[20], one of the enzymes involved in the ketogenesis.

Ketolysis

Ketolysis is the mainly extrahepatic pathway, in which AcAc and 3HB back are converted back again into acetyl-CoA (figure 4). Firstly, 3HB is reconverted into AcAc by the enzyme ß-hydroxybutyrate dehydrogenase, which was also involved in the final step of ketogenesis (see above), reducing NAD into NADH. Transfer of a CoA-group from succinyl-CoA, which is derived from the citric acid cycle, to AcAc catalyzed by succinyl-

CoA oxoacid transferase (SCOT), results in the formation of acetoacetyl-CoA and succinate. Thirdly, the mitochondrial AcAc-CoA thiolase (T2) which was also involved in the first step of ketogenesis (see above), cleaves acetoacetyl-CoA into acetyl-CoA units again. The acetyl-CoA units will enter the citric acid cycle, producing the reducing equivalents NADH and $FADH_2$, which are finally oxidized by the mitochondrial respiratory chain, resulting in the production of adenosine triphosphate (ATP) (figure 4). The rate of ketone body utilization is proportional to the plasma concentration of AcAc and 3HB. Interestingly SCOT, which has its highest activity in heart, kidney and brain, is downregulated by high concentrations of AcAc[21] and subsequently causes increasing concentrations of ketone bodies in the blood after periods of prolonged fasting (3 days to 2 weeks). However, the exact mechanism and physiologic relevance of this regulation still remains unknown.

Long-chain fatty acid oxidation disorders

Introduction

The disorders of mitochondrial FAO comprise a rapidly growing group of inherited, autosomal recessive metabolic diseases. After the first description of patients with deficiencies of enzymes involved in the carnitine cycle (the muscular form of CPT2 deficiency) in the 1970s[22,23], it remained quiet for a long period. It took until the early 1980s until the discovery of MCAD deficiency[24,25] caused a tremendous acceleration in the recognition of many other FAO disorders. The delay between the discovery of patients with disorders of the carnitine cycle and MCAD deficiency probably was caused by their completely different clinical manifestations and the lack of appropriate laboratory methods. While patients with the muscular form of CPT2 deficiency usually present in adulthood with solitary myopathic features, patients with MCAD deficiency (MCADD) present in early childhood during a minor infection with clinical signs and symptoms related to the lack of energy for metabolic functions. They usually present with what is often called a 'Reye-like syndrome' consisting of liver disease with hypoketotic hypoglycemia, raised ammonia levels, encephalopathy and severe fatty infiltration of the liver. MCAD deficiency is now known as a relatively common metabolic disease[26], and for most physicians this 'Reye-like syndrome' or hepatic type of presentation is the 'classical' clinical manifestation to trigger them to be alert for a FAO disorder.

At present, more than 10 FAO disorders are recognized. The long-chain FAO disorders are quantitatively an important group, of which the clinical presentation can be very different from the classical MCAD phenotype. Patients appear to present at younger age, sometimes already in the neonatal period. Besides, clinical presentation is more heterogeneous than the classical MCAD 'liver' phenotype, since also a 'cardiac' phenotype consisting of cardiomyopathy or arrhythmias, a 'myopathic' phenotype with muscular cramps and raised creatine kinase (CK) concentration and a 'neurological' phenotype with profound peripheral neuropathy can be distinguished. The study of longchain FAO disorders has resulted in new insights in several metabolic and pathophysiological mechanisms. So it became clear that some of the clinical features possibly can be attributed to long-chain acyl-CoA esters. These intermediates, accumulating in long-chain FAO disorders, appeared to have a role in regulating mitochondrial energy metabolism by inhibition of processes and enzymes as the mitochondrial oxidative phosphorylation[27], the adenine nucleotide translocase (ANT) which catalyses exchange of ADP and ATP across the mitochondrial inner membrane[28] and the citrate transporter[29,30]. Long-chain acylcarnitines, long-chain acyl-CoA esters

bound to carnitine, have appeared to play a role in the development of cardiac disease, since they have been demonstrated to be toxic to cultured myocytes in vitro[31]. Parallel to the accelerated clinical recognition of FAO deficient patients following the discovery of MCADD, a whole range of diagnostic tools became available to identify patients suffering from FAO disorders.

Acyl-CoA esters accumulate in the mitochondria in long-chain FAO disorders as a result of the enzyme block. These acyl-CoAs can be metabolized via alternative pathways as the aoxidation, which leads to the production of dicarboxylic acids. Demonstration of increased excretion of dicarboxylic acids, in urine by gas chromatography – mass spectrometry (GC-MS), has been the main leading biochemical feature suggesting a FAO disorder for a long time. The diagnosis can subsequently be confirmed by measuring activity of the overall FAO and the activity of the involved separate enzymes in tissues such as liver and muscle or in separate cell such as in lymphocytes or cultured skin fibroblasts. Molecular diagnosis by demonstating disease causing mutations is possible for a number of FAO disorders. The introduction of Tandem Mass Spectrometry for example, which facilitates analysis of the acylcarnitine profile (the spectrum of the accumulating acyl-CoAs conjugated with carnitine), made rapid identification of patients feasible[32,33]. The tremendous expansion in knowledge and diagnostic tools in recent years has led to the recognition of more and more patients with long-chain FAO disorders, especially of those patients presenting with nonspecific clinical features. This has resulted into a dramatic shift in our knowledge about the clinical presentation of long-chain FAO disorders, raising many new questions. As this thesis will focus on two of the long-chain FAO disorders (isolated LCHAD deficiency (LCHADD) and MTP deficiency (MTPD)), the introduction deals with the carnitine cycle and the ß-oxidation pathway in general, as well as with the known disorders of long-chain FAO.

Disorders of the carnitine cycle

Systemic or Primary Carnitine (OCTN2) deficiency (OMIM 212140)

The carnitine cycle (figure 1) is completely dependent on sufficient supply of carnitine, which can be derived from dietary intake and de novo synthesis. Although the biosynthesis of carnitine was recently completely unraveled, no patients suffering from a deficiency in the carnitine biosynthesis have been recognized yet. The clinical aspects of systemic carnitine deficiency stress the importance of the carnitine transporter (OCTN2, organic cation transporter 2), for the maintenance of carnitine homeostasis. The carnitine

transporter facilitates co-transport of sodium and carnitine across the plasmalemmal membrane into the cell, and is independent on the extracellular carnitine concentration[34]. In systemic carnitine deficiency not only intracellular carnitine concentrations are very low, but also the plasma concentration of carnitine. This is caused by failure of the kidney to reabsorb carnitine as a consequence of the OCTN2 deficiency. The spectrum of clinical presentations of systemic carnitine deficiency varies between severe cardiomyopathy on one hand and a 'Reye-like syndrome' with hypoketotic hypoglycemia on the other hand[35,36]. The clinical presentation is often accompanied by signs and symptoms of generalized disease including involvement of the heart, liver and muscle. The first clinical features of the disorder usually manifest at neonatal age, infancy or childhood. Early diagnosis is essential, because mortality is very high and can be prevented by high doses of oral carnitine supplementation. After start of therapy usually all clinical features, including cardiomyopathy, resolve[36,37]. Laboratory diagnosis Plasma and tissue concentrations of total carnitine are usually extremely low (< 5 µmol/L), thus providing a direct indication for systemic carnitine deficiency. Analysis of plasma acylcarnitines usually shows reduced levels of all acylcarnitines. Dicarboxylic aciduria often is absent or low. The diagnosis can be established experimentally by showing the deficiency of sodium driven transport of carnitine into the cells, in fibroblasts and for rapid diagnosis, in lymphocytes[38]. The gene coding for the OCTN2 has been resolved[39] and multiple private mutations have been described[40,41,42].

Carnitine Palmitoyl Transferase (CPT) 1 deficiency (OMIM 255120)

Two isoforms of CPT1, a hepatic and a muscle isoform have been recognized. Until now, only deficiency of the hepatic isoform has been described. In line with the defect, presentation of all patients reported in literature (approximately 15) is indeed limited to hepatic symptomatology. No cardiac or muscle involvement has been reported. Presentation, triggered by prolonged fasting during a period of an intercurrent illness, mostly consists of hepatomegaly and hypoketotic hypoglycemia, sometimes accompanied by the whole spectrum of 'Reye-like syndrome'[43]. Some of the patients showed renal tubular acidosis[44,45], a symptom not seen in any other defect of mitochondrial FAO. Most likely the renal involvement in CPT1 deficiency is explained by the fact that the hepatic isoform of CPT1 is also expressed in the kidney.

Acute episodes of hepatic disease often can successfully be treated by intravenous administration of glucose. Patients can be prevented from developing episodes of acute

disease by a dietary regimen, consisting of avoidance of fasting. Additionally medium chain triglycerides (MCT) can be given.

Laboratory diagnosis Liver disease with elevated transaminases is often observed in combination with hypoketotic hypoglycemia and absence of dicarboxylic aciduria. Additionally raised FFA concentrations and acidosis can be observed. Total plasma carnitine concentration is usually remarkably high, but may also be normal. Acylcarnitine profiling mostly shows reduced levels of C18:1, C18:0 and C16:0 acylcarnitines. Diagnosis of hepatic CPT1 deficiency is established by enzymatic studies in lymphocytes or fibroblasts. Only in isolated cases mutations in the gene coding for the hepatic isoform of CPT1 have been reported[46,47].

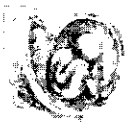
Carnitine Acylcarnitine Translocase (CACT) deficiency (OMIM 212138)

So far CACT deficiency has been reported in about 25 patients. Most had a neonatal onset of disease, with life threatening episodes often characterized by cardiac arrhythmias as a first symptom and frequently accompanied by cardiomyopathy. The neonatal disease often also included muscle disease resulting in severe hypotonia and raised CK concentrations, and liver disease with raised liver enzymes, markedly elevated ammonia levels and sometimes hypoketotic hypoglycemia[48]. Most patients died before the age of two months. Survival was only described in a few patients showing mild disease[49,50]. The underlying basis for this diversity in the clinical spectrum of the disorder remains unknown[48]. Treatment consists of a diet low in fat, supplemented with MCT and frequent meals to avoid periods of prolonged fasting. Acute episodes are handled with continuous administration of glucose thereby suppressing lipolysis and subsequent FAO.

Laboratory diagnosis Routine laboratory investigations may show hypoketotic hypoglycemia, acidosis, hyperammonemia and raised FFAs, transaminases, lactate and CK concentrations. Profiling of the acylcarnitines usually demonstrates raised long-chain C16 and C18 acylcarnitines in combination with a low total carnitine. If present, dicarboxylic aciduria is subtle. Definitive evidence for the diagnosis of CACT deficiency is provided by enzyme analysis in lymphocytes or fibroblasts followed by molecular studies which have shown a variety of different mutations[51,52].

Carnitine Palmitoyl Transferase (CPT) 2 deficiency (OMIM 255110)

CPT2 deficiency is the most common of the known disorders of the carnitine cycle. A wide spectrum of clinical presentations has been reported, often divided in two or three



groups based on the age of presentation. The most common form, referred to as the 'adult', 'muscular' or 'classical' form, presents with clinical features affecting skeletal muscle, during adolescence or adulthood. In these patients prolonged exercise and extreme environmental temperature fluctuations trigger episodes of rhabdomyolysis and myoglobinuria, eventually followed by renal failure[43]. The second, 'neonatal' or 'hepatocardiomuscular' form can be compared with the neonatal presentation seen in CACT deficiency, frequently showing cardiac arrhythmia as the first clinical feature. Most patients die within a few weeks due to complications of this initial episode, often showing a combination of severe cardiac-, liver- and muscle disease[43]. Renal dysgenesis has been described in several individuals with neonatal onset[53,54]. This phenomenon, which has been reported in multiple acyl-CoA dehydrogenase deficiency, is unique for a single enzymatic defect in the carnitine cycle or mitochondrial FAO pathway. A milder presentation of this generalized, 'hepatocardiomuscular' form of CPT2 deficiency, by some described as a separate third form, was seen in a few patients at a late infantile age and therefore called 'infantile' type[38]. Treatment, like in CACT deficiency, is based on prevention of periods of prolonged fasting and excessive muscular exercise by frequent meals and sometimes supplementation of MCT. In acute disease FAO has to be suppressed by continuous administration of carbohydrates, by intravenous glucose infusion.

Laboratory diagnosis CPT2 deficiency often shows hypoketotic hypoglycemia in combination with raised concentrations of CK and transaminases, as can be observed in many other defects of FAO. Dicarboxylic aciduria is only seen incidentally. Additionally the acylcarnitine profile resembles closely that of CACT deficiency, with accumulation of C16-C18 acylcarnitines and a very low free carnitine concentration. Definitive diagnosis only can be established with enzymatic studies in lymphocytes or fibroblasts. Although a heterogeneous group of mutations in the CPT2 gene have been reported to be causative for the disease[43], a common mutation (S113L) with an allele frequency of approximately 60% has appeared to be related to a higher residual enzyme capacity in patients[55]. This suggests a genotype/phenotype relationship in the relatively mild 'adult' or 'muscular' form of CPT2 deficiency.

Very Long-Chain Acyl-CoA Dehydrogenase (VLCAD) deficiency (OMIM 201475)

A complete overview of the incidence and clinical spectrum of VLCAD deficiency is lacking at the moment, but generally three phenotypes can be differentiated[56,57]. The first is an 'early neonatal' or 'infantile' type with profound cardiomyopathy and arrhythmias, often in combination with features of generalized disease including hepatic and muscular symptomatology. Mortality and morbidity is high among these patients, although a good result of continuous administration of glucose in acute situations has been reported. A second phenotype, showing its first symptoms in early childhood, is mainly characterized by hepatic involvement with hypoketotic hypoglycemia presenting in periods of prolonged fasting, sometimes accompanied by cardiomyopathy and muscular symptomatology. The third and relatively mild 'muscular' phenotype, presents in childhood, adolescence or even adulthood. This muscular type resembles the 'muscular' type described in CPT2 deficiency, characterized by episodes of rhabdomyolysis induced by exercise or prolonged fasting. Management consists of a dietary regimen including avoidance of fasting and replacement of long-chain triglycerides (LCT) by MCT in case of cardiac disease[58,59]. Additionally carnitine supplementation is sometimes given, despite the risk of a subsequent rise of potentially toxic intracellular long-chain acylcarnitines. Acute disease is treated with intravenous glucose administration covering energy requirements, and thus suppressing lipolysis and subsequent FAO. Laboratory diagnosis Routine plasma analysis can show elevation of transaminases and/or CK, hypoglycemia and low ketone bodies. Additional laboratory investigations have to be performed to differentiate VLCAD deficiency from many other FAO defects. Typical for VLCAD deficiency is the combination of profound medium-chain dicarboxylic aciduria (C6-C12) on organic acid analysis of the urine, and the presence of C14:1, C14:2 and C16:1 acylcarnitines on acylcarnitine profiling and a low free carnitine concentration in plasma. Definitive diagnosis is made by enzyme measurements in lymphocytes or fibroblasts. Although mutation analysis in more than fifty patients pointed out multiple private mutations, a genotype/phenotype relationship was suggested[57]. Mitochondrial trifunctional protein (MTP) and long-chain 3-hydroxyacyl-CoA dehydrogenase (LCHAD) deficiency MTP and LCHADD are disorders based on a defect of the same protein (MTP). In MTPD, the whole protein is absent, resulting in complete deficiency of all three of the enzyme activities catalyzed by MTP, which include: LCEH, LCHAD and LCKAT. In contrast, in LCHADD, the protein is normally present, but a defect in the a-subunit results in an

isolated deficiency of the LCHAD enzyme. The activities of LCEH and LCKAT are normal or slightly reduced compared to controls (60% of wild-type levels) in isolated LCHADD.

LCHAD deficiency (OMIM 143450)

Since the first description of a biochemically proven patient in 1989[60], LCHADD always has drawn a lot of interest. At first there was confusion about the precise biochemical mechanisms, until it became clear that LCHAD and MTPD have to be considered as distinct disorders. Furthermore, it later appeared that several of the clinical aspects involved in LCHADD are unique among the group of FAO defects. Over the years, more than 80 patients have been reported in literature. Clinical presentation appeared to be relatively uniform with profound liver disease, often presenting with hypoketotic hypoglycemia and hepatomegaly precipitated by prolonged fasting during a minor infectious disease in infancy or young childhood[61]. Retrospectively, some patients showed already in the neonatal period, clinical features probably related to their FAO disorder. Acute episodes of rapid progressive disease can also be observed and show more generalized pathology, including cardiomyopathy, encephalopathy and muscular disease with raised CK levels. LCHADD has been reported as a rare cause of Sudden Infant Death Syndrome (SIDS)[60]. Mortality appears to be alarmingly high (92% in series of 13 patients) mostly due to acute metabolic derangement[61]. Surviving patients often suffer from recurrent attacks of predominantly hepatic or muscular symptomatology, without showing any clinical features of the FAO defect in between. Remarkable is the slowly progressing peripheral neuropathy and pigmentary retinopathy which is seen in surviving patients. Both symptoms are not known in any other FAO defect. Nerve conduction velocity (NCV) was performed in a patient with peripheral neuropathy and was strikingly abnormal[62]. Electromyography (EMG) in this patient showed a myogenic pattern and signs of denervation. Nerve biopsy revealed demyelinisation and axonal neuropathy[62]. Pigmentary retinopathy was observed during follow up in some patients varying in age, and was suggested to be caused by low plasma concentrations of polyunsaturated fatty acids (PUFA) like docosahexanoic acid (DHA)[63]. Another, recent study of Tyni and coworkers, showed evidence for presence of FAO in retinal pigment epithelium[64]. This suggests a possible role in the etiology of the pigmentary retinopathy for potentially toxic 3-hydroxyacyl-CoA esters, the long-chain acyl-CoA esters accumulating in LCHADD. However, the exact etiology, incidence and the course of both the peripheral neuropathy and pigmentary retinopathy still remain unknown. A few additional patients have been reported presenting with a remarkable symptomatology generally not seen as a

presenting symptom in FAO defects. Cholestatic liver disease was reported in three cases[61,65]. Hypoparathyroidism was detected in one individual case and probably was caused by hypoplasia of the parathyroid glands[66]. Pathologic studies in LCHAD deficient patients show gross fat accumulation in muscle, heart and especially liver as can be seen in most other FAO disorders. Besides microvesicular hepatosis, fibrotic and cirrhotic changes have been observed in the livers of a few individual cases[67,68]. Clinical heterogeneity in LCHADD was addressed as exemplified by the family described by Schaefer et al[69]. This family showed an adult onset muscular presentation resembling CPT2 and VLCAD deficiency, with recurrent episodes of muscle pains and myoglobinuria in three sibs, eventually resulting in renal failure, and generalized areflexia as sole symptom in a fourth family member. The family members, originally reported to be MTP deficient, actually were LCHAD deficient as concluded from the biochemical data obtained from fibroblasts. All were compound heterozygous for the common LCHAD mutation.

MTP deficiency (OMIM 600890)

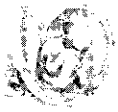
In contrast to the large number of LCHAD deficient patients, only a few MTP deficient patients were reported since its first description in 1992. At first, clinical presentation appeared to closely resemble LCHADD but with a first manifestation earlier in life. Close inspection of the symptomatology at onset however, shows a broader clinical spectrum than reported in isolated LCHADD, with a more generalized disease. This includes liver, muscular and cardiac involvement in some, and profound liver- or muscular disease as first clinical features in others. None of the patients was reported to have signs of retinopathy. Most patients died shortly after their first presentation. Some remarkable cases address the heterogeneity in the clinical manifestation of MTPD. A profound muscular presentation was described in two individual children and one adolescent[70,71]. The two children in their first report were presented to be LCHAD deficient, but additional studies showed both to be MTP deficient[72]. The first child initially was known with a delay of gross motor development and intermittent episodes characterized by bulbar weakness, hypotonia and myoglobinuria. Later in childhood he developed polyneuropathy and a slowly progressive limb girdle myopathy[70]. The second child[71] was known with cardiomyopathy in infancy, but after recovery showed a similar course as the first child. Nerve conduction velocity in both patients showed axonal neuropathy with sensory predominance[70,71]. Nerve biopsy in one of them revealed axonal degeneration and normal myelin[70]. A late onset muscular presentation as seen in the LCHAD deficient

family described by Schaefer et al[69] was reported in an MTP deficient adolescent, who experienced attacks of muscle pain and myoglobinuria after prolonged exercise since the age of 15[73]. His medical history revealed several episodes of minor infections that passed without any complication, and a normal motor development. Since fasting and prolonged exercise was avoided, he did not experience any complaints anymore. Like in LCHADD, one individual case was reported with hypoparathyroidism of unknown origin[74]. In acute episodes administration of sufficient amounts of carbohydrates is essential in both LCHAD and MTPD, but cannot always prevent lethal outcome or serious morbidity. Treatment during the intervals between acute episodes consists of a dietary regimen of frequent meals to avoid lipolysis and subsequent activation of long-chain FAO, in combination with replacement of long-chain fatty acids by MCT to prevent accumulation of potentially toxic long-chain FAO intermediates. Observations in one individual LCHAD deficient patient showed normalization of the acylcarnitines if LCT intake was limited to 10% of total energy intake. After introduction of MCT supplementation up to 15% of total energy intake, lactate concentrations of the patient completely normalized[63]. Essential Fatty Acids (EFA) should be monitored closely because of the LCT restriction, and supplemented if necessary. Major beneficial effects of carnitine supplementation were never reported in long-chain FAO disorders. Carnitine supplementation in long-chain FAO disorders is even considered inappropriate, since they probably increase long-chain acylcarnitine concentrations, which are reported to cause cardiac arrhythmias[31,75]. Laboratory diagnosis In patients presenting with the more generalized mode of presentation, hepatic involvement with elevation of transaminases and often hypoketotic hypoglycemia is seen, often in combination with increased ammonia and lactate concentrations. Also, raised CK concentrations as a result of muscle involvement are frequently involved. The biochemical diagnosis of LCHAD and MTPD is usually suggested by demonstration of 3-hydroxy-dicarboxylic aciduria by gas-chromatography analysis in the urine from periods of acute illness. In exceptional cases these dicarboxylic acids were absent[76]. The acylcarnitine profile is mostly dominated by the raised C16:0, C16:1, C18:0 and C18:1 acylcarnitines[33]. Subsequent enzyme analysis to establish the diagnosis requires measurement of all three components of MTP. LCHAD activity is absent in both isolated LCHADD and MTPD. Differentiation of the two disorders is facilitated by measurement of LCKAT activity, showing near normal activity of the enzyme in isolated LCHADD and absence of activity in MTPD. Additionally immunoblot analysis shows the normal presence of MTP in isolated LCHADD, while in MTPD the α- or ß-subunits of the MTP protein are usually markedly deficient (see chapter 4 for an introduction on MTP). In patients with isolated LCHADD a common mutation 1528G>C in the α-subunit of the protein has been reported[77,78,79] with

an allele frequency of 87%[78]. The common 1528G>C mutation inactivates the LCHAD enzyme, without affecting the formation of the α- or ß-subunit thus resulting in near normal LCEH and LCKAT activities. Molecular studies in MTP deficient patients show a wide range of private mutations in both the α- or ß-subunit. None of the patients was reported to carry the 1528G>C mutation. The two children reported to present with a more insidious disease with myopathy and slowly progressing polyneuropathy both were reported to have a mutation in exon 9 of the α-subunit[72]. Exon 9 encodes for a linker domain between the hydratase and dehydrogenase component, suggesting a genotype/phenotype relationship in these relatively mildly affected patients[72].

Pregnancy complications and LCHAD deficiency

Soon after description of the first proven LCHAD deficient patients, a striking association became clear between carriage of a LCHAD deficient child and the end stage pregnancy complications hemolysis elevated liver enzymes and low platelets (HELLP) syndrome and acute fatty liver of pregnancy (AFLP)[80,81]. HELLP syndrome often presents with clinical features of severe systemic disease including liver involvement with raised transaminases and clotting disturbances. It occurs in 0.1- 0.6% of all pregnancies and in 4 to 20% of women with severe preeclampsia[82,83,84]. AFLP is a devastating and progressive liver disease presenting with severe abdominal pains and biochemical features including raised liver enzymes, cholestasis, hypoglycemia, clotting disturbances and raised ammonia concentrations. AFLP is very rare and occurs in 1:7000- 1:13000 pregnancies, frequently in combination with features of preeclampsia or HELLP syndrome[82,83]. Both HELLP syndrome and AFLP carry a high risk for serious morbidity and even mortality for the affected mother and her child[82,83]. Histologically microvesicular or macrovesicular steatosis dominates in liver biopsies of patients suffering from HELLP syndrome as well as from AFLP. Because of their clinical and histological similarities, preeclampsia, HELLP syndrome and AFLP are suggested to present different stages of the same disease[84,85]. Their exact pathogenesis still remains unknown, although it has been suggested previously that generalized maternal endothelial dysfunction may be involved. Prompt delivery remains the treatment of choice for either of these pregnancy complications. Schoeman and coworkers suggested that the compromised oxidation of long-chain fatty acids is the common pathophysiological background causing the liver disease in both the heterozygous mother as well as her FAO deficient fetus, because of the identical observations in histological studies of the liver[80]. Later, this hypothesis was supported by two extensive studies which suggested that the common 1528G>C mutation possibly is causative for this compromised FAO[86,87]. In the first study[86] 63 pregnancies in 18 carriers



for LCHADD were studied. Preeclampsia, HELLP syndrome and AFLP occurred in 31% of the pregnancies, and were only diagnosed in mothers carrying an affected fetus. The second study[87] confirmed these observations and found 15 out of 19 (79%) pregnancies carrying a LCHAD deficient fetus, to be complicated by HELLP syndrome or AFLP. All the LCHAD deficient fetuses involved in the latter study were either homozygous or compound heterozygous for the common LCHAD mutation. Very recently this was confirmed in an extensive study from the same group, reporting the pregnancy outcomes in 35 families with MTP mutations[88]. The exceptional high incidence of AFLP (49%) and HELLP syndrome (11%) in women carrying a fetus with isolated LCHADD was addressed, and again no maternal complications were associated with heterozygous or normal fetal genotypes. Only in one study it remained uncertain if the risk for the gestational complications indeed is limited to those pregnancies in which the fetus is homozygous affected[89]. During the last years strong recommendations are given for molecular screening in the families of women suffering from HELLP syndrome or AFLP[90]. However, in another report[91] the results of prospective molecular screening for MTP mutations in a large cohort, did not justify screening of newborns in pregnancies complicated by HELLP syndrome. The same study documents a highly significant (19%) association between AFLP and LCHADD in the fetus, and concludes that prospective molecular screening for LCHADD should be implemented universally in all families of women suffering from AFLP. Five additional pregnancies in mothers carrying a MTP deficient fetus, studied in the extensive study from Ibdah and coworkers[87], were uneventful, in accordance with the observation that only women carrying a LCHAD deficient child are at risk to develop pregnancy related disease. However, both Chakrapani and coworkers (2000) and Walter (2000) reported liver disease during late gestation in three mothers carrying fetuses with complete MTPD[92,93]. Additionally several individual cases linked other fetal FAO defects than LCHAD or MTPD to maternal pregnancy complications[94,95,96]. Besides the induction of the maternal gestational complications, fetal complications such as growth retardation and prematurity are increased in LCHAD deficient patients, and is unrelated to the maternal gestational complications[86]. The precise mechanisms causing the association between maternal pregnancy complications and fetal disease remains unknown. A range of possibilities has been suggested to be of influence. At first the heterozygous state of the mother, which theoretically reduces her capacity to oxidize long-chain fatty acids to 50% of normal, has been suggested to play an important role in combination with the metabolic stress experienced at end stage pregnancy. In addition, the increase in the concentration of estrogens occurring in the later stages of pregnancy, may potentially further suppress mitochondrial FAO, as estrogens have been shown in animal studies to

compromise FAO[97]. Finally, the accumulation of potentially toxic 3-hydroxyacyl-CoA esters, which especially is observed in isolated LCHADD, is thought to play a causative role in this remarkable association between fetal and pregnancy related maternal disease.

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2

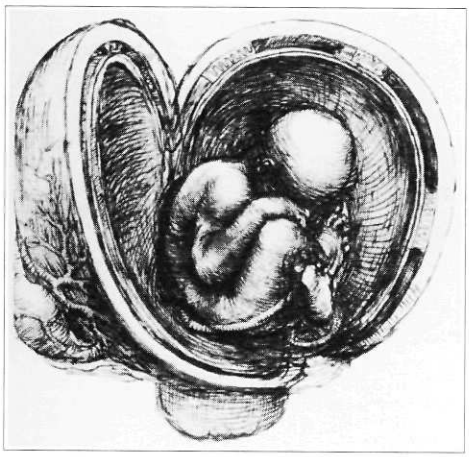
Aim of the thesis

The first aim of this thesis is to elucidate the clinical and biochemical spectrum of isolated LCHAD and MTP deficiency. Although many isolated cases and small series of LCHAD deficient patients have been reported, an overview of the clinical and biochemical features in a larger series was lacking. We therefore studied the clinical and biochemical mode of presentation, the clinical course and effects of therapy in a large cohort of unselected, mostly European LCHAD deficient patients (chapter 3). In contrast with the multiple reports on isolated LCHAD deficient patients, only a few MTP deficient patients were described, which display a heterogeneous array of symptoms and a varying age at presentation. We studied a cohort of MTP deficient patients in order to give an overview of the disease in MTP deficiency and to compare the differences in presentation and outcome with isolated LCHAD (chapter 4).

As the clinical presentation of LCHAD deficiency can be very heterogeneous, and not always resembles the classical 'MCADD- or hepatic phenotype' it is well conceivable that many patients are missed for diagnosis and that the true incidence of LCHAD deficiency is

much higher. To test this hypothesis we decided to study the prevalence of the common LCHAD mutation in the Dutch population (chapter 5).

Another aim of this thesis is to try to unravel the etiologic aspects involved in the origin of the remarkable association between a long-chain FAO disorder in the fetus and severe complications of pregnancy in their mothers. First we focused on the possible role of the heterozygousity of the mothers as a single cause. We therefore studied the prevalence of the common LCHAD mutation in a cohort of women who previously suffered from HELLP syndrome (chapter 5). Additionally we performed a LCT loading test in women heterozygous for the 1528G>C mutation, studying the in vivo capacity to oxidize fatty acids possibly causing the maternal liver disease (chapter 6). As the placenta, which genetically is of fetal origin, and the fetus are considered to be primarily dependent on glucose oxidation for all energy requirements, their potential role in the etiological mechanism causing the association between the maternal pregnancy complications and the fetal FAO disorder is still unclear. We therefore studied human placenta for the presence of multiple enzymes involved in FAO (chapter 7). Finally, expression studies for LCHAD and VLCAD were performed in human embryos, in order to observe whether FAO plays a role during intra-uterine life (chapter 8). In case of LCHAD or MTP deficiency active intra-uterine FAO could be elucidative in the explanation for both the maternal liver disease, as for the observed high incidence of intra-uterine growth retardation and prematurity.



3

Long-chain 3-hydroxyacyl-CoA dehydrogenase deficiency: Clinical presentation and follow-up of 50 patients

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Abstract

Objectives

To assess the mode of presentation, biochemical abnormalities, clinical course, and effects of therapy in patients of long-chain 3-hydroxyacyl-CoA dehydrogenase (LCHAD) deficiency.

Background

LCHAD deficiency (LCHADD) is a rare autosomal recessive inborn error of fatty acid oxidation (FAO). Though case reports and small series of patients have been published, these may not give a true picture of the clinical and biochemical spectrum associated with this disorder. To improve the early recognition and management of this potentially lethal disorder, we have reviewed a large cohort of LCHAD deficient patients.

Methods

A questionnaire was sent to the referring physicians of sixty-one unselected patients with LCHADD diagnosed in our center. The standardized questionnaire requested information about the clinical signs and symptoms at presentation, the clinical history, family history, pregnancy, biochemical parameters at presentation, treatment, and clinical outcome.

Results

Questionnaires on fifty patients (82%) were returned and included in this study. The mean age of clinical presentation was 5.8 months (range: 1 day to 26 months). Seven (15%) of the patients presented in the neonatal period. Thirty-nine patients (78%) presented with hypoketotic hypoglycemia, the classical features of a FAO disorder. Eleven patients (22%) presented with chronic problems, consisting of failure to thrive, feeding difficulties, cholestatic liver disease, and/or hypotonia. In retrospect, most (82%) of the patients presenting with an acute metabolic derangement also suffered from a combination of chronic nonspecific symptoms before the metabolic crises. Mortality in this series was high (38%), all dying before or within three months after diagnosis. Morbidity in the surviving patients is also high, with recurrent metabolic crises and muscle problems, despite therapy.

Conclusions

LCHADD often presents with a combination of chronic nonspecific symptoms. Early diagnosis is difficult in the absence of the classical metabolic derangement. Survival can

be improved by prompt diagnosis, but morbidity remains alarmingly high despite current therapeutic regimes.

Introduction

Long-chain 3-hydroxyacyl-CoA dehydrogenase (LCHAD) catalyses the third step of the mitochondrial oxidation of long-chain fatty acids, converting long-chain 3-hydroxyacyl-CoA esters into the corresponding 3-ketoacyl-CoA esters. The LCHAD enzyme is part of the mitochondrial trifuntional protein (MTP), which also harbors long-chain enoyl-CoA hydratase and the long-chain thiolase activity.

Since the first description of LCHADD in 1990[1], a number of case reports have highlighted individual clinical and biochemical features of the disorder. From these reports, the most prominent finding at presentation appears to be hypoketotic hypoglycemia, as in the commonest FAO disorder, medium-chain acyl-CoA dehydrogenase (MCAD) deficiency[2]. Additional features reported in LCHADD include cardiomyopathy, severe liver disease with cholestasis and recurrent muscle cramps with raised serum creatine kinase levels. These features also occur in other long-chain FAO disorders (such as VLCAD and CPT2 deficiency) but not in MCAD deficiency (MCADD). Finally, a number of LCHAD deficient patients develop pigmentary retinopathy and peripheral neuropathy, long-term complications which are not seen in any of the other mitochondrial FAO disorders.

Diagnosis of LCHADD is suggested by demonstrating increased secretion of 3-hydroxy-dicarboxylic acids in urine by gas chromatography - mass spectrometry (GC-MS), or by demonstrating accumulation of 3-hydroxyacyl-carnitines as measured by tandem-mass-spectrometry (Tandem MS) in plasma[3]. Confirmation of the diagnosis is possible by measuring LCHAD activity in lymphocytes, fibroblasts, muscle or liver biopsies[1,4] and by mutational analysis. A common mutation has been identified in the α-subunit of the trifunctional protein, in the domain with LCHAD activity. In the majority of LCHAD-deficient patients, at least one allele carries this point mutation (1528 G>C)[5].

Case reports and small series of patients often do not give a balanced view of the clinical and biochemical spectrum associated with inborn errors of metabolism. To provide clinical data on a large unselected affected population to allow appreciation of the clinical spectrum, we reviewed fifty patients by sending questionnaires to the relevant metabolic pediatricians.

Materials and Methods

For the last decade the metabolic center at the Academic Medical Center of the University of Amsterdam has been a referral center for the diagnosis of LCHADD. The diagnosis has been established in eighty patients from all over Europe by enzyme analysis in lymphocytes, fibroblasts, or liver tissue[1,4] and/or by demonstrating homozygosity for the common LCHAD mutation (1528G>C) by using previously described methods[5,6]. A standardized questionnaire was sent to the referring specialists of sixty-one patients for whom the referring physician was known. For the other nineteen patients, the name of the referring physician was unknown to our laboratory, and thus a questionnaire could not be sent. Patients with MTP deficiency (MTPD) were excluded from this survey.

The questionnaire requested patient initials, sex, date of birth, and clinical and biochemical parameters at time of diagnosis and during follow up, clinical history before diagnosis, family history, pregnancy, neonatal period, and current treatment. All data were analyzed anonymously, and can not be reduced to the individual patient. Frequencies and frequency distributions were calculated using the SPSS software program. Statistical analysis included Fisher's exact test.

Results

Patients

Fifty questionnaires (82%) out of the sixty-one were completed by twenty-six referring specialists. The fifty patients originated from fourty-five unrelated families. Five families had two affected siblings. Twenty-three patients were male, twenty-seven were female. Most patients (47) were of European origin, the other three coming from the USA, Australia, and Israel. Only eight of the fifty patients have been published previously[7-13].

Diagnosis

All patients were proven to be LCHAD deficient, either by enzymatic analysis (38 out of 50, 76%) and/or by mutation analysis (49 out of 50 patients). Mutation analysis demonstrated the presence of the common 1528G>C mutation in 84 out of the 98 alleles tested (allele frequency 86%). Thirty-six patients were homozygous and twelve were heterozygous for this mutation. One patient was found to be homozygous for the 583G>D mutation. All heterozygous 1528G>C and the homozygous 583G>D were enzymatically proven to be LCHAD deficient.

Pregnancies

For fourty-seven of the fifty patients, data on the pregnancies were available. Seven (15%) of the pregnancies were complicated by HELLP (hemolysis, elevated liver enzymes and low platelets) syndrome. Two pregnancies (4%) were complicated by AFLP (acute fatty liver of pregnancy).

Clinical presentation

The age of onset of clinical symptoms ranged from 1 day to 26 months, with a mean of 5.8 months (figure 1). Seven (15%) of the patients presented within the neonatal period (0-4 weeks of age).

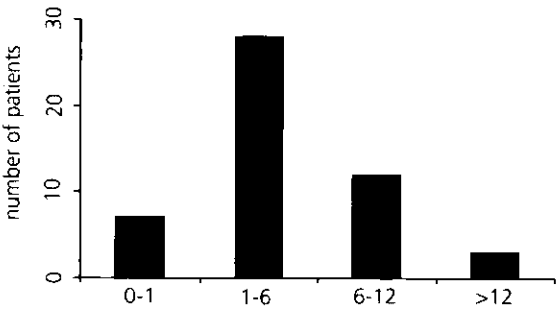

Figure 1 - Age at first presentation in months

Thirty-nine patients (78%) presented with an acute metabolic derangement with hypoketotic hypoglycemia. Clinical signs and symptoms at acute presentation are given in table 1.

Signs and Symptoms	Number of Patients	Percentage
Hepatic Dysfunction	31/39	79%
Coma	22/39	56%
Seizures	15/39	38%
Apneic spells	09/39	23%
Cardiorespiratory arrest	8/39	21%
Arrhythmias	7/39	18%
Sudden death	3/39	9%

Table 1 - Signs and symptoms in 39 LCHAD-deficient patients presenting with acute metabolic derangement

The other eleven patients (22%) presented with a more chronic disorder, consisting of liver disease, failure to thrive, feeding difficulties and/or hypotonia (table 2). These children did not suffer any acute metabolic derangement with hypoglycemia prior to diagnosis.

Signs and symptoms	11 Patients without acute metabolic derangement		39 Patients with acute metabolic derangement	
	number	percentage	number	percentage
Hepatomegaly	6/10*	60%	28/36*	78%
Hepatic dysfunction	8/10*	80%	31/39*	79%
Cholestasis	3/10*	30%	6/34*	185
Cardiomyopathy	4/11*	36%	17/35*	49%
Failure to thrive	8/11*	73%	14/35*	40%
Feeding difficulties	6/11*	55%	16/35*	46%
Vomiting	5/11*	45%	13/33*	39%
Hypotonia	7/11*	64%	22/36*	61%
Lethargy	3/10*	30%	10/35*	29%
Psychomotor retardation	3/11*	27%	9/36*	25%
Peripheral neuropathy	1/11*	9%	1/33*	3%
Microcephaly	3/11*	27%	2/33*	6%

Table 2 - Signs and symptoms before and at diagnosis in all 50 LCHAD-deficient patients
** Number of patients for whom data were available*

Careful analysis of the clinical history of those patients presenting with an acute hypoglycemic episode, revealed that thirty-two (82%) of the thirty-nine patients already had non-specific problems, probably related to LCHADD, before the hypoglycemic attack. These problems are comparable to those observed in the eleven patients who presented with chronic symptoms (table 2).

Laboratory abnormalities at presentation

Besides hypoglycemia, patients with an acute presentation showed a number of other laboratory abnormalities. These are summarized in table 3.

Test	Number of patients with abnormal values	Mean value	Range	Normal range
Glucose (mmol/L)	39/39* (100%)	1.4	0.0-2.6	3.5-7.0
Lactate (mmol/L)	14/20*(70%)	8,6	2.7-44.0	<2.0
ASAT (U/L)	25/28* (89%)	261	65-761	<65
CK (U/L)	14/21* (67%)	1178	151-9000	<150
Ammonia (µmol/L)	14/17* (74%)	150	68-400	<55
Total carnitine (µmol/L)	12/22* (55%)	14.8	8-23.5	>25

Table 3 - Laboratory values in 39 LCHAD-deficient patients presenting with acute metabolic derangement
** Number of patients for whom data were available*

Clinical outcome

Nineteen (38%) of the fifty patients were already deceased at the time of the study. Mortality in patients presenting with acute hypoglycemia (15/39, 38%) did not differ significantly from that in patients presenting with chronic non-specific symptoms (4/11, 36%). Fourteen patients died before the diagnosis of LCHADD was made. The other five children died within the first three months after diagnosis, one due to sudden infant death, one due to hepatic failure and three due to cardiomyopathy. The latter four patients had all presented with acute hypoglycemia but severe liver disease/ cardiomyopathy were already present at this time.

Follow-up for the thirty-one surviving patients (62%) ranged from 0.5 to 11 years with a median follow up of 3.4 years. Of these thirty-one patients, twenty-nine (94%) were reported to be generally "in good clinical condition". However, morbidity in this group is still high, with recurrent metabolic crises and other clinical problems (table 4). The metabolic crises were reported to be less severe than the initial acute metabolic derangement. No patient died during follow-up.

Treatment

All surviving patients (31) are treated with a low fat, high carbohydrate diet. Twenty-three patients receive a medium chain triglycerides (MCT) enriched diet, and twelve are being treated with L-carnitine (50-100 mg per kilogram body weight per day). No statistically significant difference in morbidity, as defined as the presence of recurrent metabolic attacks and/or muscle cramps, could be detected between the patients receiving and those not receiving L-carnitine supplements (Fisher's exact test: $p<0.01$).

Signs and symptoms	Number of patients
Good clinical condition	29/31(94%)
Recurrent metabolic derangement	8/31 (26%)
Recurrent muscular pains with raised CK levels	10/31 (32%)
Cardiomyopathy	0/31 (0%)
Failure to thrive	7/31 (23%)
Feeding difficulties	8/31 (26%)
Hypotonia	4/31 (13%)
Speech delay	3/31 (10%)
Motor retardation	6/31 (19%)
Peripheral neuropathy	3/31 (10%)
Visual impairment	9/31 (29%)

Table 4 - Signs and symptoms during follow-up in 31 surviving LCHAD-deficient patients

Discussion

Our study is the first to report the presentation and outcome in a large, unselected group of LCHAD deficient patients. The clinical picture revealed differs in several respects from the pattern emerging from the previous literature. Published reports suggest that most patients present with hypoketotic hypoglycemia and a Reye-like illness, after a period of prolonged fasting often during a mild illness[14-16]. Only a few isolated patients with a more chronic presentation have been described[7-9,17-21]. In our series of fifty patients, eleven patients (22%) presented with a variety of chronic problems, such as cholestatic liver disease, failure to thrive, cardiomyopathy, muscular hypotonia and feeding difficulties. Careful analysis of the clinical history of those patients presenting with acute hypoglycemia, revealed that thirty-two of the thirty-nine (82%) patients, already had a combination of non-specific symptoms probably related to LCHADD, before the hypoglycemic episode. In total, therefore, fourty-three (86%) of our fifty patients initially had chronic rather than acute problems. Unfortunately, because the chronic symptoms tend to be nonspecific, their significance is easily missed. These symptoms are also observed in other long-chain FAO disorders, such as VLCAD and CPT2 deficiency[22, 23]. Hypotonia, liver disease, cardiomyopathy and other chronic problems are probably caused by an accumulation of long-chain acyl-CoA esters, which have, for example, been shown to be toxic to cardiomyocytes in vitro[24]. Furthermore, since they are relatively poorly excreted in urine, they accumulate even in the absence of an acute metabolic crises.

Neonatal symptoms are increasingly recognized in FAO disorders. Previous studies have shown a high incidence of neonatal symptoms in MCAD and VLCAD deficiencies[25-28].

Fifteen percent of patients with LCHADD in our study presented in the neonatal period. Few patients with MCADD present between the neonatal period and the age of 6 months, the median age of presentation being twelve months in this condition[25-27]. In contrast, the median age of presentation in our patients was 5.8 months. In this respect, as in many others, LCHADD resembles VLCAD deficiency more closely than MCADD.

Mortality in LCHADD is high. In our series, 38% of the patients died before or within 3 months after diagnosis. Though this mortality rate is lower than has been reported previously[7,14], it is still much higher than the mortality in MCADD (range 16 – 19%) [26,27]. The young age at presentation and the high mortality rate in LCHADD may be explained by two mechanisms. Firstly, toxicity from long-chain acyl-CoA esters may contribute to the increased mortality, by causing cardiac rhythm disturbances and cardiomyopathy. Secondly, the block in long-chain FAO results in an almost complete inability to synthesize

ketone bodies and/or ATP from long-chain fatty acids, the most abundant energy store in man. In MCADD, there is no production of toxic long-chain acyl-CoA esters and it is still possible to oxidize long-chain fatty acids to medium-chain fatty acids, resulting in significant production of ketone bodies and ATP.

Our study reveals a high incidence of lactic acidemia during metabolic decompensation due to LCHADD (table 3). This has been reported previously in a number of case histories[10,11,14,15,18,29-32]. The cause of the lactic acidosis in LCHADD is unclear, but it can probably be attributed to the toxicity of long-chain acyl-CoA esters. Long-chain acyl-CoA esters inhibit the mitochondrial ATP/ADP carrier[33,34] and the dicarboxylate carrier[35,36] in vitro. Inhibition of these carriers will increase the intra-mitochondrial and via the malate-aspartate shuttle the cytoplasmatic NADH/NAD+ ratio, leading to lactic acidosis with an increased lactate to pyruvate ratio. A second potential mechanism would involve direct inhibition of mitochondrial oxidative phosphorylation by 3-hydroxypalmitoyl-CoA[37]. Again this would be expected to cause lactic acidosis with an increased lactate to pyruvate ratio. Finally, long-chain acyl-carnitines may inhibit the pyruvate dehydrogenase complex (PDHC)[38] and this should, however, cause lactic acidemia with a normal lactate to pyruvate ratio. It is, therefore, difficult to predict the lactate to pyruvate ratio in LCHADD. The ratio was increased in six of the ten patients with a high lactate concentration in our series and it was normal in the remaining four patients.

It is now well established that LCHADD in a fetus predisposes the mother to the gestational complications, HELLP syndrome and AFLP[15,30,39-46]. The frequency of these complications is, however, unclear. Ibdah et al reported twelve cases of AFLP and three of HELLP syndrome in a series of nineteen pregnancies in which the fetus had LCHADD. In contrast, Tyni et al found only one case of AFLP and three of HELLP syndrome in a series of twenty-nine affected pregnancies, although they also found an increased frequency of intrahepatic cholestasis, preeclampsia and pregnancy-induced hypertension. The frequencies of AFLP and HELLP syndrome in our series were closer to those reported by Tyni et al. Of the fourty-seven pregnancies for which we have data, seven were complicated by HELLP syndrome and two by AFLP. This is still much higher than the prevalence in the normal population. The most likely cause is the production of toxic long-chain acyl-CoA esters by the feto-placental unit, probably in combination with the obligatory heterozygous state of the mother. However, the fact that, at least in animal studies, the unborn fetus predominantly depends on carbohydrate degradation for energy supply[47,48], with low oxidation rates for fatty acids[49], makes a substantial production of long-chain acyl-CoA esters by the fetus unlikely. Other factors may

therefore be involved in the pathogenesis of HELLP and AFLP. HELLP syndrome and AFLP have also been reported in association with fetal MTPD[50,51], CPT1 deficiency[52], MCADD[53] and an SCAD variant[54]. The latter two case reports may, however, be chance associations, since this SCAD variant is common (6% of the normal population) and MCADD is sufficiently common for us to know that gestational complications in this disorder are very rare.

Despite dietary treatment consisting of avoidance of prolonged fasting and a carbohydrate rich, fat restricted diet, morbidity in the surviving patients with LCHADD is remarkably high, with recurrent episodes of metabolic decompensation in 26% of the patients and recurrent muscle pains with raised CK levels in 32%. This might be due to the production of long-chain acyl-CoA esters which can continue despite treatment, as has been shown by Gillingham et al[15]. Differences in outcome, however, can be due to different dietary regimens, because Gillingham et al[15] also demonstrated that changing the composition of the diet results in changes in the concentration of long-chain acyl-CoA esters. Because we have no details on the exact dietary management of the patients in this study, we can not exclude beneficial influences of a low fat, carbohydrate rich, MCT enriched diet in the treatment of LCHADD.

There is much debate about the use of L-carnitine in patients with LCHADD. There have been several anecdotal reports suggesting that carnitine supplements may improve the clinical outcome in LCHAD deficient patients. Conversely, by promoting the formation of long-chain acyl-CoA esters, L-carnitine may be harmful: there are reports suggesting that patients on L-carnitine therapy do worse than those without L-carnitine supplementation[11,14,55]. Almost half the patients in our study were receiving L-carnitine supplements but we were unable to demonstrate any significant effect on the frequency of metabolic decompensation or muscle cramps. Although this conclusion is based on retrospective data, we do not think there is sufficient evidence to support the routine use of L-carnitine in LCHAD deficient patients.

Retinopathy with progressive visual impairment, a serious long-term complication in LCHADD, was found in nine (29%) of the thirty-one patients examined in our study. This is lower than the frequency reported by Tyni et al[56]. who found retinal changes in more than 50% of their patients. The most likely explanation for this discrepancy is the relatively short period of follow up for some of our patients. This may also explain the relatively low incidence of peripheral neuropathy in our series. The causes of the retinopathy and peripheral neuropathy in LCHADD are unknown. Low plasma concentrations of docosahexanoic acid (DHA) have been found in a few LCHAD deficient

patients and, following DHA supplementation, improvement has been reported in vision and nerve conduction[15,17]. Low levels of DHA may, however, have been caused by dietary deficiency of essential fatty acids, especially a-linoleic acid, rather than LCHADD itself.

Our study demonstrates that early recognition and treatment are critical in LCHADD, since mortality is low after diagnosis is made before acute decompensation and before irreversible organic failure has occurred. It is, therefore, important to recognize that this disorder can present with chronic, nonspecific problems, such as failure to thrive, liver dysfunction, and hypotonia, as well as cardiomyopathy. Early detection will greatly benefit from newborn screening programs for FAO disorders, including LCHADD. The long-term morbidity, however, can only be improved by multicenter studies to evaluate the effects of different therapeutic regimes, such as MCT, L-carnitine, and DHA supplementation.

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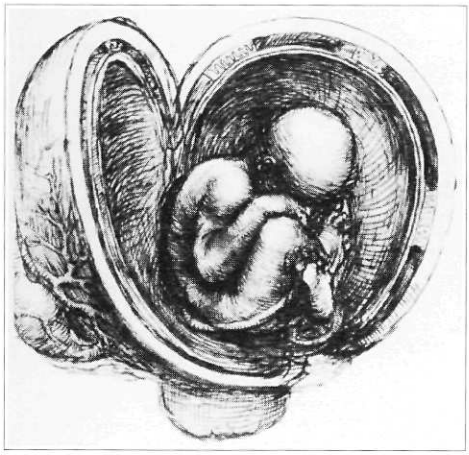

4

Mitochondrial trifunctional protein deficiency: A severe fatty acid oxidation disorder with cardiac and neurological involvement

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Abstract

Objective

To determine the spectrum of presentation, including both clinical and biochemical abnormalities, and the clinical course in a cohort of patients with complete mitochondrial trifunctional protein (MTP) deficiency, a rare inborn error of mitochondrial fatty acid oxidation (FAO).

Study design

A questionnaire was sent to the referring physicians from twenty-five unselected MTP-deficient patients.

Results

Twenty-one patients could be included. Questionnaires about four patients were not returned. Nine (43%) patients presented with rapidly progressive clinical deterioration; six of them (67%) of them had hypoketotic hypoglycemia. The remaining twelve patients presented with a much more insidious disease with non-specific chronic symptoms, including hypotonia (100%), cardiomyopathy (73%), failure to thrive, or peripheral neuropathy. Ten patients (48%) presented in the neonatal period. Mortality was high (76%), mostly attributable to cardiac involvement. Two patients who were diagnosed prenatally died despite treatment.

Conclusion

Complete MTP deficiency (MTPD) often presents with non-specific symptomatology, which makes clinical recognition difficult. Hypotonia and cardiomyopathy are common presenting features, and the differential diagnosis of an infant with these signs should include MTPD. In spite of early diagnosis and treatment, only a few patients with this condition have survived.

Introduction

Mitochondrial FAO is an important energy-producing pathway in human beings, especially during periods of prolonged fasting and exercise. The oxidation of fatty acids results in production of acetyl-coenzyme A (CoA) units that are used for adenosine triphosphate (ATP) synthesis in peripheral tissues and for ketone body synthesis in the liver. Ketone bodies that are exported from the liver can be used in all peripheral tissues, but are preferentially used in the brain and heart. The initial steps in the β-oxidation of long-chain fatty acids in mitochondria involve the activity of two proteins: very long-chain acyl-CoA dehydrogenase and the mitochondrial trifunctional protein (MPT)[1]. MTP is a multienzyme complex composed of four α- and four β-subunits bound to the inner mitochondrial membrane. It carries three distinct enzyme activities: long-chain enoyl-CoA hydratase (LCEH), long-chain 3-hydroxyacyl-CoA dehydrogenase (LCHAD) and long-chain thiolase activity[2,3]. The α-subunit harbors the LCHAD- and the long-chain enoyl-CoA hydratase activity, whereas the b-subunit contains the long-chain thiolase activity.

The biochemical diagnosis of MTPD is suggested by the demonstration of 3-hydroxy-dicarboxylic aciduria by gas-chromatography analysis. Specific abnormalities are also detectable in the plasma or blood spot acylcarnitine profile by tandem mass spectrometry, dominated by the accumulation of 3-hydroxy C16:0-, C16:1-, C18:0- and C18:1-acylcarnitines[4,5]. The diagnosis is confirmed by enzyme measurement in cultured fibroblasts[6] or lymphocytes[7].

Different biochemical phenotypes of MTPD can be recognized. In the first phenotype, called complete MTPD, all three MTP enzymes are deficient. Multiple private mutations in either the α-subunit or β-subunit have been recognized[7]. In the second phenotype, the LCHAD enzyme is primarily affected, with only partial deficiency of the thiolase enzyme. This condition is often classified as LCHAD deficiency (LCHADD). A common mutation has been identified in the α-subunit of the MTP, in the domain with LCHAD activity. In the majority of these LCHAD-deficient patients, at least one allele carries this point mutation (1528G>C)[8], which has not been detected in patients with complete MTPD. Finally, different combinations of partial defects of MTP enzymes have been described in a small number of cases[9]. Most of these cases are compound heterozygous for the 1528G>C mutation.

Since its first description in 1992, complete MTPD has been described in a total of approximately twenty patients, mostly as isolated case reports. From these reports, it appears that complete MTPD can be divided into two major clinical phenotypes: an early-

onset form, generally presenting with hypoketotic hypoglycaemia and cardiomyopathy[6,10], and a less frequent myopathic form, presenting in teenagers and adults[11,12].

Nevertheless, the clinical and biochemical phenotype of complete MTPD remains poorly defined, and little is known about its natural history. We attempted to further elucidate the clinical and biochemical spectrum of MTPD by collating data from a large cohort of patients.

Materials and methods

In the last decade complete MTPD was established in twenty-five patients at the two referral centers involved in this study (in the United Kingdom and the Netherlands). In all patients complete MTPD was confirmed by enzyme analysis. Enzyme measurement of all three MTP components (long-chain enoyl-CoA hydratase, LCHAD and long-chain thiolase) was performed using previously described methods in lymphocytes[7], if available, for rapid diagnosis, and in fibroblasts[6] in all patients. Screening for the 1528G>C mutation was performed in all patients, using previously described methods[8].

A questionnaire was sent to all thirteen referring physicians in charge of the twenty-five MTP deficient patients. The questionnaire was designed to collect information on the pregnancy, the clinical history and biochemical findings at time of diagnosis, the current treatment, follow-up and present medical status. Returned questionnaires were anonymised by permanently removing names and dates of birth. All data were further analyzed anonymously.

Results

Patients

From a total of twenty-five patients on whom questionnaires were sent out, we included in this study twenty-one patients with complete MTPD. Four patients could not be included, because the questionnaires were not returned. Five patients were enzymatically studied in the United Kingdom (Manchester), all others in the Netherlands (Amsterdam). The twenty-one patients belonged to eighteen apparently unrelated fami-lies. The male/female ratio was 8:13. Nine patients had been reported previously[6,13-16]. Twelve patients were white, five were Asian and three patients had a North-African ethnicity. One patient was of African, Caribbean-Asian and white ethnicity.

Diagnosis

In all patients the diagnosis of complete MTPD was confirmed by demonstrating deficiency of all three involved enzyme activities in cultured skin fibroblasts. The diagnosis was established prenatally in two unrelated patients, because in both the family history was strongly suggestive of a mitochondrial β-oxidation defect. None of the twenty-one patients was found to carry the 1528G>C mutation.

Pregnancy and delivery

The mean gestational age at time of delivery was 36.5 weeks (range 31-40 weeks). Five out of nineteen pregnancies for which data were available showed important maternal or fetal complications. In two pregnancies, the mother had hemolysis elevated liver enzymes and low platelets (HELLP) syndrome. None of the mothers had acute fatty liver of pregnancy (AFLP). Two other pregnancies were complicated by thyrotoxicosis. The birth weight of eight of twenty patients (40%) on whom information was available, was below the tenth percentile in relation to their gestational age. Seven out of the twenty-one patients (33%) had at least one hypoglycemic episode during the neonatal period; four of these were small for gestational age (all < 2500grams).

Clinical presentation

The median age at first presentation was three months (range, first day of life until three years). Ten out of the twenty-one patients (48%) presented within the neonatal period (0-4 weeks of age), including the two patients who were diagnosed prenatally. One of the prenatally diagnosed patients was born with signs of hydrops fetalis secondary to

cardiomyopathy, which was already detected at the end of the second trimester by ultrasound surveillance.

Nine patients (43%) presented with a rapidly progressive course with an acute life-threatening event due to metabolic decompensation, often in association with cardiac failure. The remaining twelve patients (57%) presented with a more insidious disease with non-specific symptomatology, dominated by hypotonia and other signs of muscle involvement. Mean age at presentation in the former group was sixty-three days (range 1 day - 14 months) and in the latter group 216 days (range 0 - 36 months). This difference between both groups is statistically not significant (Student t test, P = .15). Table 1 summarizes the presenting signs and symptoms of all infants.

Signs and symptoms	12 patients presenting with a slow insidious disease		9 patients presenting with rapidly progressive deterioration	
	number	percentage	number	percentage
Hypotonia	9/9*	100%	5/8*	63*
Muscular cramps	5/6*	83%	0/9*	0%
Lethargy	4/7*	57%	5/8*	63%
Cardiomyopathy	8/11*	73%		
Arrhythmia	1/10*	10%	1/7*	14%
Liver disease	6/10*	60%	4/9*	44%
Cholestasis	1/8*	13%	1/7*	14%
Feeding difficulties	9/10*	90%		
Failure to thrive	7/10*	70%		
Absent tendon reflexes	7/9*	78%		
Peripheral neuropathy	7/10*	70%	4/4*	100%
Pigmentary retinopathy	1/9*	11%	1/7*	14%

Table 1 – Signs and symptoms prior to and at diagnosis in all 21 MTP deficient patiens
** Number of patients on whom data were available*

Biochemical data

Rapidly progressive deterioration was always accompanied by lactic acidosis, often in combination with hypoglycemia. Six of seventeen patients had low calcium levels at presentation. In one patient this hypocalcaemia was demonstrated to be due to hypoparathyroidism[14]. The origin of the hypocalcaemia in the other five patients was unclear, but there was complete recovery after calcium administration in all these cases.

The acylcarnitine profile revealed abnormalities characteristic of MTPD in all tested patients. However, the abnormalities were occasionally very subtle. For instance, analysis of plasma acylcarnitines in two brothers presenting with the relatively mild phenotype consisting of severe peripheral neuropathy revealed 3-hydroxy C16:0-, C16:1-, C18:0- and C18:1-acylcarnitine concentrations only just above the normal range. The relevant biochemical parameters at presentation are listed in table 2.

Test	Patiens presenting with slow, insidious disease (n=12)		Patients presenting with rapidly progressive deterioration (n=9)		
	Patients with abnormal values, n (%)	Range	Patients with abnormal values, n (%)	Range	Normal Range
Glucose (mmol /L)	0/10 *(0)	3.9-6.9	6/9* (67)	0.7-2.6	3.5-7.0
Lactate (mmol/L)	5/9* (56)	3.5-7.5	8/8* (100)	3.7-36	<2.0
CK (U/L)	3/3* (100)	459-608	4/5* (80)	2937-12,280	<150
ASAT (U/L)	4/8* (50)	86-479	3/7* (43)	271-2190	<65
Ammonia (mmol/L)	2/8* (25)	69-112	6/7* (86)	140-503	<60
Total carnitine (mmol/L)	16* (17)	7	4/6* (67)	10-21	>25
Acylcarnitines	6/6* 100	-	6/6* (100)	-	-
Calcium (mmol/L)	4/10* (40)	0.93-1.70	2/7* (29)	1.29-1.86	2.1-2.5

Table 2 - Laboratory values at diagnosis in all 21 MTP-deficient patients
** Number of patients on whom data were available*

Clinical outcome

Overall mortality in this series of complete MTP patients was 76% (16 of 21). Eight of the nine patients who presented with a rapidly progressive disorder died of cardiac complications within eight weeks of presentation. One of them was diagnosed prenatally with complete MTPD and was started immediately after birth on frequent feedings. She suddenly presented with severe cardiomyopathy at three weeks of age and died of cardiac failure within twenty-four hours. The ninth patient in this group died of liver failure, four weeks after clinical presentation. In the group of twelve patients who presented with a more slow insidious course, seven (58%) died: five from progressive cardiomyopathy resulting in cardiac failure, one from an acute infection complicated by severe metabolic derangement and one died suddenly almost fourteen years after the first clinical presentation probably due to cardiac arrhythmia. The patient who was

antenatally diagnosed with cardiomyopathy and born with fetal hydrops died within twelve hours after birth despite intensive treatment including glucose infusions.

The five surviving patients have been followed-up for 0.5, 0.5, 1.25, 5 and 8.5 years respectively. All five are in relatively good clinical condition without any signs of cardiomyopathy. The first of these has persistent hypotonia and psychomotor retardation, but she has not suffered any acute metabolic derangement or muscular complications so far. Two of the survivors are brothers who originally presented with severe neuropathy with subsequent delay in motor development. Both have recurrent episodes of acute metabolic derangement with rhabdomyolysis. The other two surviving patients do not show any significant developmental delay. However, both have symptoms related to progressive peripheral neuropathy and regularly suffer from mild episodes of metabolic acidosis and/or muscular pains with associated myoglobinuria. Retinopathy was documented in two patients at the ages of 3 months and 14 months respectively. Both died within one year after diagnosis. Retinopathy was not been documented in any of the surviving patients. Even the patient who died suddenly fourteen years after diagnosis, did not have signs of retinopathy when examined in the last year of life.

Treatment

All five surviving patients have been on frequent feeds with a carbohydrate rich, low fat diet since diagnosis. Two of these patients additionally receive MCT- and carnitine supplementation and two are also treated with docosahexanoic acid (DHA). Calcium and vitamin D were prescribed in one patient because of persistent hypoparathyroidism. This individual also appears to have benefited from creatine supplementation, with a significant reduction in the frequency and severity of metabolic decompensation after starting creatine.

Discussion

Mitochondrial long-chain FAO disorders like very-long-chain acyl-CoA dehydrogenase deficiency, carnitine palmitoyl transferase 1 (CPT1) deficiency, and LCHADD can present with myopathy, cardiomyopathy and liver disease, with or without hypoketotic hypoglycemia, the classical metabolic derangement of FAO disorders.[1,7] Because complete MTPD includes LCHADD, it may be expected that patients with complete MTPD will present with a clinical picture closely resembling that of isolated LCHADD. Indeed, a number of patients have been reported with the same presentation as has been described in LCHADD. However, several case reports suggest that complete MTPD is generally a more severe disorder, with earlier presentation and severe cardiac involvement.

In our study, which is the first to describe a large cohort of complete MTP-deficient patients, the median age of presentation was 3 months, with 48% of the patients presenting within the neonatal period. This finding contrasts with isolated LCHADD, in which the median age at clinical presentation is six months.[17] An important observation in this regard is the development of hydrops fetalis due to severe cardiac failure in one of the two prenatally diagnosed cases. Prenatal onset of symptoms due to a FAO disorder is unusual, because it is generally assumed that fetal metabolism largely depends on carbohydrates as the main metabolic fuel. The antenatal onset of cardiac failure strongly suggests that, at least in myocardial tissue, FAO may also play a prominent role even during prenatal life. The potential role of FAO during prenatal life is also stressed by the large percentage (40%) of intrauterine growth retardation as observed in our series. A similar observation has been reported by Ibdah et al[18], who described a significantly lowered birth weight in MTP deficient mice, unrelated to maternal influences and not associated with placental abnormalities.

The severe pregnancy complications HELLP syndrome and AFLP have frequently been reported to be associated with the carriage of LCHAD deficient fetuses[19,20]. This association was recently also reported in other long-chain FAO disorders such as carnitine palmitoyl transferase 1 deficiency and complete MTPD[16,21]. In our study, two (11%) out of nineteen pregnancies on which information was available, were complicated by HELLP syndrome. The most likely cause of the high incidence of this pregnancy-related complication in mothers carrying a fetus with a long-chain FAO defect is the formation of toxic intermediates such as long-chain acyl-CoA esters by the affected fetal-placental unit.

Our study shows that cardiac involvement is very common in complete MTPD. Long-chain acyl-CoA esters have been implicated in the development of cardiac disease, because it

has been demonstrated that acylcarnitines are toxic to cultured cardiomyocytes in vitro[22]. Positron emission tomography (PET) studies in patients with long-chain FAO disorders have recently shown decreased myocardial palmitate oxidation with accumulation of fatty acids in a slow turnover pool as result of decreased oxidative capacity compatible with the cardiac involvement in this group of disorders[23].

Peripheral neuropathy with low to absent tendon reflexes has been reported in a few patients with isolated LCHADD[17]. Again, accumulating toxic long-chain acyl-CoA esters, are considered of etiologic significance. Our study reveals that peripheral neuropathy is a remarkably common and early sign in complete MTPD, as eleven out of the fourteen patients on whom information was available, had peripheral neuropathy at diagnosis (table 1 on page 63).

Pigmentary retinopathy, which has been reported as a late complication in 29% to 50% of the patients with LCHADD due to the common 1528G>C) mutation[17,24],was present in our series on complete MTPD in only two (13%) of the sixteen patients on whom information was available. This low incidence of retinopathy was also observed in a large family with adult presentation of MTPD[9]. However, in our study the low incidence of retinopathy may well be due to the high mortality and the consequently short period of follow-up in many patients.

Overall, complete MTPD appears to be a more severe disorder than isolated LCHADD. The reason for this is unclear, but may be related to the deficient activity of the MTP enzyme enoyl-CoA hydratase, which is normal in isolated LCHADD. The additional deficiency of enoyl-CoA hydratase can result in the formation of other metabolites, such as long-chain enoyl-CoA esters, which may contribute to, and exacerbate the toxic effect of the accumulating long-chain acyl-CoA esters. However, plasma acylcarnitine analysis, even during severe metabolic derangement, does not differentiate between isolated LCHADD and complete MTPD. It is probable that other yet unknown factors determine the different evolution of these two long-chain FAO disorders.

Treatment of complete MTPD, as in the other long-chain FAO disorders, generally consists of avoidance of fasting with frequent meals and supplementation with medium-chain triglycerides to prevent activation of long-chain FAO. Unfortunately, in complete MTPD this approach seems to be ineffective, because several patients in our series deteriorated with progressive cardiomyopathy despite dietary treatment. Even the two patients in whom the diagnosis was made before birth, died in spite of early treatment. A possible explanation may be that myocardial energy metabolism remains dependent on long-chain

fatty acids irrespective of high carbohydrate intake. Ongoing production of toxic intermediates may thus result in progression of the cardiomyopathy.

The reported beneficial effects of creatine in a patient with isolated LCHADD[25] and the remarkable results of creatine supplementation in one complete MTP deficient patient in this study are encouraging. Because phosphocreatine represents the most immediate reserve for the rephosphorylation of adenosine triphosphate (ATP) within the skeletal muscle cell, high doses of oral creatine may result in an increased energy reserve, causing clinical improvement. However, more research is needed to establish the value of this therapy in MTPD.

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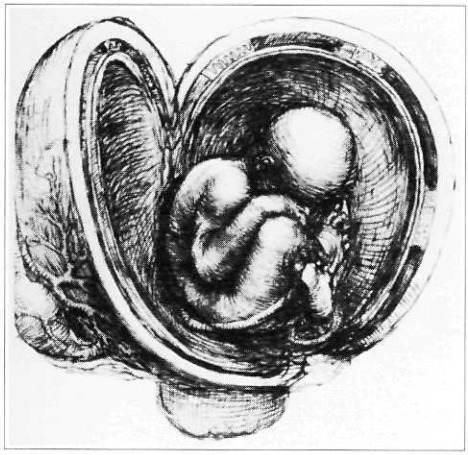

5

Heterozygosity for the common LCHAD mutation (1528G>C) is not a major cause of the HELLP syndrome and the prevalence of the mutation in the Dutch population is low

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Abstract

Long-chain 3-hydroxyacyl-CoA dehydrogenase (LCHAD) deficiency is an autosomal recessive disorder of mitochondrial fatty acid oxidation (FAO). Apart from life-threatening metabolic derangement with hypoketotic hypoglycemia, patients often show liver disease, cardiomyopathy and neuropathy. A common mutation (1528G>C) in the gene coding for the α-subunit of mitochondrial trifunctional protein harboring LCHAD activity is found in 87% of the alleles of patients. LCHAD is considered a rare disorder with only sixty-three patients reported in literature. Whether this is due to a truly low prevalence of the disorder or because many patients remain unrecognized as a result of aspecific symptomatology is not clear. A remarkable association between LCHAD deficiency (LCHADD) and the hemolysis, elevated liver enzymes and low platelets (HELLP) syndrome, which is a severe complication of pregnancy, has been reported. Because of this, we studied the frequency of the common LCHAD mutation in the Dutch population by analyzing 2047 Guthrie cards and one hundred and thirteen women who had suffered from HELLP syndrome. To be able to perform this large scale study in dried bloodspots we developed a new sensitive PCR-restriction fragment length polymorphism method. The carrier frequency for the common LCHAD mutation in the Dutch population was found to be low (1:680), consistent with the observed low incidence of the disorder. In the group of women with a history of a HELLP syndrome, the prevalence of the common LCHAD mutation was also low (1:113). We conclude that LCHADD is, indeed, a rare disorder and that heterozygosity for the common mutation is not a major cause of the HELLP syndrome.

Introduction

LCHADD is one of the thirteen inborn errors of mitochondrial FAO currently known. In patients with LCHADD the oxidation of long-chain fatty acids is impaired, due to mutations in the gene coding from the α-subunit of the mitochondrial trifunctional protein. The latter protein is an octamer of four α- and four β-subunits. The α-subunit harbors the enoyl-CoA hydratase and 3-hydroxyacyl-CoA dehydrogenase activities whereas the β-subunit carries the thiolase activity.

LCHADD is an autosomal recessive disorder. Patients usually present in infancy with recurrent attacks of hypoketotic hypoglycemia provoked by prolonged fasting often during a minor intercurrent illness such as a gastroenteritis[1]. In addition, cardiomyopathy[1,2] and hepatomegaly with cholestatic jaundice, which can sometimes progress to fulminant liver failure, is regularly observed[2,3]. Peripheral neuropathy and pigmentary retinopathy can occur during the course of the disease[2-5]. LCHADD can also present as sudden infant death even in the neonatal period[2,6].

The diagnosis of LCHADD is suggested by demonstrating the presence of large amounts of 3-hydroxy-dicarboxylic acids in the urine and by assessment of the acylcarnitine profile in plasma by tandem mass spectrometry[7]. Definitive diagnosis requires enzymatic studies which may be performed in liver, muscle, lymphocytes and fibroblasts[8-10].

The gene for the α-subunit of mitochondrial trifunctional protein carrying LCHAD activity is located on chromosome 2, and the genomic structure of the gene has been clarified[11]. Most remarkable is the occurrence of a common point mutation (1528G>C) in LCHADD[12-14] accounting for 87% of the affected alleles in seventy patients investigated[12]. This common mutation makes diagnosis at the molecular level feasible[12]. Remarkably, severe complications during pregnancy, including HELLP syndrome and acute fatty liver of pregnancy (AFLP) have been reported in mothers who are heterozygous carriers of the LCHAD mutation[14-16]. HELLP syndrome is a serious complication of pregnancy occuring in approximately 4% to 20% of women with severe preeclampsia[17]. AFLP is another complication of pregnancy, characterized by severe progressive liver disease, in approximately 50% of cases complicated by preeclampsia and sometimes observed in combination with HELLP syndrome[15,18]. HELLP syndrome carries a high risk for serious morbidity and even mortality for the affected mother and her child[17]. Although several mechanisms have been proposed, the exact pathophysiological mechanism(s) causing HELLP syndrome and AFLP still remain unclear[17,18]. The similarities between the liver disease seen in LCHADD and in HELLP syndrome as well as in AFLP, with microvesicular or

macrovesicular steatosis, in combination with the reported high incidence of these gestational complications in mothers heterozygous for the LCHAD mutation, suggest a causal relationship between a compromised long-chain FAO and HELLP and AFLP[14-16,19,20]. However, it is uncertain whether the risk of these gestational complications is limited to those pregnancies in which the fetus is homozygous for the LCHAD mutation. Nevertheless, in some centers mutation screening is offered to all mothers who suffered from HELLP syndrome or AFLP in order to allow presymptomatic diagnosis of LCHAD deficient newborns.

We decided to study the prevalence of the common LCHAD mutation (1528G>C) in women who suffered from HELLP syndrome during pregnancy and to compare this prevalence with the frequency of the 1528G>C mutation in the Dutch population in order to determine whether screening of mothers or their offspring for the LCHAD mutation is justified.

A PCR-RFLP method using a *Pst*I restriction site has previously been described, making detection of heterozygous individuals possible[12]. However, an important drawback of this method is that the sensitivity is not high enough when samples with a relatively low DNA concentration are used, because of the large amplified fragment (640 bp). To be able to perform large scale sensitive screening for the common LCHAD mutation in dried bloodspots, we developed a novel improved PCR-RFLP method and applied the method to establish the frequency of the 1528G>C mutation in bloodspots from control persons and women with a history of HELLP syndrome.

Materials and Methods

DNA extraction from bloodspots

DNA was extracted from bloodspots using Chelex (BioRad) essentially as described before[21] with some modifications. To this end a sample (3 mm diameter) was taken from a dried bloodspot and washed with 1 mL sterile water for 30 minutes at 50°C in a 1.5 mL Eppendorf tube. Thereafter 200 µL Chelex (50 g/L, pH 10.5) was added and incubated at 56°C for 30 minutes. Subsequently the samples were mixed for 10 seconds and centrifuged (3 min., 10,000 x g) followed by a 8-min. incubation in a boiling water bath. After cooling to room temperature, the samples were mixed for 10 sec. and centrifuged (3 min., 10,000 x g). 10 mL of sample was used in a 25 µL PCR reaction.

PCR-RFLP for the common LCHAD (1528G>C) mutation

To increase sensitivity of the used PCR-RFLP method we aimed to amplify a fragment smaller than the fragment of 640 bp, used before[12]. To prevent interference of the pseudogene as identified by Zang and Baldwin[11], a new primer had to be selected in intron 15. Since the sequence of this intron has not been published, we sequenced intron 15 completely (data not shown). Based on the obtained sequence, different primersets were selected. Only with the primerset used here (see below) a specific PCR fragment with high yield was obtained. The product contains a predicted *PstI* site which can serve as a convenient internal control.

Exon 15 and part of intron 15 were amplified in a 25 µL PCR reaction containing 10 mM Tris-HCl (pH 8.4 at 25°C), 1.2 mM $MgCl_2$, 50 mM KCl, 0.1 mg/mL BSA, dNTP (0,2 mM each), 2.5 U *Taq* polymerase (Promega) and the following primerset (12.5 pmol each): sense primer 5'-CCC TTG CCA GGT GAT TGG C-3', antisense primer 5'-ACA AGC CTG GAG GTA AAA GG-3'. DNA amplification was performed in a PTC-100 thermocycler from M.J. Research, Inc., programmed as follows:

120 s at 96 °C initial to cycling, 5 cycles of 30 s at 96 °C, 30 s at 55 °C and 30 s at 72°C followed by 25 cycles of 30 s at 94°C, 30 s at 55 °C and 30 s at 72 °C and the end of cycling 10 min. at 72°C. The amplified fragment (224 bp) was directly digested after addition of 2.5 mL buffer M and 5 U *PstI* (Boehringer Mannheim).

The restriction fragments were analyzed on a 2% (wt/vol) agarose gel with ethidiumbromide staining. To validate this method we performed PCR-RFLP in dried bloodspots from a control subject, a homozygous 1528G>C patient and both parents.

Population screening for the common ICHAD mutation

In the Netherlands approximately 99% of all newborns (± 200.000 live births yearly) are tested for phenylketonuria and congenital hypothyroidism in a nationwide screening program by means of Guthrie cards. For this study 2047 Guthrie cards were anonymously obtained from the screening laboratories representing the twelve Dutch provinces and the two largest cities (Amsterdam and Rotterdam), after approval by the Dutch Health Authorities. The total number of cards selected from each of the fourteen screening areas and used in our population screening was proportional to the number of live births in each of these regions, which guarantees a demographic representation of the Dutch population

Confidence intervals (CI) were calculated using the method for estimating the population carrier rate when some carriers are not detected as described by Parker and Philips[22], accounting for the 87% allele frequency of the common 1528G>C mutation in LCHAD deficient patients.

Prevalence of the common LCHAD mutation in HELLP syndrome

HELLP syndrome was defined as hemolytic anemia (LDH > 600 U/L), elevated liver enzymes (ASAT > 70 U/L) and thrombocytopenia (thrombocytes < 100 x 10^9/L) during pregnancy[23]. A total of one hundred and thirteen women who had suffered from HELLP syndrome during at least one of their pregnancies were included in this study. Inclusion was irrespective of the outcome of the affected pregnancy. Bloodspots on Guthrie cards were collected from all one hundred and thirteen women. This study was approved by the Institutional Medical Ethical Committee.

Results

In order to be able to do the studies described in this article, we had to set up a new sensitive method allowing unequivocal identification of the 1528G>C mutation in bloodspots. The result of this new procedure is shown in figure 1.

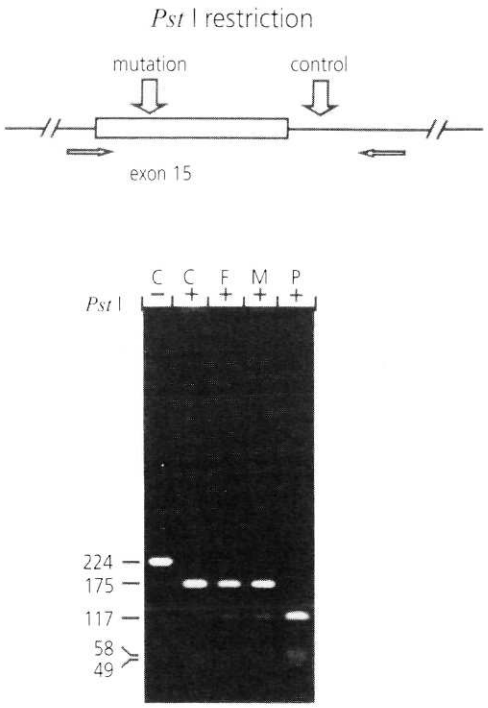

Figure 1 - PCR-RFLP analysis of the 1528G>C mutation.
Upper part: Schematic representation of a part of the gene.
The primers are indicated by horizontal arrows,
positions of the PstI restriction sites are indicated by vertical arrows.
Lower part: Ethidium bromide stained agarose gel showing PCR-RFLP analysis using DNA extracted from dried bloodspots of a control subject [C], a LCHAD deficient patient [P] and the father [F] and the mother [M] of the patient.
PCR products were either directly loaded (-), or digested with PstI (+) before electrophoresis.

After restriction of the amplified fragment from a control bloodspot, the predicted restriction fragment of 175 bp (and 49 bp) was obtained. In a patient known to be homozygous for the 1528G>C mutation a smaller restriction fragment of 117 (58 and 49 bp) was found, indicating that *Pst*I has cut at both the control site and at the position of the mutation. Both fragments of 175 bp and 117 bp were visible using material from both parents, which is compatible with heterozygosity (figure 1, lower panel). The PCR product contains a predicted *Pst*I site which can serve as a convenient internal control (figure 1, upper panel).

Screening for the common LCHAD mutation (1528G>C), using the new PCR-RFLP method, in the 2047 Guthrie cards obtained form the neonatal screening centres, detected three carriers for this mutation. No homozygous deficient samples were found. The prevalence of the common (1528G>C) LCHAD mutation in the Dutch population is thus estimated to be 1: 680 (95% confidence interval of 1: 325 to 1: 1400).

Among the one hundred and thirteen women who suffered from HELLP syndrome during at least one of their pregnancies, one carrier for the 1528G>C mutation was identified (prevalence 1:113, 95% confidence interval of 1:18 to 1:560).

The prevalences of the common LCHAD mutation among the general Dutch population and among women who suffered from the HELLP syndrome are not statistically different (two-tailed Fisher exact test, p value 0.19).

Discussion

Inborn errors of mitochondria FAO are often diagnosed with considerable delay because of the aspecific symptomatology. For instance, 20% of the MCAD deficient patients were diagnosed after death, and in 25% a sibling had suddenly died without a proper diagnosis[24] but probably due to MCAD deficiency (MCADD). While MCADD is a relatively common disorder among Caucasians with a prevalence of the common mutation (985G>A) ranging from 1:333 in Italy[25] to as high as 1:55 in the Netherlands[26], LCHADD seems to be a much rarer disorder. While in the Netherlands every year approximately fourteen patients are diagnosed with MCADD in accordance with the carrier frequency for the common mutation, only six patients with LCHADD have been identified in the last ten years. However, since LCHADD can present with misleading signs and symptoms such as cholestatic jaundice and severe cardiomyopathy, it may well be that the diagnosis of LCHADD is even more frequently missed than MCADD. For this reason population screening for the common LCHAD mutation (1528G>C, allele frequency 87%) was performed.

Because the previously described PCR-RFLP method[12] has a low sensitivity for the heterozygous detection in samples with relatively low concentration of DNA such as bloodspots, a new, more sensitive method was developed. Recently Ding et al[27]. described a sensitive nested PCR-RFLP method. The origin of the PCR product was then confirmed by a gene (and not a pseudogene) specific restriction site for *PvuI*. This two-step amplification method works well but is too laborious for processing large numbers of samples. The method described here is a simple, one-step PCR-RFLP method which only amplifies the coding gene. Furthermore the method uses a second *PstI* site as internal control for the restriction, preventing false-negative results. Therefore this PCR-RFLP method is superior to the previously described methods and allows heterozygous detection in dried bloodspots, making our carrier frequency studies possible.

The observed prevalence of the carrier frequency for the common LCHAD mutation of 1:680 (CI 1:325 - 1:1400) in the Dutch population is indeed much lower than that of the MCAD mutation (1:55) and with approximately 200,000 live births yearly, corresponds well with the low number of patients diagnosed in the last decade. It is therefore unlikely that many patients are missed because of an aspecific presentation.

In order to study the relation between HELLP syndrome and AFLP on the one hand and LCHADD on the other hand, we compared the observed prevalence with the frequency of the 1528G>C mutation in a group of women who had suffered from HELLP syndrome.

It is important to keep in mind that our study only allows conclusions related to HELLP syndrome. Although AFLP is regarded as part of the spectrum of the group of gestational disorders which includes HELLP syndrome, further studies are necessary to see whether heterozygosity for the common LCHAD mutation is an important risk factor for the development of AFLP.

Our results clearly show that virtually all women with a history of HELLP syndrome were homozygous normal for the 1528G>C mutation. These results are important especially since it has been suggested to perform mutation screening in women suffering from the HELLP syndrome. The results of our study provide no justification for this.

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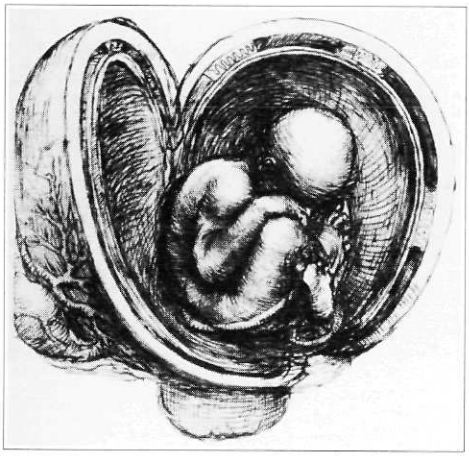

6

Long-chain triglyceride loading test in female LCHAD heterozygotes

submitted

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Abstract

Toxic long-chain acyl-CoA esters produced by the foeto-placental unit are considered to be causative in the remarkable and still unravelled association between severe complications of pregnancy in the mother carrying a fetus with a fatty acid oxidation (FAO) disorder. Transported to the mother in the form of long-chain acylcarnitines, these long-chain acyl-CoA esters are considered to accumulate and cause severe liver disease. In order to study the capacity of these women heterozygous for the common LCHAD mutation to metabolise long-chain fatty acids, we performed long-chain triglyceride (LCT) loading tests and compared them with healthy controls. The results show a normal capacity to metabolize fatty acids. The hypothetical accumulating long-chain acyl-carnitines do not appear to affect the mothers directly by entering their circulation, but possibly by causing auto-intoxication of the placenta which can result in shedding of microparticles entering the maternal circulation and causing multi-organ disease such as haemolysis elevated liver enzymes and low platelets (HELLP) syndrome or acute fatty liver of pregnancy (AFLP).

Introduction

In the last decade a remarkable association between several inborn errors of long-chain fatty acid oxidation, and the maternal complications of end-stage pregnancy, HELLP syndrome, and AFLP has become clear. These gestational complications, characterized by progressive maternal liver disease with potential life-threatening complications for both mother and child, are rare in the normal population with an incidence of 0.1 to 0.6 percent for HELLP syndrome and < 0.01 percent for AFLP in all pregnancies. A very high risk of such complications, ranging from 19 to 79 percent, has been well documented in long-chain 3-hydroxyacyl-CoA dehydrogenase (LCHAD) deficiency (McKusick 600890)[1,2] and mitochondrial trifunctional protein (MTP) deficiency [3,4].

The etiology of this puzzling association has remained unclear. Following the demonstration that mothers only had an increased risk for HELLP and AFLP when they were carrying a LCHAD deficient fetus, and not when carrying an unaffected sib, it was proposed that the interaction between the affected fetus and the heterozygous mother is a prerequisite for the increased risk for one of these pregnancy complications[2]. It has been hypothesized that the affected feto-placental unit produces excessive amounts of long-chain acyl-CoA esters[5,6]. These are presumed to be cytotoxic by inhibition of a number of intramitochondrial processes such as the mitochondrial ATP/ADP carrier, the dicarboxylate carrier and the mitochondrial oxidative phosphorylation system[7]. These long-chain acyl-CoA esters may be transported via the placenta towards the mother as long-chain acylcarnitine esters. Finally, the liver of the mother is thought to be unable to rapidly metabolise these long-chain acylcarnitines, due to its decreased long-chain FAO capacity as a consequence of its heterozygous state. The accumulating toxic long-chain acyl-CoA esters would thus cause the maternal liver disease. However, as there have been no reports on any clinical signs or symptoms due to a diminished long-chain FAO capacity in individuals heterozygous for the 1528G>C mutation, it is unclear whether mitochondrial FAO is indeed compromised in heterozygous women. In order to study the in vivo long-chain fatty acid oxidation capacity, we therefore performed LCT loading tests in two women heterozygous for the common LCHAD mutation as well as in four healthy female controls.

Materials and methods

Subjects

Two females, 39 and 27 years old, both heterozygous for the common 1528G>C mutation, were studied. One of them is the mother of three children, of whom two are LCHAD deficient, both homozygous for the 1528G>C mutation and one is homozygous normal. During her first pregnancy, carrying a LCHAD deficient fetus, she suffered from severe HELLP syndrome. The two subsequent pregnancies, resulting in one unaffected and one LCHAD deficient baby, were uncomplicated. The second mother has given birth to a single LCHAD deficient child with homozygosity for the 1528G>C mutation. This pregnancy was uncomplicated.

Four healthy controls (27-30 years old), all on oral contraceptives as sole medication, were studied as controls. Heterozygosity for the common 1528G>C mutation was excluded in all controls using established techniques[8].

The LCT loading test

After an overnight fast (12 hours an indwelling, nonheparinized, catheter was placed into the forearm vein for blood collection. The LCT load, consisting of 1.5 ml of sunflower oil (fatty acid composition: linoleic acid 68%, oleic acid 21%, palmitic acid 7%, stearic acid 4%) per kg bodyweight, was ingested orally within a period of 10 minutes. The subjects were allowed to drink only water during the test. Blood samples were taken before and 120, 240 and 480 min. after ingestion, and immediately placed on ice and deproteinised for measurement of ketone bodies (acetoacetate and ß-hydroxybutyrate).

Analytical procedures

Glucose, ketone bodies, triglycerides and FFA-concentrations, were measured in plasma using standard methods. Acylcarnitine analysis was performed by tandem-mass spectrometry, using previously described method[9].

This study was approved by the local Medical Ethical Committee and written informed consent was obtained from all included women.

Results and discussion

The LCT loading test is presumed to be a sensitive tool for diagnosing disturbances in the oxidation of long-chain fatty acids[10,11]. It is less hazardous than a prolonged fasting test. The sunflower oil used for the LCT loading, is rich in linoleic acid, a preferred substrate for the LCHAD enzyme[12].

In our study, the LCT load was well tolerated by all six tested subjects. Blood glucose concentrations remained normal in all subjects during the test. As expected, plasma triglyceride concentrations increased mildly during the test in all subjects, reflecting adequate absorption of the LCT load. The C14:2-acylcarnitine concentration rose in all subjects during the test, from a mean value of 0.05 µmol/L before loading to 0.14 µmol/L 480 minutes after loading. C14:2 is one of the intermediate products of the ß-oxidation of linoleic acid, the main component of sunflower oil (68%).

The long-chain hydroxy-acylcarnitines (C16, C16:1, C18, C18:1), which are considered to be the characteristic metabolites in LCHAD deficiency[9], did not rise significantly during the test in either of the two groups. Furthermore, the identical rise in plasma ketone body concentrations, in both the heterozygous mothers and the healthy controls, demonstrates normal activation and activity of long-chain FAO in both groups, even under conditions of a fatty acid overload.

As there have never been any reports on clinical signs or symptoms related to a decreased capacity of long-chain fatty acid oxidation (FAO), such as hypoketotic hypoglycaemia, cardiomyopathy, rhabdomyolysis or peripheral neuropathy in individuals heterozygous for a long-chain FAO disorder, normal results of a LCT loading test might be expected. However, the very high incidence of HELLP syndrome and AFLP during end stage pregnancy in mothers carrying a LCHAD deficient child has led to the hypothesis that an interaction between the affected fetus and the heterozygous mother is a prerequisite[2].

To our opinion however, the normal LCT loading tests in heterozygous mothers as presented in this study, strongly suggests that long-chain FAO capacity is normal and that these mothers should be able to rapidly metabolise any foetally produced long-chain acylcarnitines, entering their circulation. We therefore postulate that the heterozygous state of the mother in itself does not play a major role, if any, in the pathogenesis of the gestational complications. An alternative explanation may be provided by the recent observation that the human placenta has a high activity of the long-chain FAO enzymes[13,14]. High activity of FAO in the placenta, which genetically is of foetal origin, can result in the production of toxic long-chain acyl-CoA esters in case of a feto-placental unit

deficient in long-chain FAO. Auto-intoxication of the affected placenta by the accumulating long-chain acyl-CoA esters, may result in excessive shedding of placental microparticles into the maternal circulation, activating the cytokine system, causing multi-organ disease[15] including HELLP syndrome and AFLP.

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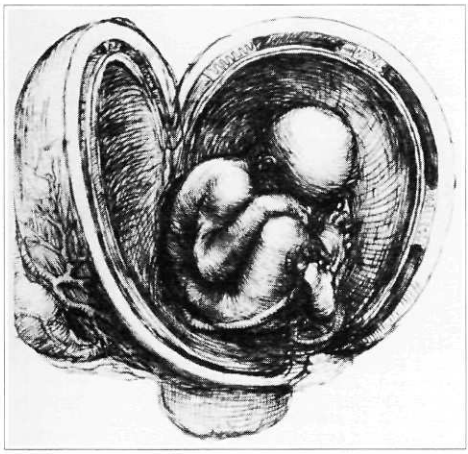

7

High activity of fatty acid oxidation enzymes in human placenta: Implications for fetal-maternal disease

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Abstract

As the human fetus and placenta are considered to be primarily dependent on glucose oxidation for energy metabolism, the cause of the remarkable association between severe maternal pregnancy complications and the carriage of a fetus with an inborn error of mitochondrial long-chain fatty acid oxidation (FAO) has remained obscure. We analyzed human term placenta and chorionic villus samples for the activities of a variety of enzymes involved in FAO, and compared the results with those obtained in human liver. All enzymes were found to be expressed, with a very high activity of two enzymes involved in the metabolism of long-chain fatty acids (CPT2 and VLCAD), whereas the activity of medium-chain acyl-CoA dehydrogenase (MCAD) was found to be low, when compared to liver. These results suggest that FAO may play an important role in energy generation in human placenta, and that a deficiency in the placental oxidation of long-chain FAO may result in placental dysfunction, thus causing gestational complications.

Introduction

Mitochondrial β-oxidation of fatty acids plays an essential role in energy metabolism in humans. In muscle, during moderately severe exercise, energy is mainly produced from FAO. The heart preferentially uses fatty acids as a substrate for energy production. During fasting, FAO in the liver is used to produce ketone bodies that are exported and can be used by all peripheral tissues, but are preferentially used in the brain and in the heart. Mitochondrial β-oxidation involves the concerted action of a multitude of enzymes, starting with the carnitine-mediated transfer of long-chain fatty acids over the mitochondrial inner-membrane via carnitine palmitoyl-CoA transferase 1 (CPT1) (EC 2.3.1.21), carnitine acyl-carnitine translocase (CACT) and carnitine palmitoyl-CoA transferase 2 (CPT2). Medium-chain and short-chain fatty acids can enter the mitochondrial matrix independent of this carnitine cycle. Once inside the mitochondria, fatty acyl-CoA esters undergo β-oxidation via the classical four-step mechanism, involving dehydrogenation, hydration, dehydrogenation and thiolytic cleavage. The importance of the mitochondrial FAO is stressed by the existence of a variety of different genetic diseases in man in which mitochondrial β-oxidation is impaired[1]. Clinical signs and symptoms are in part related to the lack of energy for metabolic functions, resulting in hypotonia, hypoketotic hypoglycemia and multiple organ failure. In addition, patients with inborn errors of the mitochondrial long-chain β-oxidation, such as very long-chain acyl-CoA dehydrogenase (VLCAD) deficiency (McKusick 201475), long-chain 3-hydroxyacyl-CoA dehydrogenase (LCHAD) deficiency (McKusick 600890) and mitochondrial trifunctional protein (MTP) deficiency may present with a variety of severe clinical problems, such as cardiomyopathy, retinopathy and peripheral neuropathy. Accumulation of toxic long-chain acyl-CoA esters is considered to be involved in the pathogenesis.

In recent years it has become clear that there is a striking association between the severe pregnancy complications hemolysis, elevated liver enzymes and low platelets (HELLP) syndrome and acute fatty liver of pregnancy (AFLP) and the carriage of a fetus with a long-chain FAO disorder[2-12]. These complications of pregnancy are very rare in the normal population, but have been reported to occur with a high frequency, up to more than 10%, in mothers carrying a fetus with a long-chain FAO disorder[11,12] The mechanism behind this association has remained obscure. As the fetus is considered to be primarily dependent on glucose oxidation for energy production[13], it is unlikely that fetal production of toxic long-chain acyl-CoA esters causes the HELLP syndrome or AFLP in the

mother. However, since the human placenta is for the largest part of fetal origin, and since the placental mass represents a relatively high proportion of the fetal-placental unit at term, an alternative explanation would be that the defective oxidation of fatty acids in placental tissue is directly responsible for HELLP syndrome or AFLP in the mother. Recently, Rakheja and coworkers[14] were the first to demonstrate activity of the FAO enzymes LCHAD and short-chain 3-hydroxyacyl-CoA dehydrogenase (SCHAD) in human placenta, giving support to this hypothesis. In order to expand on these results, we studied the activity of a whole range of different enzymes involved in FAO in normal term placenta, as well as in normal chorionic villus biopsies.

Materials and Methods

Human placentas were collected, after written informed consent from the mother, from six uncomplicated term pregnancies. All deliveries were spontaneous and without complication. Placentas were biopsied (0.5 cm^3) within one hour after delivery on the fetal side, and multiple biopsies were snap frozen in liquid nitrogen and stored at -70°C until biochemical studies were done. Chorionic villus samples were obtained by standard procedures at gestational ages ranging from 10 - 14 weeks for prenatal diagnostic purposes. For this study biopsies that were found to be normal were used. Biopsies were stored at -70°C for further studies.

The activities of VLCAD and of medium-chain acyl-CoA dehydrogenase (MCAD) (EC 1.3.99.3) were measured as described elsewhere[1], using phenylpropionyl-CoA and palmitoyl-CoA as substrates, respectively. The production of the α- and β-unsaturated and 3-hydroxyacyl-CoA species were determined by HPLC and used to calculate acyl-CoA dehydrogenase activities. The activities of short-chain and long-chain enoyl-CoA hydratase (SCEH and LCEH) (EC 4.2.1.17), SCHAD and LCHAD (EC 1.1.1.35) and long-chain 3-hydroxyacyl-CoA thiolase (LCTHIO) were determined as previously described[15]. The activity of CPT2 was measured radiochemically, essentially as described by Demaugre et al[16].

Control values for enzymatic activity in human liver were measured in our laboratory, using the same techniques as used for the study in placental tissue.

Results and Discussion

In order to investigate the capacity of placental tissue for fatty acid β-oxidation, we measured the activity of a range of different enzymes involved in FAO. Surprisingly, there is very little information on this point in literature[14,17]. All eight enzymes studied were found to be expressed in term human placental tissue as well as in chorionic villus samples. Remarkable high activities of FAO enzymes involved in the oxidation of long-chain fatty acids were detected. The activities of all enzymes, as measured in placenta, chorionic villus samples and liver are shown in table 1. We compared the acitivities of the FAO enzymes in placenta and chorionic villus samples with the activities as measured in control human liver. Interestingly, the mean enzymatic activity of both CPT2 and VLCAD were found to be even higher in placental tissue and chorionic villus samples than in human liver tissue, which is known for a very high capacity for FAO.

	Placenta			Chorionic villus samples			Liver		
Enzymes	Mean	SEM	n	Mean	SEM	n	Mean	SEM	n
CPT2	88	21	6	88	12	4	57	19	3
VLCAD	11.9	1.7	6	22.8	2.3	3	7.1	0.9	3
LCHEH	32.2	5.4	6	167	6.0	2	197	21	4
LCHAD	36.9	5.0	6	120	7.1	19	313	22	10
LCTHIO	19.8	3.4	6	22.3	2.1	12	44	9	11
MCAD	0.13	0.02	6	0.8	-	1	6.94	0.71	5
SCEH	295	55	6	836	9.2	2	1200	139	4
SCHAD	77.1	17.0	6	175	15.7	20	857	123	19

Table 1 - Mean activities and SEMs of FAO enzymes in human placenta at term, in chorionic villus samples gestational (ages 10-14 weeks) and in control human liver. Activities are expressed as nmol/min/mg protein. (Abbreviations of enzymes: see text materials and methods)

The human placenta has a high rate of oxygen consumption at term[18], in order to sustain a high production rate of ATP, which is expended in the synthesis of a variety of placental proteins and hormones, in placental cation transport and in placental synthesis of essential poly-unsaturated fatty acids. While in sheep placenta oxidative phosphorylation depends on glucose as a substrate[19], our results suggest that in human placenta FAO may play an important role in energy metabolism. Long-chain fatty acids as substrate for

placental FAO are abundantly available in maternal plasma during the last trimester of pregnancy, when maternal lipid metabolism switches to a catabolic state resulting in increased concentrations of plasma triglycerides and free fatty acids (FFA)[20,21]. Rat placenta has been shown to express heart fatty acid binding protein (FABP), fatty acid translocase (FAT) as well as fatty acid transport protein (FATP), pointing to the ability of placental tissue for fatty acid uptake, and transplacental movement of fatty acids to fetal compartments[22]. Our results clearly show that the FFA produced by enhanced maternal lipolysis during end-stage pregnancy may be important as fuel for placental metabolism.

Comparing the activities of the different enzymes involved in placental and chorionic villi FAO in relation to the activities in liver (table 1), a striking difference between the enzymes involved in long-chain FAO and the enzyme involved in medium-chain FAO (MCAD) becomes clear. Mean VLCAD activity in placenta was 11.9 nmol/min/mg protein and in liver 7.05, resulting in a ratio of placental activity to liver activity of 1.7. In contrast, mean MCAD activity in placenta was found to be 0.13 nmol/min/mg protein with an activity in liver of 6.94, resulting in a placenta to liver ratio of 0.02. For the other enzymes studied, involved in long-chain FAO (CPT2, LCEH, LCHAD and LCTHIO), the calculated placenta to liver activity ratios were 1.55, 0.16, 0.12 and 0.45 respectively, all substantially higher than the activity ratio for MCAD (0.02). The same pattern was found for the FAO enzymes in the chorionic villus samples. As in liver complete fatty acid degradation proceeds normally from long-chain fatty acids as the main substrate, via medium-chain fatty acids and short-chain fatty acids to acetyl-CoA units, our data from placental tissue and chorionic villus samples, with a skewed pattern of enzyme expression as compared to liver, suggest that medium-chain acyl-CoA esters are produced as an intermediate metabolite. Accumulating medium-chain acyl-CoA esters produced in the mitochondrial matrix can be converted into medium-chain acyl-carnitine esters by CPT2, which we showed to have a very high activity in placental tissue, followed by transport out of the mitochondria to the cytosol and subsequently into the fetal circulation. FAO of medium-chain fatty acids is, in contrast to FAO of long-chain fatty acids, independent of the CPT1-CACT-CPT2 system and therefore not controlled by malonyl-CoA. Since malonyl-CoA strongly inhibits CPT1 in the fetus[23], medium-chain fatty acids produced by the placenta can thus, in contrast to long-chain fatty acids, be used as an important metabolic fuel, reducing the fetal dependency of glucose as substrate. This hypothesis is substantiated by the remarkably high concentration of C8-carnitine as detected by tandem-MS in mid-term amniotic fluid of healthy pregnancies[24].

It has been well-established that the presence of an inherited disorder of long-chain FAO in the fetus predisposes the mother for gestational complications such as HELLP syndrome and AFLP[2-12]. The prevalence of these rare disorders with substantial neonatal and maternal morbidity and mortality varies between 1:250 for HELLP syndrome and 1:13.000 for AFLP. In contrast, the combined prevalence is more than 1:10 in pregnancies in which the mother carries a fetus with a long-chain FAO defect[11,12]. The most likely cause of these complications is the production of long-chain acyl-CoA esters by the feto-placental unit. It has been demonstrated that long-chain acyl-CoA esters inhibit the mitochondrial ATP/ADP carrier, the dicarboxylate carrier and the pyruvate dehydrogenase complex in vitro[25-27]. In addition, 3-hydroxypalmitoyl-CoA is an inhibitor of mitochondrial oxidative phosphorylation[28]. However, because the unborn fetus predominantly depends on carbohydrate degradation for energy supply, a substantial production of toxic long-chain acyl-CoA esters by the fetus seems highly unlikely. As the placenta at term accounts for more than 15% of the total weight of the feto-placental unit, and because placental tissue is almost completely of fetal origin, our observation that the enzymes involved in long-chain FAO are highly expressed in term-placenta strongly suggests an important role for placental production of toxic long-chain acyl-CoA esters in the pathogenesis of HELLP syndrome and AFLP in mothers carrying a fetus with a long-chain FAO disorder. This hypothesis was already suggested by Rakheja and coworkers[14] on the basis of the reported activities of LCHAD and SCHAD in human placenta. In addition, they demonstrated higher activities of these two enzymes in early gestation. In line with their observation, our study in normal human chorionic villus samples also demonstrated higher activities of all studied FAO enzymes, except CPT2, in the very early stages of pregnancy.

There are two possible explanations for the observed high prevalence of these pregnancy complications. One is that long-chain acyl-CoA esters produced by the placenta are transferred as long-chain acylcarnitine esters to the maternal circulation and taken up by maternal liver and other tissues. The maternal liver, which might be compromised for long-chain FAO capacity due to its obligatory heterozygous state, is then unable to rapidly oxidize the produced long-chain acyl-CoA esters, resulting in maternal liver disease (AFLP). Another and presumably additional explanation is that accumulation of long-chain acyl-CoA esters in the placenta directly results in placental damage, due to inhibition of a variety of essential metabolic processes. Indeed, a high percentage of placental infarctions has been demonstrated in pregnancies of LCHAD deficient fetuses[8,29]. Inhibition of oxidative phosphorylation by long-chain acyl-CoA esters might also result in the production of reactive oxygen species (ROS) and enhanced lipid peroxide formation,

which could lead to endothelial dysfunction which is considered as an important pathophysiologic factor for preeclampsia or HELLP[30,31]. Moreover, the disturbed energy metabolism in the placenta as a result of accumulating long-chain acyl-CoA esters, may promote shedding of micro-particles into the maternal circulation, resulting in activation of the cytokine system and thereby in multi-organ disease[32].

In summary, this report demonstrates the expression of eight different enzymes involved in FAO in human term placenta and chorionic villus samples, with remarkably high activities of enzymes involved in long-chain FAO. A decreased capacity for placental long-chain FAO, as is the case during pregnancies with a fetus suffering from an inborn error of long-chain FAO, may result in placental production of toxic long-chain acyl-CoA esters, presumably causing the high prevalence of gestational complications in mothers carrying a long-chain FAO deficient fetus. Finally, the observation of a very low activity of MCAD in placenta as compared to the long-chain FAO enzymes, adds to the understanding of fetal energy metabolism during end-stage pregnancy.

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Long-chain fatty acid oxidation during early human development

Submitted

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Abstract

Patients with very long-chain acyl-CoA dehydrogenase (VLCAD) and long-chain 3-hydroxyacyl-CoA dehydrogenase deficiency / mitochondrial trifunctional protein deficiency (LCHADD/MTPD), disorders of the mitochondrial long-chain fatty acid oxidation (FAO), can present with hypoketotic hypoglycemia, rhabdomyolysis and cardiomyopathy. In addition, patients with LCHADD/MTPD may suffer from retinopathy and peripheral neuropathy. Until recently there was no indication of intra-uterine morbidity in these disorders. This observation was in line with the widely accepted view that FAO does not play a significant role during fetal life. However, the high incidence of the gestational complications acute fatty liver of pregnancy (AFLP) and hemolysis elevatid liver enzymes and low platelets (HELLP) syndrome observed in mothers carrying a LCHAD/MTP deficient child and the recent reports of fetal hydrops due to cardiomyopathy in MTP deficiency (MTPD), as well as the high incidence of intra-uterine growth retardation (IUGR) in children with LCHADD/MTPD, suggest that FAO may play an important role during fetal development. In this study, using in situ hybridization of the VLCAD and the LCHAD genes, we report on the expression of genes involved in the mitochondrial of long-chain fatty acids during early human development. Furthermore, we measured the enzymatic activity of the VLCAD and LCHAD enzymes in different human fetal tissues. Human embryos (at days 35 and 49 of development) and separate tissues (5-20 weeks of development) were used. The results show a strong expression of VLCAD and LCHAD mRNA and a high enzymatic activity of VLCAD and LCHAD in a number of tissues, such as liver and heart. In addition, high expression of LCHAD mRNA was observed in the neural retina and central nervous system. The observed pattern of expression during early human development is well in line with the spectrum of clinical signs and symptoms reported in patients with VLCAD or LCHADD/MTPD.

Introduction

Mitochondrial oxidation of fatty acids plays a critical role in energy metabolism after birth. The heart preferentially uses fatty acids as substrate for energy production. In addition, during moderately severe exercise, skeletal muscle predominantly utilizes fatty acids as energy source. In the liver, FAO is used during fasting to produce ketone bodies that are exported from the liver and can be used for energy production by peripheral tissues such as the brain. Mitochondrial FAO of long-chain fatty acids involves the concerted action of a multitude of enzymes. This process starts with the carnitine-mediated transfer of the long-chain fatty acids, activated to their CoA esters, over the mitochondrial inner-membrane. This involves the activity of three enzymes: carnitine palmitoyl-CoA transferase 1 (CPT1), carnitine acyl-carnitine translocase (CACT) and carnitine palmitoyl-CoA transferase 2 (CPT2). Once inside the mitochondria, the fatty acyl-CoA esters undergo β-oxidation via a four-step mechanism, involving dehydrogenation, hydration, another dehydrogenation and thiolytic cleavage. Oxidation of long-chain fatty acids starts with the first dehydrogenation catalyzed by the very long-chain acyl-CoA dehydrogenase (VLCAD) enzyme. The next three steps are catalyzed by the mitochondrial trifunctional protein (MTP). MTP is a hetero-octamer of 4 α- and 4 β-subunits. It harbors the activity of three out of the four enzymes required for the oxidation of long-chain fatty acids: the long-chain enoyl-CoA hydratase (LCEH), the LCHAD and the long-chain thiolase. The α-subunit carries the LCEH and the LCHAD activities, whereas the β-subunit harbors the long-chain 3-ketoacyl-CoA thiolase activity. The importance of the mitochondrial FAO is stressed by the existence of a variety of different genetic disorders in which mitochondrial FAO is impaired[1]. In general, clinical signs and symptoms of FAO disorders are related to the lack of energy for metabolic functions, resulting in hypoketotic hypoglycemia and multiple organ failure. In addition, patients with inborn errors of long-chain FAO, such as VLCAD deficiency[2,3], isolated LCHAD deficiency (LCHADD)[4], as well as complete MTPD[5] present with a variety of severe clinical problems, such as cardiomyopathy, retinopathy and peripheral neuropathy, presumably due to the accumulation of toxic long-chain acyl-CoA esters.

In contrast, before birth, the fetus is considered to be primarily dependent on glucose oxidation for energy production[6]. There are four reasons for this.
Firstly, glucose is abundantly supplied by the mother and rapidly crosses the placenta. Secondly, in animal studies (e.g. in rat, rabbit and lamb) a low mRNA expression and a low activity of FAO enzymes is detected in fetal heart and liver, with a rapid rise of mRNA

levels and subsequent increase in enzymatic activity directly after birth[7-9].
Thirdly, the abundance of glucose as a substrate for the fetus results in high concentrations of malonyl-CoA, which inhibits CPT1. This prevents the entry of long-chain fatty acids via the CPT1-CACT-CPT2 system into the mitochondria and thus results in a low activity of long-chain FAO. In addition, fetal CPT1 levels are low and highly sensitive to malonyl-CoA[8]. Fourthly, although inborn errors of mitochondrial FAO can present with clinical signs and symptoms immediately after birth, fetal disease has, until recently, not been reported in this group of disorders[1].

In the last decade, however, several reports have linked the presence of two defects in the mitochondrial long-chain FAO in the fetus, namely isolated LCHADD and MTPD, to the severe pregnancy complications AFLP and the HELLP syndrome[4,10-13]. In addition, recent studies have noticed a higher frequency of prematurity, IUGR and intra-uterine death in association with isolated LCHADD and MTPD[5,11,14]. These findings suggest that FAO plays an important role in the human fetal-placental unit, which would be in contrast to the results obtained in animal studies.

As to our knowledge, the role of mitochondrial long-chain FAO has not been investigated during early human development, we investigated the expression of two genes involved in long-chain FAO, VLCAD and LCHAD, in the human embryo during development, using in situ hybridization as well as enzymatic studies.

Materials and methods

Sections

Human embryos and fetal tissues were collected from legally terminated pregnancies in agreement with the French law as well as the recommendations of the local Ethics Committee. Written informed maternal consent was obtained after termination of pregnancy. Tissues were prepared as described previously[15]. For the in situ hybridization studies in sections of intact embryos, two embryos, at Carnegie stage 14 (day 35 of development) and stage 18 (day 49 of development), were used. For later hybridization studies, heart, lung and eye from 3 fetuses (8, 9 and 20 weeks of development) were used. Also, separate frozen organs from 5 different fetuses (5, 6, 7.5, 8 and 8.5 weeks of development) were used for enzymatic studies.

Hybridization probes

Templates used for the generation of hybridization probes for VLCAD and LCHAD were amplified by PCR from human genomic DNA using the following oligonucleotide primers: VLCAD-forward 5'-AAT TGT GGT GGA GAG GGG C-3'; VLCAD-reverse 5'-AAA CTG GGT ACG ATT AGT GGC-3'; LCHAD-forward 5'-AAT TCT TCC TGT ACG ATT GGG G-3'; LCHAD-reverse 5'-AAT CTA ATG GTC TTA ATT CAG GC-3'. After amplification, the DNA fragments were purified and subcloned into the pGEM-T vector (Invitrogen, The Netherlands), which contains both a T7 and a SPG promoter. The inserts were verified by sequencing to exclude PRC-introduced errors. To generate a sense or an antisense RNA probe, the pGEM-T vector containing VLCAD and LCHAD was digested with SalI for the T7 promoter or SphI for the Sp6 promoter, respectively. The linearized plasmids were purified with phenol/chloroform extraction and dissolved in TE at 200 ng/ml. α[35S]UTP labeled probes were generated from these templates as described previously[15].

Hybridization

Hybridization and post-hybridization washes were carried out according to standard protocols[16]. Slides were dehydrated, exposed to Biomax MR X-ray films (Amerscham) for 3 days, dipped in Kodak NTB2 emulsion for 3 weeks at +4°C. Developed and toluidine blue counterstained slides were analyzed with dark and bright field illumination. Adjacent slides were hematoxylin/eosin/saffron stained for histological studies.

No hybridization signal was detected with the α[^{35}S]-labeled sense probes.

Enzymatic studies

Tissue samples, stored at –70 °C, were thawed. The activity of VLCAD was measured as described elsewhere[17], using phenylpropionyl-CoA and palmitoyl-CoA as substrates, respectively. The activity of LCHAD was determined as previously described[18].

Control values for enzymatic activity in human liver were measured in our laboratory, using the same techniques as used for embryonic studies.

Next pages:

Fig. 1: VLCAD and LCHAD gene expression in human embryos.

a,d,g,j,m are hematoxylin-eosin (HE) stained sections, adjacent to the slides hybridized with the VLCAD (*b,e,h,k,n*) and LCHAD (*c,f,i,l,o*) genes respectively.

a-f: sagittal sections of a CS14 (35 days) human embryo (*d-f* are enlarged part of *a-c*), showing the ubiquitous VLCAD gene expression, with a strong signal in liver (*e*, arrow), and a weak but specific LCHAD expression in heart and liver (*f*, arrows).

g-o: Transverse sections through a CS18 (49 days) human embryo at the abdominal (*g-l*) and caudal region level (*m-o*). *j-l* are enlarged part of *g-i*. The VLCAD gene is still ubiquitously expressed with a strong signal in liver (*h, k*).

i-o: LCHAD gene expression is observed in the metanephros (metaN), gonads (gon, arrow in *l*), gut epithelium (*i, o* open arrows), liver, dorsal root ganglia (DRG), and anterior part of the spinal cord (Sp) as shown by arrowheads in *o*.

Ad: adrenal glands
DR: dorsal root
mes: mesencephalon
pro: prosencephalon
rh: rhombencephalon
vert: vertebrae.

Fig. 2: VLCAD and LCHAD gene expression in fetal tissues.

a,e,i,m are hematoxylin-eosin (HE) stained sections, adjacent to the slides hybridized respectively with the VLCAD (*b,f,j*), LCHAD (*c,g,k,n*) antisense and sense (*d,h,l,o*) probes.

Transverse sections of a 9 weeks fetal heart (*a-d*) and lung (*e-h*). VLCAD is ubiquitously expressed throughout the heart and great vessels (*b*), the lung (*f*). In contrast, no expression is detected in the great vessels (*b*). In the lung, LCHAD expression is restricted to the epithelium.

i-j: parasagittal section through a 8 weeks eye. In the neural retina, LCHAD gene expression is slightly higher than the VLCAD gene expression. Note the false positive signal given by the pigmented retina also observed with the sense probe.

m-o: Transverse section through the spinal cord of a 20 weeks fetus, showing strong expression of the LCHAD gene in motoneurons of the anterior horn (arrows), compared with the sense probe (figure 2*o*).

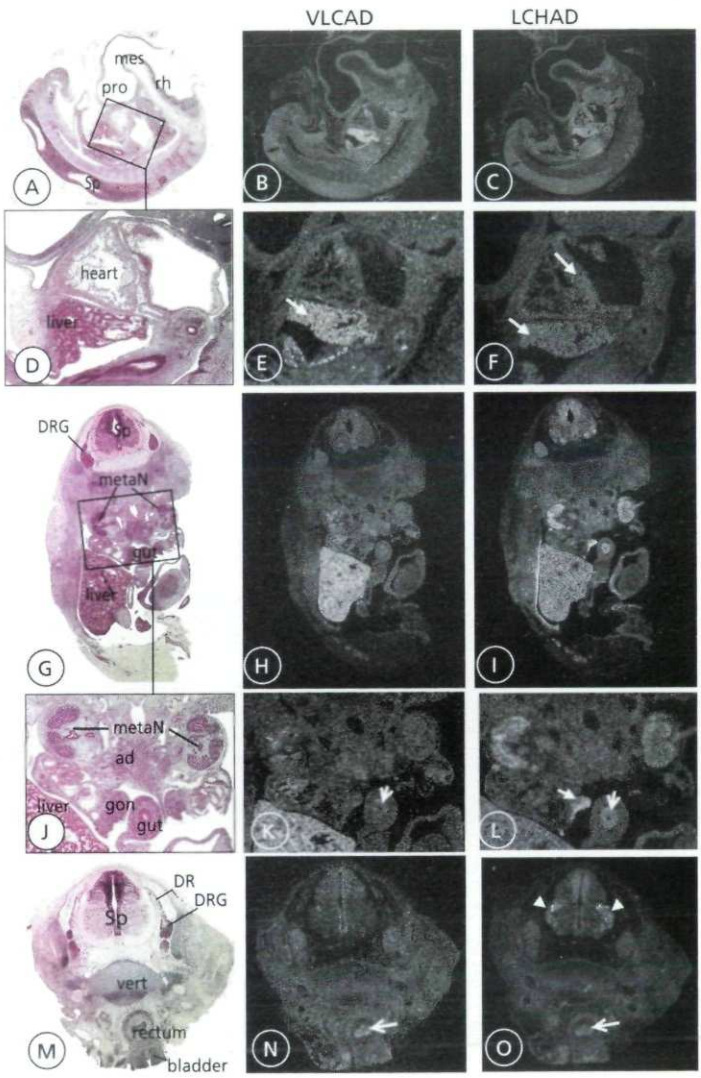

figure 1- VLCAD and LCHAD gene expression in human embryos.

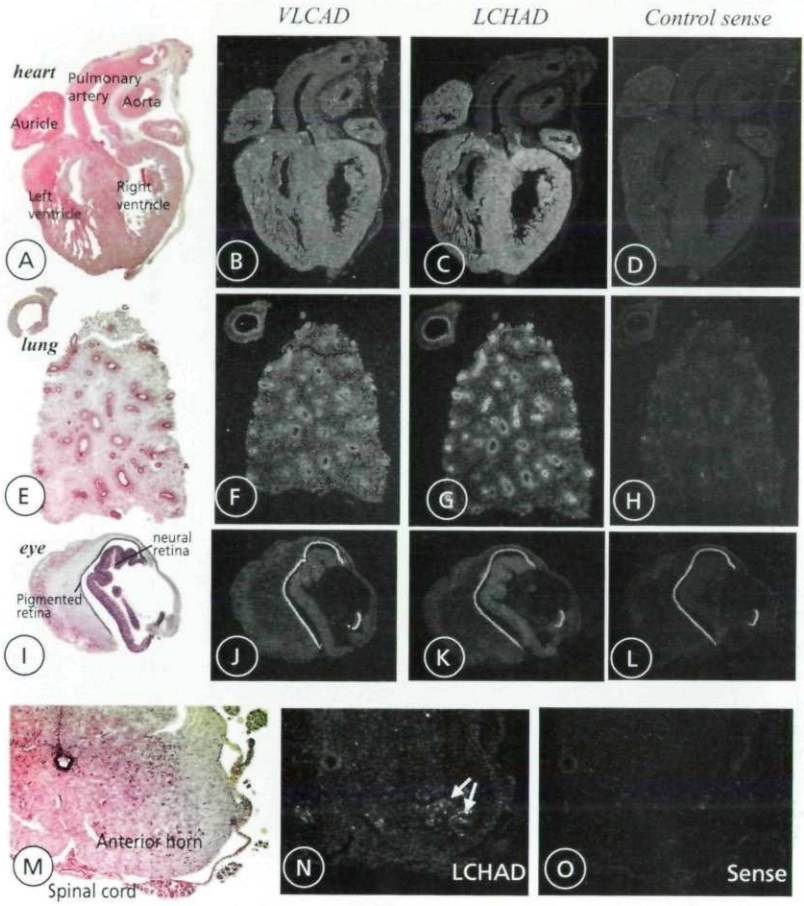

Figure 2 - VLCAD and LCHAD gene expression in human embryos.

Results

We studied the expression of the genes encoding the long-chain FAO enzymes VLCAD and LCHAD, using in situ hybridization of human embryos at 35 and 49 days of development (Carnegie stages (CS) 14 and 18) and of tissue sections of fetuses at 8, 9 and 20 weeks of development. In addition, VLCAD and LCHAD activities were measured enzymatically in fetal heart, liver and brain, between 5 and 8.5 weeks of development. The expression pattern of VLCAD and LCHAD genes during human development is presented in figures 1 and 2.

VLCAD

At CS14, 35 days of development, the VLCAD gene was ubiquitously expressed with a strong expression in liver (figure 1*b,e*). At CS 18, 49 days of development, its strong liver expression was still conserved (figure 1*h,k*). A weak expression was also observed in the developing kidney (figure 1*k*), heart (not shown) and digestive tract epithelium (figure 1*k*). At early fetal stages (figure 2), VLCAD was ubiquitously expressed in all tissues examined. The expression was particularly high in the heart and great vessels (figure 2*b*), the lung (figure 2*f*) and the neural retina (figure 2*j*) when compared with the control sense probes (figure 2*d,h,l*).

LCHAD

Interestingly, the LCHAD gene expression was different from the VLCAD gene expression. At 35 days of development, a weak LCHAD gene expression was found in both heart and liver (figure 1*c,f*). At CS18, in addition to the liver (figure 1*i*) and heart (not shown), LCHAD was specifically and strongly expressed in the metanephros, gonads (figure 1*l*) and the developing gut epithelium (figure 1*l,o*). In the nervous system, LCHAD was expressed in developing brain and neural retina (data not shown), as well as in a subpopulation of cells of the anterior horn of the spinal cord, and in dorsal root ganglia, (figure 1*i,o*). This pattern of LCHAD gene expression was again observed during fetal stages (figure 2). In the heart, LCHAD was strongly expressed in the myocardial tissue, but no signal was detected in the great vessels (figure 2*c*). In the lung, LCHAD expression was restricted to epithelial cells (figure 2*g*). In the central nervous system, a strong expression of the LCHAD gene was observed in the neural retina (figure 2*k*) and the motorneurons of the anterior horn of the spinal cord (figure 2*n*) when compared to controls (figure 2*o*).

Enzymatic studies

The enzymatic activity of VLCAD and LCHAD could be clearly detected in heart, liver and brain tissue of human embryos (table 1). Although enzyme activity was generally higher in adult human liver, which has a high FAO capacity, the difference was only small.

Tissue developmental week	VLCAD	LCHAD
heart, 5 weeks	0.253	121
heart, 6 weeks	0.327	129
heart, 7.5 weeks	0.318	151
heart, 8 weeks	0.402	142
Liver, 5 week	0.337	169
Liver, 6 weeks	0.760	207
Liver, 8.5 weeks	0.418	126
Brain, 6 weeks	0.11	95
Brain 7.5 weeks	0.054	47
Control adult liver	0.720	313

Table 1. Activities of FAO enzymes during early human development and in control human liver. Activities are expressed as nmol/min/mg protein. Control human liver: n=3 for VLCAD and n=10 for LCHAD.

Discussion

In contrast to the widely accepted view, the present study indicates that FAO plays an important role during early human development. Recent studies in human placenta already demonstrated a remarkable high activity of FAO enzymes[19,20]. In placenta, the activity of the enzymes LCHAD and short-chain 3-hydroxyacyl-CoA dehydrogenase (SCHAD) were inversely correlated with gestational age[19]. Furthermore, the activity of CPT2 and of VLCAD was higher in term human placenta than in adult human liver[20]. As the human placenta is of embryonic origin, these results indicate that FAO takes place in fetal tissue, suggesting a possible role for a disturbed placental FAO in the pathogenesis of the pregnancy complications AFLP and HELLP syndrome. The results of our study demonstrate mRNA expression as well as enzymatic activity of the long-chain FAO enzymes VLCAD and LCHAD during early human development and are well in line with these observations in human placenta and strongly suggest that not only the placenta, but also the human embryo utilizes fatty acids as a substrate for energy production.

Studies by Ibdah and coworkers[21] revealed that MTP deficient knockout mice suffer from IUGR. IUGR has also been reported in humans as a consequence of fetal MTPD [5,14,22]. Although in humans the high incidence of IUGR may be associated with the maternal complications HELLP syndrome and AFLP, IUGR in MTP deficient mice could not be attributed to placental nor to maternal disease[21]. Our study revealed significant mRNA expression and high enzymatic activity of LCHAD and VLCAD in numerous tissues during early human development. Therefore it is not surprising that isolated LCHADD, MTPD, or VLCAD deficiency, can result in fetal disease with IUGR as a consequence.

Cardiomyopathy is a frequent and often fatal complication of long-chain FAO enzyme defects, including VLCAD deficiency, isolated LCHADD and complete MTPD. Presumably it results from the accumulation of arrhythmogenic long-chain acylcarnitines[24]. In addition, fetal hydrops due to intra-uterine cardiomyopathy was reported recently in a child with MTPD[5,25]. Hydrops fetalis due to cardiomyopathy was also reported in relation to carnitine deficiency[25]. These observations suggest that long-chain FAO is taking place in the myocardium during intra-uterine life. Our study, conducted in human embryos shows a strong expression and high activity of LCHAD and VLCAD in myocardial tissue (figure 2*b,c* and table 1). If the fetal heart uses fatty acids as an important substrate for the production of ATP, it is likely that intra-uterine cardiomyopathy can result from a defective long-chain FAO. In that respect, however, it is surprising that fetal hydrops has not been reported more frequently in relation to long-chain FAO deficiency.

The observed expression pattern of LCHAD mRNA in human embryos correlates very well with clinical signs and symptoms observed in patients with isolated LCHADD and complete MTPD. Pigmentary retinopathy is an important feature of LCHADD, and has not been reported in any other FAO defect. Recently, Tyni and co-workers demonstrated that FAO is taking place in cultured porcine retinal pigment epithelium cells. This suggests that FAO may play an important role in the retina[26]. Although pigmentary retinopathy has not been observed at birth, it has been detected in LCHAD deficient patients at 4 months of age[22]. The LCHAD mRNA expression we observed in the neural retina (Figures 3*k*), suggests that long-chain FAO plays a role in the developing human retina. Therefore, retinal damage observed in LCHAD and MTP deficient patients may already have started in utero.

Another unique feature of LCHAD and MTPD is the presence of a progressive peripheral neuropathy, reported in more than 50% of MTP deficient patients[5]. This symptom is not reported in any other FAO defect. Nerve conduction velocity was determined in a few patients with peripheral neuropathy and showed axonal neuropathy with sensory predominance[27-29]. Nerve biopsy was normal in one patient, but revealed demyelination and axonal neuropathy in two others[22,29]. Our human embryo studies showed LCHAD mRNA expression in the developing central nervous system. In particular, LCHAD mRNA could be detected in the anterior horn of the spinal cord at 49 days of development. In addition, the LCHAD gene was also clearly expressed in the motor neurons of the anterior horn of the spinal cord at 20 weeks of development (figure 2*n*), while VLCAD expression was very weak (data not shown).

It is difficult to explain the discrepancy between the LCHAD and VLCAD expression patterns as observed in the central nervous system of the developing human embryo, as the mitochondrial FAO involves the concerted action of all enzymes. An explanation might be that MTP has an additional metabolic role in the developing central nervous system, including the retina, as already suggested by Tyni and coworkers[26]. However, such an additional role has never been detected at the biochemical level.

Finally, our study also demonstrates that long-chain FAO is present in other tissues during early human development. In particular, a strong expression of LCHAD is observed in lung, gut, gonadal tissue and metanephros. We are not aware of any involvement of these tissues in patients with long-chain FAO disorders. Nevertheless, we believe that patients with inborn errors of long-chain FAO should also be monitored for pulmonary, gastrointestinal, gonadal and renal complications during long-term follow up.

In summary, here we describe a detailed study of the expression of the long-chain FAO VLCAD and LCHAD genes as well as their enzymatic activity, during early human development. In contrast to the widely accepted view that embryologic development depends on glucose as the major source of metabolic energy, our results clearly show that long-chain FAO is also taking place in the human embryo. Our observations are well in line with the pattern of clinical signs and symptoms observed in patients with VLCAD and LCHADD/MTPD. Additional studies on the role of the LCHAD enzyme in retinal, nervous, renal, and gonadal tissues may well reveal developmental pathways using alternative metabolic functions of the LCHAD/MTP enzyme.

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9

Summary and discussion

The disorders of mitochondrial fatty acid oxidation (FAO) present an important group of inherited, autosomal recessive metabolic diseases. Probably the most common and widely known FAO disorder is medium-chain acyl-CoA dehydrogenase deficiency (MCADD). MCAD deficient patients usually present in infancy or early childhood, often during a minor infectious disease, with clinical signs and symptoms related to the lack of energy for metabolic functions. This results in what is often named a 'Reye like syndrome' consisting of liver disease with hypoketotic hypoglycemia, raised ammonia levels, encephalopathy and severe fatty infiltration of the liver. At present, more than ten FAO disorders are recognized. Quantitatively, the long-chain FAO disorders are an important subgroup of FAO disorders. Their clinical presentation can be very different from the classical 'Reye-like' MCADD phenotype. Presentation is much more heterogeneous with a cardiac, myopathic, neurological and a MCADD-like phenotype. Patients usually present at younger age than in MCADD, frequently already in the neonatal period. Since new diagnostic tools like acylcarnitine profiling using Tandem Mass Spectrometry have made

the diagnostics of patients presumably suffering from a long-chain FAO disorder easier, more patients presenting with nonspecific signs and symptoms have been identified.

This thesis focuses on inborn errors of the mitochondrial trifunctional protein (MTP), an enzyme complex, that harbors the activity of three (LCEH, LCHAD and LCKAT) of the four enzymes involved in the mitochondrial oxidation of long-chain fatty acids. Two forms of MTP deficiency (MTPD) can be recognized: complete MTPD, in which the whole protein is absent, resulting in complete deficiency of all three enzyme activities, and isolated long-chain 3-hydroxyacyl-CoA dehydrogenase (LCHAD) deficiency in which the protein is normally present, but a defect in the α-subunit results in solitary deficiency of the LCHAD activity. A common mutation (1528G>C), causing this defect in the α-subunit, has been identified in LCHAD deficient patients with an allele frequency of 87%. Isolated LCHAD deficiency (LCHADD) was reported in association with a MCADD like phenotype, but often results in a more generalized disease including cardiomyopathy and skeletal myopathy. Reported mortality is alarmingly high. In the course of the disease retinopathy and peripheral neuropathy, symptoms not seen in any of the other FAO defects, occur in some of the patients. Complete MTPD has until now only been described in a small number of patients, demonstrating a more heterogeneous mode of presentation. In general, complete MTP deficient patients appear to present earlier in life and with more severe disease as compared to LCHAD deficient patients.

A striking association has been observed between the complications of end stage pregnancy, hemolysis, elevated liver enzymes and Low Platelets (HELLP) syndrome and acute fatty liver of pregnancy (AFLP) on the one hand and the carriage of a LCHAD deficient child on the other hand. A common pathophysiological background causing the liver disease in the child after birth as well as in its heterozygous mother during pregnancy has been suggested because of the identical observations in histological studies of the liver. The precise mechanisms causing this association between maternal pregnancy complications and fetal disease remain unknown, but several mechanisms are considered to play a role. The most important mechanisms that are thought to be involved, are the heterozygous state of the mother, theoretically resulting in a reduction of the capacity to oxidize long-chain fatty acids as well as the accumulation of potentially toxic 3-hydroxyacyl-CoA esters produced by the fetus as a result of its metabolic defect. However, the clinical and biochemical phenotype of both isolated LCHADD and complete MTPD remain poorly defined, and a little is known about their natural history.

Chapter 3 and 4

In order to try to elucidate the clinical spectrum of both disorders, we studied a large cohort of LCHAD deficient patients (N=50) and a cohort of MTP deficient patients (N=21). All included patients presented in infancy or in childhood, with a mean age of presentation within the first six months of life (5.8 months in LCHADD and 3 months in complete MTPD). Complete MTPD often presents already in the neonatal period: almost half of the patients (48%) presented before the age of 6 weeks. Surprisingly, many patients (22% in LCHADD and 57% in MTPD) initially did not present with the rapidly progressive clinical deterioration which is generally considered to be 'classical' for FAO disorders, namely the MCADD-like liver phenotype resulting in a 'Reye-like' syndrome. In contrast, they presented with a more insidious disease with nonspecific chronic problems, such as cholestasis, failure to thrive and hypotonia, making the clinical recognition of patients suffering from a long-chain FAO disorder very difficult.

Hypotonia and signs of cardiomyopathy were the main presenting symptoms in many of the MTP deficient patients showing slowly progressing disease. Therefore the differential diagnosis of a child with otherwise unexplained nonspecific symptoms such as failure to thrive, cholestasis, hypotonia and cardiomyopathy, should include MTPD and appropriate diagnostic tests to rule out a long-chain FAO disorder should be performed in these patients. Additional laboratory studies may help in the initial diagnostic workup of a patient with a suspected MTPD. Increased plasma CK- and lactate levels were found to be present in many of the studied patients. Mortality in our series was high (38% in LCHAD and 76% in MTPD). In survivors, recurrent episodes of clinical deterioration were observed, but these were less severe and less frequent than before the diagnosis was made. Retinopathy was observed in isolated LCHADD as well as in complete MTPD (respectively 29% and 12.5%). Peripheral neuropathy was detected in 12% of the LCHAD deficient patients and in 79% of the MTP deficient patients. In complete MTPD neuropathy seen at a younger age than in isolated LCHADD. It is not easy to explain the differences in clinical presentation and course between isolated LCHADD and complete MTPD. Presumably, accumulation of 2-trans enoyl-CoA esters and 3-ketoacyl-CoA esters in complete MTPD, due to the additional enzymatic defects in LCEH and LCKAT, causes the observed differences. However, the plasma acylcarnitine profile does not make a differentiation possible, even during acute metabolic decompensation, between the two disorders.

Also in our studies, we observed a significant association between isolated LCHADD and complete MTPD and the pregnancy complications HELLP syndrome and AFLP, although

both had a lower incidence in our series than in other reports. Remarkable is the observation that 40% of the MTP deficient patients in our series were small for gestational age, not related to maternal pregnancy complications.

Most LCHAD and MTP deficient patients were treated with a LCT restricted diet in combination with avoidance of fasting and supplementation with medium-chain triglycerides (MCT). However, this dietary therapy could not prevent clinical deterioration in all patients, as some of the patients, including the two prenatally diagnosed neonates, died despite treatment. A possible explanation may be that myocardial energy metabolism remains dependent on FAO, irrespective of the carbohydrate intake. Ongoing accumulation of toxic long-chain acyl-CoA esters, which are presumed to be arrhythmogenic and toxic to cardiomyocytes, may thus result in progression of the cardiomyopathy despite dietary treatment.

The remarkable effect of creatine supplementation in one complete MTP deficient patient in our study, which has also been reported in a patient with LCHADD, is encouraging. Recent studies by Roe and coworkers (J Clin Invest 2002;100:259-269) demonstrated encouraging results of treatment of patients with long-chain FAO defects, with a medium-chain triglyceride with heptanoic acid rather than octanoic acid, as in MCT. In contrast to octanoic acid, which only produces acetyl-CoA units, heptanoic acid generates acetyl-CoA and proprionyl-CoA. It is hypothesized that proprionyl-CoA increases gluconeogenesis by acting as an anaplerotic substrate for the citric acid cycle in all tissues, since proprionyl-CoA generates oxaloacetate.

More research is needed to establish the value of the different therapeutic options in both isolated LCHADD and complete MTPD. Therapy should be aimed at both the prevention of recurrent episodes of clinical deterioration as well as the prevention of long-term sequelae such as retinopathy and peripheral neuropathy.

In recent years expansion of neonatal screening programs by Tandem Mass Spectrometry, including long-chain FAO disorders have been introduced in a number of countries. Our results demonstrate that this will not result in a truly diagnosis in all patients with MTPD, as some of them already present with a devastating disorder in already the first days after birth. Furthermore, we have shown for MTPD that the current mode of therapy will not always prevent morbidity and even mortality. On the other hand, this does not hold true for LCHADD as a timely diagnosis seems to result in increased survival.

Chapter 5

As from our study the clinical presentation of LCHADD appears to be more heterogeneous than initially was reported, it may well be that many patients are missed for diagnosis, and that the true incidence of LCHADD is much higher. We therefore tested the prevalence of the common LCHAD mutation in the Dutch population by analyzing Guthrie cards and found the carrier frequency to be low. The observed prevalence of 1:680 was consistent with the observed low incidence of the disorder in the Netherlands. LCHADD thus remains a very rare inborn error, and it is not likely that many patients are not properly diagnosed.

The etiology of the remarkable association between a long-chain FAO disorder in the fetus and severe complications of pregnancy in their mothers has long remained unclear. In order to try to unravel (parts of) the involved pathophysiological mechanisms, we first focused on the possible role of the heterozygosity of the mothers as a single cause. We therefore performed analysis for the common LCHAD mutation in an otherwise unselected group of women who had previously suffered from the HELLP syndrome. Only one of the 113 women tested was found to be heterozygous, which does not differ significantly from the observed low carrier frequency in the Dutch population. Heterozygosity for the common LCHAD mutation is therefore not a major cause for the HELLP syndrome. Recently, Yang and coworkers (JAMA 288:2163-2166) made the recommendation to screen all families from women suffering from AFLP for the presence of the common LCHAD mutation, excluding mothers suffering from only a HELLP syndrome. Although our results support this recommendation, we still believe that in the case of a very severe HELLP syndrome screening for long-chain FAO disorders is indicated, since HELLP syndrome and AFLP are suggested to present different stages of the same disease.

Furthermore, screening for the common 1528G>C mutation will fail to detect HELLP syndrome and or AFLP due to a MTP deficient unborn child. We therefore prefer the use of acylcarnitine profiling by Tandem Mass Spectrometry in the child, in combination with molecular screening.

Chapter 6

Additionally we performed a long-chain triglyceride (LCT) loading test in female carriers for the common LCHAD mutation, studying their in vivo capacity to metabolize fatty acids and compared them with healthy controls. The results indeed showed a normal capacity to metabolize fatty acids.

This strongly suggests that long-chain FAO capacity is normal and that these mothers should be able to rapidly metabolize any fetally produced long-chain acylcarnitines, entering their circulation. We therefore concluded that the heterozygous state of the mother in itself does not play a major role, if any, in the pathogenesis of the gestational complications.

Chapter 7

An alternative explanation for the association between the carriage of a child with a long-chain FAO defect and the maternal HELLP syndrome and AFLP therefore is the possible production of long-chain acyl-CoA esters by the feto-placental unit. As the fetus is considered to be primarily dependent on glucose oxidation for energy production, it is unlikely that fetal production of long-chain acyl-CoA esters plays an important role. However, since the human placenta is mainly of fetal origin, and since the placental mass represents a relatively high proportion of the fetal-placental unit at term, an alternative explanation would be that the defective FAO in placental tissue is directly responsible for the severe pregnancy complications in the mother. Recently, Rakheja and coworkers (Placenta 2002;23: 447-450) were the first to demonstrate activity of the FAO enzymes LCHAD and SCHAD in human placenta, giving support to this hypothesis. In order to expand on these results, we studied the activity of a whole range of different enzymes involved in FAO (CPT2, VLCAD, LCEH, LCHAD, LCKAT, MCAD, SCEH and SCHAD) in normal term placenta, as well as in chorionic villus biopsies and compared them with the activities in control human liver. All enzymes studied were found to be expressed in term human placenta as well as in chorionic villus samples. Interestingly, the mean activity of the enzymes CPT2 and VLCAD, both involved in long-chain FAO, was found to be even higher than in human liver tissue, which is known for a very high capacity for FAO. These results strongly suggest an important role for placental production of toxic long-chain acyl-CoA esters in the pathogenesis of HELLP syndrome and AFLP in mothers carrying a fetus with a long-chain FAO disorder. The long-chain acyl-CoA esters produced by the placenta can be transferred as long-chain acyl-carnitine esters to the maternal circulation and taken up by maternal liver and other tissues. The first suggested explanation for the maternal liver disease is the inability of the liver of the heterozygous mother to rapidly metabolize these long-chain acyl-CoA esters, but our study in chapter 4 suggests that this explanation is less likely. A second explanation is that accumulation of long-chain acyl-CoA esters in the placenta directly results in placental damage, due to inhibition of a variety of essential metabolic processes. Inhibition of the oxidative phosphorylation for example, might result in the production reactive oxygen species (ROS) and enhanced lipid

peroxide formation, which could lead to endothelial dysfunction, which has been considered as an important etiological factor for preeclampsia and the HELLP syndrome. Moreover, placental damage by long-chain acyl-CoA esters may promote shedding of microparticles into the maternal circulation, resulting in a systemic immunological response causing multiorgan disease, as this has been described as an important pathophysiological mechanism for the development of preeclampsia and the HELLP syndrome.

Chapter 8

A second observation suggesting an important role for FAO in the feto-placental unit is the high frequency of intra-uterine growth retardation (IUGR) and prematurity reported in patients with isolated LCHADD or complete MTPD. This observation is in line with the reported IUGR in MTP deficient knock-out mice, which can not be attributed to maternal pregnancy complications. As the role of FAO had never been studied in the human embryo, we investigated the expression of two enzymes involved in the long-chain FAO, VLCAD and LCHAD, during in situ hybridization as well as by enzymatic studies and demonstrated significant mRNA expression and high enzymatic activity of LCHAD and VLCAD in a number of different tissues such as myocardium and the developing nervous system including the retina. The observed IUGR in LCHAD deficient and in MTP deficient patients can thus be well explained by defective FAO prenatally. The fetal hydrops due to intra-uterine cardiomyopathy reported in one patient from our MTP study (chapter 4), in combination with the high expression of VLCAD and LCHAD in fetal heart, makes it likely that intra-uterine cardiomyopathy can be the result of a defective FAO. Therefore, the differential diagnosis of unexplained hydrops fetalis should include long-chain FAO disorders. Another unexplained complication of LCHAD and MTPD, pigmentary retinopathy which has been observed in some patients is in line with the high expression of LCHAD mRNA in early human development as detected by our study (chapter 8), suggesting that this retinal damage may already start in utero. Remarkable are the observed discrepancies between the high expression of LCHAD on the one hand and the low of expression of VLCAD in the developing central nervous tissue on the other hand, as the mitochondrial FAO involves the concerted action of all involved enzymes. An explanation might be that the MTP or one of its constituting enzyme activities (LCEH, LCHAD and LCKAT) has an additional role in the (developing) nervous system, including the retina.

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Samenvatting en discussie

Vetzuuroxidatie (FAO) is het biochemische proces dat in het menselijk lichaam zorgt voor het vrijmaken van de energie die is opgeslagen in vet. FAO is vooral actief tijdens perioden van langdurig vasten en tijdens matig zware lichamelijke inspanning.

FAO-stoornissen vormen een snel groeiende groep erfelijke ziektebeelden. Alle tot dusver bekende FAO-stoornissen erven autosomaal recessief over. De meest bekende en waarschijnlijk ook meest voorkomende FAO-stoornis is een defect in de oxidatie van middenketen lange vetten: medium-chain acyl-CoA dehydrogenase deficiëntie (MCADD). MCADD presenteert zich meestal op de peuterleeftijd, vaak als er sprake is van een milde infectieuze ziekte. De symptomen weerspiegelen het tekort aan energie dat nodig is voor stofwisselingsfuncties. Dit uit zich in een combinatie van symptomen dat bekend is geworden onder de naam 'Reye-like syndroom' en gekenmerkt wordt door leverfunctiestoornissen met verhoogde ammoniakwaarden en verhoogde transaminases (leverenzymen) in het bloed, stapeling van vet in de lever, hersenoedeem en verlaagde bloedsuikerspiegels.

Er zijn op dit moment meer dan tien verschillende FAO-stoornissen bekend. Aangeboren, erfelijke defecten van de oxidatie van lang-keten vetzuren vormen een snel in omvang toenemende groep FAO-stoornissen. De presentatie bij een lange-keten FAO-stoornis wijkt af van het klassieke 'Reye-like beeld' dat meestal bij MCADD wordt gezien. Lange-keten FAO-stoornissen worden gekenmerkt door een spectrum aan verschillende aandoeningen, waarbij het hart, de skeletspieren, het zenuwstelsel en de lever betrokken kunnen zijn. De lange-keten FAO-stoornissen presenteren zich vaak op een jongere leeftijd dan midden-keten FAO-stoornissen, vaak al direct na de geboorte. De mogelijkheid om bij patiënten die worden verdacht van een FAO-defect snel en betrouwbaar de diagnose te stellen is in de afgelopen jaren sterk verbeterd, met name door de introductie van nieuwe technologische mogelijkheden zoals de bepaling van het acylcarnitine-profiel, uitgevoerd met behulp van Tandem Mass Spectrometry. Hierdoor worden steeds meer patiënten herkend die zeer aspecifieke symptomen vertonen, waarvan eerder niet duidelijk was dat ze ook tot de verschijningsvormen van een FAO-stoornis konden behoren.

Dit proefschrift richt zich op aangeboren deficiënties van het *mitochondrial trifunctional protein* (MTP), een enzymcomplex dat de activiteit herbergt van drie (LCEH, LCHAD en LCKAT) van de vier enzymen die betrokken zijn bij de oxidatie van lange-keten vetten. Twee vormen van MTP-deficiëntie zijn beschreven: de totale MTP-deficiëntie, waarbij het hele MTP afwezig is, leidend tot deficiëntie van alle drie de genoemde enzymen, en de geïsoleerde deficiëntie van het *long-chain 3-hydroxyAcyl-CoA dehydrogenase* (LCHAD)-enzym. Bij geïsoleerde LCHAD deficientie (LCHADD) is MTP wel aanwezig, maar leidt een mutatie in een bepaald deel van het gen dat codeert voor MTP tot een geïsoleerde deficiëntie van uitsluitend het LCHAD-enzym. Een frequent voorkomende mutatie in het MTP-gen (1528G>C), die wordt gezien in 87% van de allelen van patiënten met een geïsoleerde LCHADD, is met name verantwoordelijk voor de geïsoleerde LCHADD. LCHADD werd aanvankelijk geassocieerd met een MCADD-achtige presentatie, maar inmiddels is gebleken dat er frequent een veel uitgebreider symptomencomplex bij voorkomt, waarbij ook het hart en de skeletspieren zijn betrokken. Het sterftepercentage is zeer hoog bij LCHADD. In de loop van de ziekte kan zowel retinitis pigmentosa, een degeneratieve aandoening van het netvlies aantasting van het netvlies, als perifere neuropathie, een aandoening van het zenuwstelsel ontstaan, beelden die bij andere FAO-stoornissen nooit werden beschreven. Totale MTP-deficiëntie (MTPD) was tot dusver slechts bij een zeer klein aantal patiënten beschreven. In het algemeen zijn patiënten met totale MTPD jonger bij de eerste klinische presentatie van de ziekte dan patiënten met een geïsoleerde LCHADD en lijkt de ziekte een ernstiger beloop te hebben.

Opvallend is de associatie tussen het zwanger zijn van een LCHAD deficiënt kind en de ernstige zwangerschapscomplicaties *hemolysis, elevated liver enzymes and low platelets* (HELLP-syndroom) en *acute fatty liver of pregnancy* (AFLP)) bij de moeder. Op grond van sterk vergelijkbare afwijkingen bij pathologisch anatomisch onderzoek van de lever van de moeder met een dergelijke zwangerschapscomplicatie en die van het FAO-deficiënte kind, wordt aangenomen dat er een vergelijkbaar pathofysiologisch mechanisme is. Er zijn twee hypotheses. Ten eerste zou het dragerschap van de moeder voor de FAO-stoornis een rol kunnen spelen, omdat hierdoor bij haar een verminderde FAO-capaciteit (50%) zou kunnen bestaan. Een andere hypothese is dat de toxische invloed van 3-hydroxy acyl-CoA esters, stofwisselingsproducten die zich stapelen ten gevolge van het FAO-defect, van invloed zijn op het ontstaan van de leverziekte bij de moeder.

Ondanks alle kennis die in de afgelopen jaren is opgedaan, zijn er nog veel vragen over het klinisch beloop staan er nog veel vragen open. Allereerst is de kennis over het klinisch beloop van zowel LCHADD als MTPD fragmentarisch en veelal gebaseerd op geïsoleerde casuïstiek of kleine series van patiënten. Daarnaast is de pathofysiologie van de hoge incidentie van het HELLP syndroom en AFLP bij deze lang-keten FAO-defecten nog onduidelijk.

Hoofdstuk 3 en 4

Met het doel om een completer overzicht te krijgen van de presentatievormen van zowel LCHADD als MTPD, bestudeerden wij de gegevens van zowel een cohort van patiënten met geïsoleerde LCHADD (N=50) als een cohort van patiënten met totale MTPD (N=21). Alle patiënten presenteerden zich voor de leeftijd van 3 jaar, gemiddeld voor de leeftijd van zes maanden (LCHADD: 5.8 maanden, MTPD: 3 maanden). Totale MTPD presenteert zich vaker in de neonatale fase: bijna de helft van de patiënten presenteerde voor de leeftijd van 6 weken. Opvallend is dat veel van de patiënten zich niet presenteerden met een snel progressieve ziekte, zoals bij de klassieke MCADD-achtige presentatie, maar met meer sluipende weinig specifieke verschijnselen zoals *failure to thrive* (achterblijvende groei), hypotonie (spierzwakte) en cholestase (galstuwing in de lever). Aangezien deze klachten bij een veelheid aan andere ziektes voor kunnen komen, is het moeilijk om op deze basis patiënten die lijden aan FAO-stoornis te herkennen.

Hypotonie en cardiomyopathie (ziekte van de hartspier) waren de belangrijkste presentatievormen bij MTPD-patiënten die zich niet met een acuut, snel verslechterende ziekte presenteerden. MTPD moet daarom altijd overwogen worden bij kinderen met anderszins niet verklaarde, weinig specifieke symptomen zoals *failure to thrive*,

cholestase, hypotonie en cardiomyopathie. Diagnostiek om een FAO-stoornis aan te tonen, zoals het bepalen van een acylcarnitine-profiel, moet dan altijd worden verricht. Aanvullend bloedonderzoek naar de CK- en lactaatconcentratie kan behulpzaam zijn, aangezien beide parameters bij de meeste LCHADD en MTPD patiënten verhoogd blijken te zijn. De mortaliteit (sterftecijfer) van beide ziektebeelden blijkt zeer hoog te zijn (LCHADD: 38%, MTPD: 76%). Daarnaast is er sprake van een hoge morbiditeit (ziektecijfer). Veel patiënten lijden aan recidiverende episodes van metabole ontregeling, veelal uitgelokt door een milde infectieuze ziekte. Retinopathie (beschadiging van het netvlies) blijkt frequent voor te komen bij zowel LCHADD als MTPD (respectievelijk 29% en 12.5%). Perifere neuropathie (ziekte van het zenuwstelsel) had een duidelijk hogere incidentie in patiënten met MTPD (79%) dan in patiënten met LCHADD (12%), maar lijkt zich bij MTPD op een jongere leeftijd te manifesteren. Het is niet eenvoudig de verschillen tussen LCHADD en MTPD te verklaren. Mogelijk dat de stapeling van stofwisselingsproducten zoals 2-transenoyl-CoA esters en 3-ketoacyl-CoA esters, die optreden ten gevolge van deficientie van de twee enzymen (LCEH en LCKAT) die naast LCHAD afwezig zijn in het geval van totale MTPD, hierop van invloed zijn. Anderzijds is het plasma acylcarnitine-profiel, dat een weerspiegeling geeft van de stapelende vetten die tengevolge van het FAO-defect niet kunnen worden afgebroken en zich hebben gebonden aan carnitine, bij LCHADD en MTPD niet van elkaar te onderscheiden, zelfs niet tijdens acute ontregeling van de ziekte.

De associatie tussen het dragen van een LCHAD- of MTP-deficiënt kind en de ernstige zwangerschapscomplicaties HELLP-syndroom en AFLP bij de moeder, werd ook in onze studie bevestigd, hoewel beiden een lagere incidentie vertoonden dan in eerdere publicaties. Opvallend is dat in ons onderzoek (hoofdstuk 4) bij MTPD een hoge incidentie (40%) werd gezien van foetale groeiachterstand (intra-uteriene groei retardatie, IUGR), die niet toe te schrijven is aan het optreden van zwangerschapscomplicaties. Over de mogelijke oorzaken hiervan wordt ingegaan in de hoofdstukken 7 en 8.

De behandeling van LCHADD en MTPD bestaat meestal uit een vetbeperkt dieet in combinatie met het voorkomen van vasten en suppletie van midden-keten vetten (medium-chain triglycerides, MCT). Desondanks blijkt uit ons onderzoek, kunnen snel progressieve en dodelijke ziekteverschijnselen hiermee niet altijd worden voorkomen. Mogelijk dat de stofwisseling van de hartspier, onder alle omstandigheden afhankelijk blijft van FAO als de belangrijkste energiebron, zelfs als veel koolhydraten toegediend waarvan verwacht mag worden dat die in principe activiteit van het FAO proces zullen onderdrukken. Hierdoor blijven de lang-keten acyl-CoA esters, die worden gevormd ten

gevolge van het FAO-defect en als verantwoordelijk beschouwd voor het ontwikkelen van hartritmestoornissen en voor de schade aan de hartspiercel, zich stapelen en blijven progressie van de cardiomyopathie veroorzaken, ondanks dat therapie wordt gegeven.

Dat een van de door ons beschreven MTP-deficiënte patiënten veel baat had bij het toedienen van creatine, zoals ook een eerder beschreven patiënt met LCHADD, is veelbelovend. Recent werd een nieuwe therapie voor patiënten met lange-keten FAO-stoornissen beschreven door Roe en collegae (J Clin Invest 2002;100:259-269), welke bestaat uit een dieet met triheptanoinezuur, een vetzuur met een oneven ketenlengte dat door het lichaam wordt omgezet in acetyl-CoA en propionyl-CoA. Ook deze therapie laat in preliminair klinisch onderzoek veelbelovende resultaten zien bij toediening aan patiënten. Hoewel het niet duidelijk is hoe de therapie exact werkt, wordt gesuggereerd dat het proprionyl-CoA de gluconeogenese (de nieuwvorming van glucose in de lever) stimuleert en citroenzuurcyclus, een essentiële cyclus in de stofwisseling, herstelt.

Meer en uitgebreider onderzoek is nodig om de verschillende therapeutische mogelijkheden voor LCHADD en MTPD beter te kunnen bestuderen.

In verschillende landen werd recent door middel van hielprik-screeningskaartjes gestart met neonatale screeningsprogramma's die verschillende metabole ziekten bevatten, waaronder ook lang-keten FAO-stoornissen. De screening wordt uitgevoerd door middel van Tandem Mass Spectrometry. Ons onderzoek duidt er op dat deze screeningsmethode voor een subgroep van de MTP-deficiënte patiënten niet op tijd komt, aangezien zij al in de eerste dagen na de geboorte ernstige symptomen van de ziekte kunnen vertonen. Bovendien heeft ons onderzoek aangetoond dat de huidige therapie bij deze neonatale presentatie van MTPD niet altijd afdoende is om morbiditeit en zelfs mortaliteit te voorkomen. Dit geldt alleen voor MTPD en niet voor LCHADD, aangezien in ons onderzoek (hoofdstuk 3) het tijdig stellen van de diagnose in LCHADD wel degelijk mortaliteit leek te voorkomen.

Hoofdstuk 5

Zoals blijkt uit onze studie naar de presentatie vormen van LCHADD (hoofdstuk 3), is er een subgroep van patiënten die zich presenteert met langzaam progressieve, niet specifieke symptomen, waarvan eerder niet bekend was dat ze tot de uitingsvormen van een FAO-defect konden behoren. Dit zou kunnen betekenen dat de diagnose LCHADD frequent zou kunnen worden gemist en dat de daadwerkelijke incidentie van LCHADD veel hoger is dan tot nu toe werd aangenomen. Om deze reden hebben wij met behulp van hielprik-screeningskaartjes onderzoek gedaan naar de prevalentie in de Nederlandse

bevolking van de veelvoorkomende mutatie (1528G>C) die LCHADD veroorzaakt. De prevalentie van deze mutatie blijkt laag (1:680) en consistent met de lage incidentie van LCHADD in Nederland. LCHADD is dus inderdaad een zeer zeldzame aangeboren aandoening en op basis van ons prevalentie onderzoek is het onwaarschijnlijk dat de diagnose bij veel patiënten wordt gemist.

De etiologie van de associatie tussen een FAO-stoornis in het kind en ernstige zwangerschapscomplicaties in de moeder is onduidelijk. Om meer te weten te komen over de mogelijke pathofysiologische mechanismen, hebben wij in eerste instantie onderzoek gedaan naar de mogelijke rol van het dragerschap bij de moeder, als een op zichzelf staande etiologische factor. Hiervoor werd prevalentie onderzoek verricht naar de veelvoorkomende mutatie (1528G>C) die LCHADD veroorzaakt, onder een groep vrouwen die in het verleden het HELLP syndroom doormaakten. Bij de selectie van deze vrouwen speelde de uitkomst van de zwangerschap geen rol. Slechts een van de 113 geteste vrouwen blijkt drager te zijn voor deze mutatie, overeenkomend met de prevalentie van het dragerschap voor deze mutatie onder de Nederlandse bevolking. Dragerschap voor deze mutatie lijkt daarmee geen belangrijke factor te kunnen zijn in de etiologie van het HELLP syndroom.

Recent werd door Yang en collegae dringend geadviseerd (JAMA 288: 2163-2166) in om de gezinnen van alle vrouwen die AFLP doormaken te screenen op de veel voorkomende mutatie (1528G>C) die LCHADD veroorzaakt. Voor de gezinnen van vrouwen die aan het HELLP-syndroom lijden acht hij dit advies niet zinvol. Hoewel de resultaten van ons onderzoek zijn advies ten aanzien van vrouwen met AFLP ondersteunen, menen wij dat het wel geïndiceerd is om de gezinnen van vrouwen die lijden aan een zeer ernstig HELLP-syndroom te screenen voor lang-keten FAO-stoornissen. Immers, HELLP-syndroom en AFLP lijken tot hetzelfde spectrum van ziektebeelden te behoren en zijn soms moeilijk van elkaar te onderscheiden.

Bovendien zal screening alleen door middel van louter de veel voorkomende LCHAD mutatie (1528G>C) in deze gezinnen onvoldoende zijn, aangezien het HELLP-syndroom en AFLP ten gevolge van MTPD hiermee niet kunnen worden opgespoord. Naar onze mening zou hierom naast de screening op de veelvoorkomende LCHAD mutatie (1528G>C), ook een acylcarnitine-profiel van het kind bepaald moeten worden.

Hoofdstuk 6

Aansluitend werden belastingstesten met lange-keten triglycerides (LCT) verricht bij vrouwelijke dragers van de 1528G>C mutatie en bij gezonde controles, om te bestuderen of de FAO-capaciteit, die als gevolg van dragerschap theoretisch 50% van normaal zou kunnen zijn, in vivo ook daadwerkelijk gestoord is. De resultaten tonen een volledig normale capaciteit om vetzuren te oxideren, leidend tot de conclusie dat vrouwelijke dragers voldoende in staat zouden moeten zijn om door de foetus geproduceerde lange-keten acylcarnitines die haar eigen bloedcirculatie bereiken, snel te kunnen metaboliseren. Het is daarom niet waarschijnlijk dat het dragerschap van de moeder een op zichzelf staande rol speelt in de etiologie van de gerapporteerde zwangerschaps-complicaties.

Hoofdstuk 7

Een andere mogelijke verklaring voor de associatie tussen een FAO-stoornis in het kind en de ernstige zwangerschapscomplicaties bij de moeder zou de productie van lange-keten acyl-CoA esters door de foeto-placentaire unit (de eenheid foetus en placenta) kunnen zijn. Echter, in het algemeen wordt aangenomen dat de foetus vrijwel uitsluitend glucose gebruikt om in zijn energiebehoeften te voorzien en geen aanspraak doet op de FAO-capaciteit. Daarom lijkt het niet waarschijnlijk dat de foetus een belangrijke producent is van lange-keten acyl-CoA esters. De placenta daarentegen is vrijwel geheel van foetale oorsprong en maakt aan het einde van de zwangerschap kwantitatief een belangrijk deel uit van de foeto-placentaire unit. In het geval van een FAO-defect zou de placenta dus direct verantwoordelijk kunnen zijn voor de productie van lange-keten acyl-CoA esters. Recent toonden Rakheja en collegae (Placenta 2002;23: 447-450) als eersten aan dat twee enzymen betrokken bij de FAO, LCHAD en SCHAD, inderdaad in placentair weefsel aanwezig zijn, passend bij deze hypothese. Hierop verder gaand bestudeerden wij de activiteit van een reeks aan enzymen die betrokken zijn bij de FAO (CPT2, VLCAD, LCEH, LCHAD, LCKAT, MCAD, SCEH en SCHAD) in a terme, humane placenta en chorionvlokken en vergeleken de activiteit met die in normale humane lever. Alle gemeten enzymen blijken zowel in placenta als in chorionvlokken, verkregen bij vlokkentesten, tot expressie te komen. Een opvallende bevinding is dat de activiteit van de enzymen CPT2 en VLCAD, beiden betrokken bij de oxidatie van lange-keten vetten, nog hoger blijkt te zijn in placenta dan in controle lever, het orgaan dat bij uitstek bekend staat om zijn hoge activiteit van FAO-enzymen. Deze resultaten maken het waarschijnlijk dat placentaire productie van toxische lange-keten acyl-CoA esters een rol speelt in de etiologie van het HELLP-syndroom en AFLP bij moeders die zwanger zijn van een kind met

een lange-keten FAO-stoornis. De onder deze omstandigheden door de placenta geproduceerde lange-keten acyl-CoA esters kunnen als lange-keten acylcarnitine esters via het bloed naar de moeder worden getransporteerd en worden opgenomen in de lever en andere organen. Het mechanisme waardoor de lange-keten acyl-CoA esters leverziekte veroorzaken bij de moeder, blijft vooralsnog onduidelijk. Er zijn drie mogelijke hypotheses. De eerste hypothese, die stelt dat de leverziekte ontstaat door het onvermogen om lange-keten acyl-CoA esters snel te metaboliseren als gevolg van dragerschap voor de FAO-stoornis, is gezien de resultaten van de in hoofdstuk 5 gepresenteerde studie niet waarschijnlijk. De tweede hypothese stelt dat de geproduceerde lang-keten acyl-CoA esters direct schade berokkenen aan het placentaire weefsel doordat diverse metabole processen worden geremd door deze esters. Zo zou endotheliale (vaatwand) dysfunctie, wat beschouwd wordt als een van de belangrijke factoren in de etiologie van preeclampsie en het HELLP-syndroom, veroorzaakt kunnen worden door verminderde functie van de mitochondriale ademhalingsketen. Daarnaast kan schade aan de placenta door lang-keten acyl-CoA esters ook het loslaten van micropartikels van placentaweefsel in de bloedstoom van de moeder tot gevolg hebben. Dit mechanisme, dat wordt beschreven als een belangrijke mechanisme in het ontstaan van preeclampsie en het HELLP-syndroom, leidt tot een hevige afweerreactie van het lichaam van de moeder en zou zo ziekte van de lever en andere organen tot gevolg kunnen hebben.

Hoofdstuk 8

Een andere observatie die een belangrijke rol voor de FAO suggereert in de foeto-placentaire unit, is de hoge incidentie van foetale groeiachterstand (intra-uteriene groei retardatie, IUGR) and prematuriteit in patiënten met LCHADD of MTPD. Deze observatie komt overeen met de bevindingen die eerder werden gepubliceerd in muizen met MTPD. Aangezien de rol van FAO nooit eerder was bestudeerd in humane embryo's, bestudeerden wij de expressie van twee enzymen die betrokken zijn bij de lange-keten FAO, VLCAD en LCHAD, zowel met behulp van in situ hybridisatie, als door middel van meting van de enzymactiviteit. Zowel de mRNA expressie als meting van de enzymactiviteit toonde in verschillende weefsels, zoals het myocard en het zich ontwikkelende zenuwstelsel, inclusief de retina (netvlies), significantie expressie, hetgeen bewijst dat deze enzymen in het embryo al actief worden aangemaakt. De IUGR bij LCHADD en MTPD kinderen kan hieruit worden verklaard. In overeenstemming met de bevindingen in het humane embryo waar door ons expressie van lang-keten FAO enzymen in het hart gevonden werd, is de diagnose MTPD bij één patiënt met hydrops

foetalis als gevolg van reeds intra-uterien vastgestelde cardiomyopathie (ziekte van de hartspier) (hoofdstuk 4). Een andere tot nu toe onverklaarde complicatie van LCHADD en MTPD is retinitis pigmentosa, een degeneratieve aandoening van het netvlies, die bij deze ziekte al op jonge leeftijd op kan treden. De bevinding dat LCHAD een hoge expressie heeft in foetale retina tijdens de vroege ontwikkelingsfasen past hierbij en suggereert dat retinitis pigmentosa wellicht al intra-uterien zou kunnen ontstaan. Opvallend is de door ons gevonden discrepantie tussen enerzijds de hoge expressie van LCHAD en anderzijds de lage expressie van VLCAD in het zich ontwikkelende zenuwweefsel. Een mogelijke verklaring zou kunnen zijn dat de MTP enzymen (LCEH, LCHAD en LCKAT) naast hun rol in de FAO nog een andere, tot nu toe onbekende functie hebben in het (zich ontwikkelende) zenuwweefsel.

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Curriculum Vitae

Lisette den Boer wordt op 7 november 1969 geboren in Gouda. In 1988 slaagt zij voor haar eindexamen aan het Coornhert Gymnasium in diezelfde gemeente. Vervolgens studeert zij geneeskunde aan de Universiteit van Amsterdam. Tijdens haar studie is Lisette student assistent bij de afdeling huisartsgeneeskunde en participeert zij via de wetenschapswinkel van de universiteit in het schrijven van medisch voorlichtingsmateriaal voor verschillende patiëntenverenigingen.

In oktober 1995 behaalt zij haar artsexamen. Vervolgens werkt, nu *dokter* den Boer als AGNIO kindergeneeskunde op verschillende afdelingen van het Emma Kinderziekenhuis AMC en werkt zij een jaar fulltime aan het onderzoek dat leidt tot dit proefschrift. In 1998 start Lisette met de opleiding kindergeneeskunde in het Emma Kinderziekenhuis AMC (opleider prof. dr. H.S.A. Heymans). In het kader van deze opleiding verhuist zij gedurende een jaar naar het Onze Lieve Vrouwe Gasthuis te Amsterdam (opleider E.M.A. van der Veer). Tijdens haar opleiding wordt zij bestuurslid van de junior afdeling van de Nederlandse Vereniging voor Kindergeneeskunde (NVK), waarvoor zij gedurende enkele jaren deel uitmaakt van de Commissie Wetenschappelijke Vergaderingen van de NVK. In juli 2002 wordt Lisette geregistreerd als kinderarts en start zij met een fellowship metabole ziekten (opleider dr. H.D. Bakker) in het Emma Kinderziekenhuis AMC. Dit fellowship zal zij niet afronden, aangezien zij per februari 2004 als algemeen kinderarts in dienst treedt van Medisch Spectrum Twente te Enschede.

Lisette is getrouwd met Alfons Wegdam. Sinds augustus 2003 hebben zij een dochter, genaamd Maaike Hendrikje.

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