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KAIRIT JOOST

Selective screening of metabolic diseases in Estonia: the application of new diagnostic methods

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Department of Biochemistry, University of Tartu, Tartu, Estonia

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Supervisors: Professor Ursel Soomets, PhD, Department of Biochemistry, University of Tartu, Estonia

Professor Katrin Õunap, MD, PhD, Department of Paediatrics, University of Tartu, Tartu, Estonia

Riina Žordania, MD, PhD, Department of Genetics, United Laboratories, Tartu University Hospitals, Tallinn, Estonia

Kalle Kilk, PhD, Department of Biochemistry, University of Tartu, Estonia

Reviewers: Professor Allen Kaasik, PhD, Department of Pharmacology, University of Tartu, Tartu, Estonia

Associate Professor Oivi Uibo, MD, PhD, Department of Paediatrics, University of Tartu, Tartu, Estonia

Opponent: Professor Elisabeth Holme, MD, PhD, Clinical Chemistry Department, Sahlgrenska University Hospital, Gothenburg University, Gothenburg, Sweden

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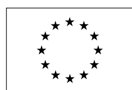

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To Karl-Markus
and his family

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LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications referred to in the text by Roman numerals (I–IV), and previously unpublished data:

I K. Joost, K. Õunap, R. Žordania, M.-L. Uudelepp, R.K. Olsen, K. Kall, K. Kilk, U. Soomets, T. Kahre The high prevalence of long-chain 3-hydroxyacyl-CoA dehydrogenase deficiency in Estonia JIMD Reports 2011; 2:80–85.

II K. Joost, R. J. Rodenburg, A. Piirsoo, L. van den Heuvel, R. Zordania, H. Põder, I. Talvik, K. Kilk, U. Soomets, K. Õunap Developing a diagnostic algorithm for mitochondrial disorders presenting in childhood in Estonia. (Submitted).

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IV K. Joost, P. Tammur, R. Teek, O. Žilina, M. Peters, M. Kreile, B. Lace, R. Žordania, I. Talvik, K. Õunap Whole Xp deletion and skewed X-chromosome inactivation in a girl with OTC deficiency Mol Syndromol. 2011 Sep;1(6):311–315.

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My contributions to the original articles are:

Publication I: developed the method and performed tandem MS analysis of acylcarnitines, interpretation of the results, diagnoses and treated a patient with LCHAD deficiency, wrote/co-wrote the manuscript.

Publication II: designed the study and led the clinical investigation of patients and the clinical investigation of family members. Interpreted the results and wrote/co-wrote the manuscript.

Publication III: led the clinical investigation of the patient and the clinical investigation of family members. Interpreted the results and wrote/cowrote the manuscript.

Publication IV: conducted specification of phenotype, interpreted the results and wrote/co-wrote the manuscript.

ABBREVIATIONS

ACAD	acyl-CoA dehydrogenases
ACADM	acyl-CoA deydrogenase, medium-chain
aCGH	array comparative genomic hybridization
ACP	acylcarnitine profiling
AD	autosomal dominant
ADP	adenosine diphosphate
AFLP	acute fatty liver of pregnancy
ALT	alanine aminotransferase
AMP	adenosine monophosphate
AMPK	AMP-activated protein kinase
AR	autosomal recessive
ASL	argininosuccinate lyase
ASS	argininosuccinate synthase
ATP	adenosine triphosphate
CACT	carnitine acylcarnitine translocase
cDNA	complementary DNA
CI	confidence interval
CNS	central nervous system
CoQ	coenzyme Q; ubiquinone
COX	cytochrome oxidase
CPS1	carbamylphosphate synthase 1
CPT1	carnitine palmitoyltransferase 1
CPT2	carnitine palmitoyltransferase 2
CSF	cerebrospinal fluid
DCMA	dilated cardiomyopathy with ataxia
DIDMOAD	diabetes insipidus, diabetes mellitus, optic atrophy, deafness
EMA	ethylmalonic acid
EMG	electromyography
ETF	electron transfer flavoprotein
ETC	electron transfer chain
FA	fatty acid
FAD	flavinadenine dinucleotide
FADH	semireduced flavineadenine dinucleotide
FADH2	reduced flavineadenine dinucleotide
FAO	fatty acid β-oxidation
FAOD	fatty acid β-oxidation defects
FFA	free fatty acids
FGF-21	fibroblast growth factor-21
FMN	flavin mononucleotide
GC/MS	gas chromatography/mass spectrometry
GRACILE	growth retardation, aminoaciduria, cholestasis, iron overload, lactic acidosis, early death

HADH	3-hydroxyacyl-CoA dehydrogenase deficiency
HELLP	hemolysis, elevated liver enzymes, low platelets
HHH	hyperammonaemia, hyperornithinaemia, homocitrullinaemia
HPLC	high-performance liquid chromatography
IEM	inborn errors of metabolism
IUGR	intrauterine growth retardation
KSS	Kearns-Sayre syndrome
LCAD	long-chain acyl coenzymeA dehydrogenase
LC-FA	long-chain fatty acid
LCHAD	long-chain hydroxyacyl-CoA dehydrogenase
LCHADD	long-chain hydroxyacyl-CoA dehydrogenase deficiency
LCEH	long-chain 2,3-enoyl-CoA hydratase
LCKAT	long-chain 3 ketoacyl-Co-thiolase LDH lactate dehydrogenase
LHON	Leber hereditary optic neuropathy
LS	Leigh syndrome
MAD	multiple acyl-CoA dehydrogenase
MADD	multiple acyl-CoA dehydrogenase deficiency
MCAD	medium-chain acyl-CoA dehydrogenase
MCADD	medium-chain acyl-CoA dehydrogenase deficiency
MC-FA	medium-chain fatty acid
MCKAT	medium chain-ketoacyl-CoA thiolase
MCT	medium chain triglycerides
MD	mitochondrial disorders
MERFF	myoclonic epilepsy, ragged red fibres
MIDD	maternally inherited diabetes and deafness
MLASA	myopathy, lactic acidosis, sideroblastic anaemia
MPS	mucopolysaccharidoses
MRI	magnetic resonance imaging
MRM	multiple reaction monitoring
MRS	magnetic resonance spectroscopy
MS/MS	tandem mass spectrometry
mtDNA	mitochondrial DNA
MTP	mitochondrial trifunctional protein
NADH	nicotine amide adenine dinucleotide
NAGS	N-acetylglutamate synthase
NARP	neuropathy, ataxia, *retinitis pigmentosa*
NBS	newborn screening
nDNA	nuclear DNAOCTN2 sodium ion-dependent carnitine transporter
OTC	ornithine transcarbamylase
OXPHOS	oxidative phosphorylation system
PEO	progressive external ophthalmoplegia
PKU	phenylketonuria
PDH	pyruvate dehydrogenase
RC	respiratory chain
Ref	reference range

ROS	reactive oxygen species
RRF	ragged red fibres
rRNA	ribosomal ribonucleic acid
SANDO	sensory ataxia, neuropathy, dysarthria, ophtalmoplegia
SCAD	short-chain acyl-CoA dehydrogenase
SCADD	short-chain acyl-CoA dehydrogenase deficiency
SCHAD	short-chain hydroxyacyl-CoA dehydrogenase
SCHADD	short-chain hydroxyacyl-CoA dehydrogenase deficiency
SCO2	synthesis of cytochrome c oxidase 2
SDH	succinic dehydrogenase
SIDS	sudden infant death syndrome
SRM	selective reaction monitoring
UC	urea cycle
UCD	urea cycle disorders
TCA	tricarboxylic acid
TFP	trifunctional protein
tRNA	transfer ribonucleic acid
VLCAD	very-long-chain acyl-CoA dehydrogenase
VLCADD	very-long-chain acyl-CoA dehydrogenase deficiency
XCI	X-chromosome inactivation

1. INTRODUCTION

Inborn errors of metabolism (IEM) are caused by biochemical inbalance in the organism resulting from pathogenic mutations. Many of these present in neonatal age or in infancy, but the number of diseases diagnosed in childhood and the teen years is increasing, including congenital metabolic disorders causing health problems in adulthood (Saudubray 1995).

Historically, the hypothesis of "chemical physiology and pathology" in man was first presented by Sir Archibald Garrod in 1908 in his four Croonian lectures (Garrod 1908). He was the first to use the term "inborn errors of metabolism" and to apply Mendel's law of segregation to humans, including the description of recessive inheritance while pointing out the importance of consanguinity. Although not appreciated during his lifetime, during the 20th century Garrod's ideas accumulated supporting scientific evidence, and each of his chapter has acquired its own genomic locus, a cloned gene, a repertoire of annotated phenotype-modifying alleles, a gene product with known structure and function and altered function in Mendelian variant (Scriver 2008).

The first systematic review in the field of IEM in Estonia was published in 1996 by Õunap et al. (Õunap *et al.,* 1996). This work established the incidence of phenylketonuria (PKU) and made possible the development of a newborn screening program for the early detection of PKU in children. In 1998 it was shown that the incidence of PKU in Estonia identified during retrospective studies is lower than the incidence identified prospectively during newborn screening (NBS) (Õunap *et al.,* 1998). Early detection of PKU allows for presymptomatic treatment of patients, and the development of mental retardation is avoided. In 2012 the Estonian guidelines for PKU treatment were published (Uudelepp *et al.,* 2012).

Due to the non-specific clinical picture characteristic of most IEMs, the diagnostics is largely dependant on the availability of specific laboratory investigations. In 2007, when this project was initiated, the following analytical methods were available in the diagnosis of IEM in Estonia: simple urine screening tests, high-performance liquid chromatography (HPLC) of amino acids, and gas chromatography/mass spectrometry (GC/MS) of organic acids. In addition, urinary creatine and guanidinoacetate was measured for the diagnosis of defects of creatine metabolism, and quantitative analysis of very long chain fatty acids is available for the diagnosis of peroxisomal diseases (GC/MS method). Lactate, ammonia, homocysteine and total sialotransferrine measurement was used in the primary screening of related diseases. There were, however, several groups of IEMs where specific analyses were not available in Estonia, and these were performed in other European laboratories in the case of clinical suspicion. These included energy deficiency disorders such as fatty acid β-oxidation defects (FAOD) and mitochondrial disorders (MD).

Early identification of FAOD is clinically important, as the clinical outcome in those disorders is generally favourable, but they are related with significant

mortality if they remain undiagnosed (Sim *et al.,* 2002). The first diagnostic test of FAOD to become available in Estonia was molecular genetic testing for the common mutations in genes responsible for medium-chain acyl-CoA dehydrogenase (MCAD) deficiency and long-chain acyl-CoA dehydrogenase (LCHAD) deficiency. Lilleväli *et al* have shown that carrier frequency for the mutation c.985A>G (p.K329E) in the *ACADM* gene responsible for the MCAD deficiency (MCADD) is 1:220 in Estonia, and the frequency of possibly affected homozygotes is 1:193,000 (Lilleväli *et al.,* 2000), but no MCADD patients were known until 2007. LCHAD deficiency (LCHADD) was diagnosed retrospectively in one family. Mutational analysis in FAOD has limited informativeness, as common mutations are not entirely responsible for the development of the disease. In addition, the clinical phenotype of MCADD and LCHADD overlaps to a great extent, and therefore the detection of the mutations related with both forms of the disease was generally necessary and was time-consuming and expensive. Acylcarnitine analysis using tandem mass spectrometry (MS/MS) in bodily fluids as developed by Millington *et al* in 1989 has been shown to be highly informative in the diagnosis of FAOD, and allows simultaneous and quick detection of all FAODs from one sample (Millington *et al.,* 1989). It has been successfully applied in expanded NBS, allowing presymptomatic testing of >20 congenital conditions (Lindner *et al.,* 2011; Lindner *et al.,* 2010; Schulze *et al.,* 2003; Wilcken *et al.,* 2003).

MD are probably one of the most common IEMs, and have an estimated frequency of 1:5000–10,000 (Skladal *et al.,* 2003). The diagnosis of these disorders is, however, complex, as there is no single test with sufficient accuracy to prove or exclude the presence of a mitochondrial disorder in the patient. MD presenting in childhood in Estonia has previously been diagnosed in only one patient with Leigh syndrome (LS) caused by respiratory chain complex I deficiency due to a mutation in the mitochondrial *ND3* gene.

The "gold standard" in diagnostics is morphological and enzymatic investigations performed on muscular tissue, but as muscular biopsy is invasive, time-consuming and some tests are not available in Estonia, the careful pre-selection of patients suggestive of a MD is essential. Therefore in 2004 we developed a diagnostic algorithm for MD which takes into account our local diagnostic possibilities and medical organization.

In order to improve the diagnostics of IEM in Estonia, we undertook this study with the following aims: to introduce acylcarnitine analysis by MS/MS into the clinical diagnostics of FAOD and to evaluate the effectiveness of a diagnostic algorithm for MD in Estonia and to establish the live-birth prevalence of those disorders in our population. During our study we also characterise patients with rare metabolic diseases.

2. LITERATURE REVIEW

2.1. An overview of metabolic diseases

The basic biochemical lesions in monogenic disorders involve defects in a wide variety of proteins, mostly enzymes, receptors, transport proteins, peptide hormones, immunoglobulins, collagens and coagulation factors. The vast majority of IEMs involve abnormalities in enzymes and transport proteins, and the basic biochemical lesion usually affects either one metabolic pathway common to a large number of cells or several organs or is restricted to one organ but gives rise to humoral and systemic consequences. According Saudubray and Charpentier, IEMs can be subdivided into 3 groups from the pathophysiological perspective (Saudubray 1995).

Group 1 – storage type. This group includes diseases that disrupt the synthesis or catabolism of complex molecules, leading to storage phenomena and chronic toxicity. Clinical symptoms are permanent, progressive, independent of intercurrent events and unrelated to food intake. Lysosomal storage disorders, peroxisomal biogenesis disorders, congenital glycosylation defects and cholesterol biosynthesis defects belong to this group. The diagnosis of this group relies largely on measurement of the activities of specific enzymes in tissues, but the first line tests used in the selective screening of the diseases of this group are the measurement of serum very long chain fatty acids (peroxisomal biogenesis defects) or serum cholesterol precursors (cholesterol biosynthesis defects) or urinary excretion of glycosaminoglycans and oligosaccharides (lysosomal storage disorders). Isoelectric focussing of serum transferrins is indicated in suspicion of congenital glycosylation defects.

Group 2 – intoxication type. This group includes diseases where the accumulation of substrate of the deficient enzyme reaction (or transporter) will result in toxicity and disease. The accumulating substrates may be metabolised via alternative pathways that may yield further toxic compounds that normally occur at low concentrations, if present at all. This metabolic disturbance may cause acute toxicity or chronic toxicity in the long term. All of the conditions in this group present clinical similarities, including a symptom-free interval, clinical signs of intoxication, which may be either acute (vomiting, lethargy, coma, liver failure, or thromboembolic complications) or chronic (progressive psychomotor retardation) and recurrent metabolic disturbances (acidosis, ketosis, and/or hyperammonemia). This group includes most of the aminoacidopathies, most of the organic acidurias, urea cycle defects (UCD) and sugar intolerances. Biochemical diagnosis is easy and relies mostly on plasma and urine amino acid or organic acid chromatography. Treatment of these disorders requires removal of the toxic compounds through special diets, exchange transfusion or hemodialysis.

Group 3 – energy deficiency type. Compromised mitochondrial energy production at the levels of fatty acid oxidation (FAO), tricarboxylic acid cycle and respiratory chain enzymes fall into this group. Energy utilisation disorders

such as disorders of glyconeogenesis and glycogen storage diseases also belong to this group. These diseases present an overlapping clinical spectrum that sometimes results from both the accumulation of toxic compounds and deficient energy production. These disorders predominately affect organs with great energy consumption (brain, muscle, liver). Symptoms common to this group include hypoglycaemia, lactic acidosis, severe generalised hypotonia, myopathy, cardiomyopathy, failure to thrive, cardiac failure, circulatory collapse, sudden infant death syndrome (SIDS), and malformations (the latter suggesting that the abnormal processes affect the foetal energy pathways (Illsinger and Das 2010). The extreme clinical diversity of these disorders illustrates the ubiquitous role of energy processes at every age and in every organ. Treatment of these disorders would generally require adequate energy replacement.

More than 700 different IEMs are known (Illsinger and Das 2010). These are individually rare, but as a group their incidence is significant – approximately 1:2500 live births (Applegarth *et al.,* 2000). The overview of the more frequent disorders among Caucasians and their known frequencies among Estonians are given in table 1.

Table 1. More frequent IEMs presenting in childhood.

Disease or group	**Prevalence among Caucasians**	**Prevalence in Estonia**
PKU	1:10,000 (Mathias and Bickel 1986)	1:6010 (Õunap *et al.,* 1998; Õunap 1999)
Classical galactosaemia	1:23,000 – 1:44,000 (Bosch 2006; Schweitzer-Krantz 2003)	1:19,700 (Õunap *et al.,* 2010)
Urea cycle defects	1:8000 – 1:39,000 (Braissant 2010; Keskinen *et al.,* 2008)	Unknown
Mucopolysaccharidoses (MPS)	1:25,000 (Nelson 1997; Poorthuis *et al.,* 1999)	1:24,687 (Krabbi *et al.,* 2012)
MCADD	1:15,000 (Lindner *et al.,* 2010)	1:193,000 (Lilleväli *et al.,* 2000)
LCHADD	1:250,000 (Lindner *et al.,* 2010)	**1:94,864 (this study) Publication I**
MD	1:5000 – 1:10,000 (Skladal *et al.,* 2003)	**1:20,764 (this study) Publication II**

2.2. Fatty acid oxidation defects

2.2.1. An introduction to FAO

Fatty acids (FA) represent a major source of energy in man, providing up to 80% of total energy requirements during fasting (Zschocke and Hoffmann 2011). FAO is a key metabolic pathway for energy homeostasis in organs such as the liver, heart and skeletal muscle. It becomes essential during fasting, when glucose supply becomes limited and most tissues use FA directly to create energy. Furthermore, FA are converted into ketone bodies in the liver and can subsequently function as an additional energy source that is used by all tissues, including the brain.

Classification of fatty acids:

FA are classified: a. on the basis of chain length (table 2);
b. on the basis of chemical structure: FA without double bonds are saturated, and FA without double bonds are unsaturated.

Table 2. Classification of FA on the basis of chain length.

FA	No. of carbons
Short-chain fatty acids	<6
Medium-chain fatty acids (MC-FA)	6–12
Long-chain fatty acids (LC-FA)	14–20
Very long chain fatty acids	>22

FAO was discovered by Georg Franz Knoop, whose experiments led to the conclusion that the metabolism of FA proceeds through the successive removal of two carbon fragments, and postulated that the oxidation takes place on the β-carbon atom. Knoop published the results of his experiments in 1904. In 1955 Irving B. Fritz discovered the role of carnitine in FAO (Houten and Wanders 2010).

Palmitoyltransferase I deficiency was the first defect in an FAO pathway that was clinically described in a patient with familial recurrent myoglobinuria in 1973 by DiMauro and DiMauro (1973). Carnitine deficiency in skeletal muscle in association with lipid storage myopathy was first described in the same year (Engel and Angelini 1973). Karpati *et al* described the syndrome of systemic carnitine deficiency in a patient with recurrent hepatitis and cerebral dysfunction and underdeveloped musculature with impaired ketogenesis and no sign of oxidation LC-FA (Karpati *et al.,* 1975). In 1976 Gregersen *et al* described the first patient with MCADD in a patient with unexplained episodes of lethargy and consciousness with elevated urinary excretion of suberylglycine and C6-C10 decarboxylic acids. Long-chain acyl coenzymeA dehydrogenase (LCAD) deficiency and LCHADD were described in 1985 and 1989 respectively (Hale *et al.,* 1985; Wanders *et al.,* 1989).

2.2.2. The metabolism of FA

During fasting or prolonged exercise, LC-FA stored as triglycerides in fat tissue are released by lipases. Free fatty acids (FFA) move into the blood stream and are transported there by serum albumin. In the plasma membrane FA are translocated into cytosol by fatty acid transport proteins, which are transmembrane proteins that possess acyl-CoA synthetase activity (Houten and Wanders 2010).

The carnitine shuttle: The inner mitochondrial membrane is not permeable to LC-FA, which form the major part of dietary fatty acids. They are transported into the mitochondria via the carnitine shuttle, which consists of three enzymes (carnitine palmitoyltransferase 1 (CPT1), carnitine acylcarnitine translocase (CACT, *SLC25A20*), carnitine palmitoyltransferase 2 (CPT2)) and a small, soluble molecule, carnitine, to transport FA as their long-chain fatty acylcarnitine esters. CPT1 converts acyl-CoA into an acylcarnitine. Subsequently CACT exchanges acylcarnitine for free carnitine inside the mitochondria. Finally CPT2 reconverts the acylcarnitines into their CoA esters, which then enter β-oxidation.

β-oxidation: After transport across the plasma membrane, FA are activated to acyl-CoAs at the cytosolic site. β-oxidation takes place in mitochondria and consists of a series of cycles, each of which shortens the fatty acids by 2 carbon atoms. Each cycle consists of 4 enzymatic reactions. First acyl-CoA is dehydrogenated to a trans-2-enoyl-CoA, which is hydrated to form L-3-hydroxyacyl-CoA in the next step. The third reaction in this cycle is dehydrogenation to form 3-keto-acyl-CoA. Finally, thiolytic cleavage produces an acyl-CoA shortened by two carbons and acetyl-CoA. Shortened acyl-CoA enters into the new cycle and acetyl-CoA enters into the citric acid (Krebs) cycle. In addition to acyl-CoA and acetyl-CoA, each cycle yields one nicotinamide adenine dinucleotide (NADH) and one flavin adenine dinucleotide ($FADH_2$), which are later reoxydized in the mitochondrial respiratory chain. The electron transfer from FAD dependant dehydrogenases is mediated by electron transfer flavoprotein (ETF). The characterisation of β-oxidation is schematically illustrated in figure 1.

Long chain acyl-CoAs are metabolised by membrane-bound enzymes very-long-chain acyl-CoA dehydrogenase (VLCAD) and mitochondrial trifunctional protein (MTP). MTP has hydratase, LCHAD and thiolase activity (LCKAT). After 2–3 FAO cycles using these enzymes, the resulting medium and short chain acyl-CoAs are metabolised in the mitochondrial matrix via a cycle that consists of similar enzymatic steps.

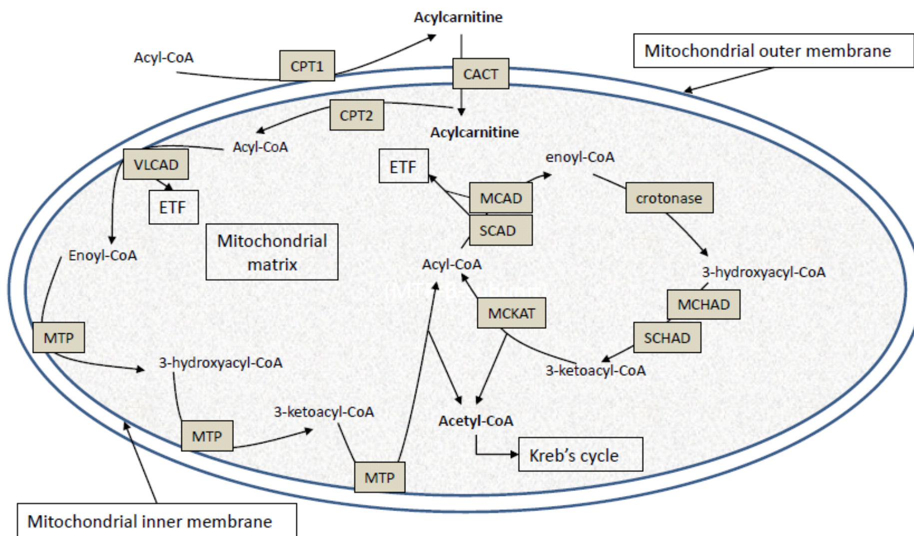


Figure 1: β-oxidation in the mitochondria.

Inherited deficiencies of most of the enzymes involved in carnitine transport, the carnitine shuttle and β-oxidation are known (table 3).

Regulation of FAO and substrate use in energy homeostasis: The reciprocal relationship between the oxidation of FA and glucose is known as the glucose-fatty acid or Randle cycle. This cycle explains the mechanisms through which glucose is spared for usage in brain tissue. In the fasted state, activation of lipolysis provides tissues with FA. In the liver, β-oxidation of FA fulfils the local energy needs and may lead to ketogenesis. Ketone bodies are preferentially oxidized in extrahepatic tissues. By inhibiting glucose oxidation, FA and ketone bodies contribute to a glucose-sparing effect, which is an essential survival mechanism for the brain during starvation (Hue and Taegtmeyer 2009).

Substrate availability is regulated by hormonal and metabolic factors. Insulin increases glucose transport and inhibits lipolysis, which decreases substrate availability for FAO. The release of FFA into the circulatory system is, however, increased by adrenalin, noradrenaline, glucagon and adrenocorticotropic hormone, which all induce lipolysis.

CPT1 activity is regulated by malony-CoA. As the level of adenosine triphosphate (ATP) decreases and the level of adenosine monophosphate (AMP) increases, the AMP-activated protein kinase (AMPK) is activated. Activated AMPK leads to acetyl-CoA decarboxylase inactivation, possibly together with malonyl-CoA decarboxylase activation, which decreases malonyl-CoA concentration and hence favours FAO (Hue and Taegtmeyer 2009).

Additionally, pyruvate dehydrogenase (PDH) catalyses the oxidative decarboxylation of pyruvate to acetyl-CoA and links glycolysis with oxidative

metabolism. Metabolites can also affect the trancriptional regulation of genes involved in metabolism (Houten and Wanders 2010).

Table 3. Inherited deficiencies of the enzymes involved in carnitine transport, the carnitine shuttle and β-oxidation, the genes responsible and the OMIM numbers of clinical phenotypes.

Enzyme	Deficiency	Gene	Phenotype OMIM
Sodium ion-dependent carnitine transporter (OCTN2)	Carnitine transporter deficiency	*SLC22A5*	212140
CPT1	CPT1 deficiency	*CPT1A*	255120
CACT	Carnitine-acylcarnitine translocase deficiency	*SLC25A20*	212138
CPT2	CPT2 deficiency	*CPT2*	608836 (lethal neonatal) 600648 (infantile) 255110 (late onset
VLCAD	VLCAD deficiency	*ACADVL*	201475
MTP	LCHAD (MTP β-subunit)	*HADHA*	600890
	LCKAT (MTP-β-subunit)	*HADHB*	609015
LCHAD	Isolated LCHADD	*HADHB*	600890
LCKAT	Isolated LCKAT deficiency	*HADHB*	143450
MCAD	MCADD	*ACADM*	201450
MCKAT	MCKAT deficiency	Unknown	602199
SCAD	SCAD deficiency	*ACADS*	201470
HADH*	3-hydroxy-acyl dehydrogenase deficiency (HADH) deficiency	*HADH*	601609
ETF dehydrogenase	Multiple acyl-CoA dehydrogenase (MAD) deficiency	*ETFDH*	231680
ETF-α-subunit		*ETFA*	
ETF β-subunit		*EFTB*	

*formerly known as SCHAD

2.2.3. The biochemical diagnosis of FAOD

Initial laboratory studies usually include serum glucose, electrolytes, lactate, ammonia, transaminases, creatine kinase and urine analysis. Hypoglycaemia is usually present, but normal to borderline levels may be observed. Serum lactate may be moderately increased, and mild elevations of plasma ammonia levels (up to 100 μmol/l; reference range (ref. <50 μmol/l)) may be observed. Liver dysfunction and elevated creatine kinase activity is more prominent in LCHADD patients, being present in 89% and 67% of patients respectively (den Boer *et al.,* 2002).

Biochemical diagnosis of FAOD commonly begins with a search for diagnostic metabolites in physiological fluids. This is followed by in vitro functional studies if the initial findings are inconclusive, and is confirmed by enzymology and molecular analyses (den Boer *et al.,* 2002).

2.2.3.1. Organic acid analysis

Methods to identify abnormal metabolites of FAs using gas chromatography coupled to mass spectrometry (GC/MS) have been available since the mid-1970s (Roe 1995). Changes in urinary organic acid analysis are detectable in β-oxidation defects, where the accumulation of chain-specific metabolites can be detected. In the case of carnitine cycle defects, no abnormalities are detected in the GC/MS analysis of urinary organic acids (Roe 1995).

The general changes characteristic of most FAO defects are the absence or low excretion of ketones. Key metabolites detected in the urinary organic acid analysis suggestive of FAO defect are dicarboxylic acids arising from microsomal ω-oxidation, and acylglycines (Roe 1995). The changes suggestive of specific blockage in β-oxidation are given in table 4.

Table 4. The changes characteristic of FAO defects observed in GC/MS analysis of organic acids.

Defect	Dicarboxylic acids	Acylglycines
VLCADD	C12-C14 dicarboxylic acids	Not present
LCHADD	3-OH dicarbocylic acids (C6-C14) C6-C10 dicarboxylic acids	Not present
MCADD	C6-C10 dicarboxylic acids	Hexanoylglycine Suberylglycine
SCADD	Ethylmalonate Methylsuccinate	Butyrylglycine
MADD	Isovaleric acid Isobutyric acid 2-methylbutyric acid Glutaric acid Ethylmalonate 3-OH-isovaleric acid 2-OH-glutaric acid 5-hydroxyhexanoic acid C6-C12 dicarboxylic acids	Isovalerylglycine Isobutyrylglycine 2-methylbutyrylglycine

It must be emphasised that dicarboxylic acid excretion is not pathognomic to FAOD, as they can also be observed in diabetic ketoacidosis and after the dietary administration of medium chain triglycerides (MCT). The concentrations of abnormal urinary metabolites vary greatly: they may be below detection level if the patient is well. Therefore samples obtained during an acute episode are most informative (Roe and Coates 1995).

2.2.3.2. Tandem mass-spectrometry

MS/MS can be applied in different fields of biochemical genetics. The technique is most commonly performed using a triple quadrupole mass spectrometer, which consists of an ionisation source, three mass filters connected in tandem and a detector. Ions produced in the source are selected by the first quadrupole (Q1) for transmission to the second quadrupole, which is designated as the collision cell. In this region, the ions are accelerated and collide with molecules of an inert collision gas and undergo collision-induced dissociation. The fragments produced are transmitted to the final quadrupole (Q3), where they are again selected for transmission to the detector. The MS/MS can be operated to scan for all fragments produced from a single precursor (product ion scan), all precursors producing a single product (precursor ion scan), or to scan both filters of a fixed mass separately to select all precursors undergoing a loss of common noncharged moiety (neutral loss scan). Multiple reaction monitoring (MRM), also referred to as selective reaction monitoring (SRM), is used. In this mode Q1 is fixed to the precursor ion and Q3 to a characteristic fragment (Carpenter and Wiley 2002).

The biochemical diagnosis of FAOD relies on acylcarnitine profiling (ACP) using MS/MS. The defect in β-oxidation will lead to an increased accumulation of fatty acyl-CoA molecules of chain-specific length that cannot be further metabolised. These metabolites are not able to exit mitochondria, but esterifying to carnitine and formation of acylcarnitines allows transportation across compartments, making measurement possible.

All disorders of the carnitine cycle and β-oxidation can be detected on ACP (table 5). This analysis is also informative in some disorders of organic acid metabolism, which cause accumulation of acyl-CoAs (not given).

Carnitine and its esters are found in virtually all biological fluids. The preferred sample type for the testing of symptomatic patients and asymptomatic at risk individuals is plasma or serum (Rinaldo *et al.,* 2008). Testing from dried blood spots is used in NBS. ACP from urine can be used when there is a suspicion of organic aciduria. Post-mortem screening can be performed on dried-blood spots or bile (Chace *et al.,* 2001), and amniotic fluid can occasionally be used for prenatal diagnosis (Rinaldo *et al.,* 2001).

Table 5. The key metabolites detected during ACP by MS/MS. * Mass to charge ratio $[M+H]^+$ is given for molecules where the carnitine carboxyl group has been derivatised into butyl esters.

Disorder	Acylcarnitine	Designation	$[M+H]^{+*}$	Associated acylcarnitines
CPT1 deficiency	Free carnitine ↑	C0	218	C16, C18↓
SCADD	Butyrylcarnitine↑	C4	288	–
MADD	Butyrylcarnitine↑	C4	288	C5+ other longer chain species
SCHADD	3-hydroxybutyryl-carnitine↑	C4-OH	304	
MCADD	Octanoylcarnitine↑	C8	344	C6, C10, C10:1
VLCADD	Tetradecenoylcarnitine↑	C14:1	426	C14, C14:2
CPT2 deficiency	Palmitoylcarnitine↑	C16	456	C18:2, C18:1, C18
CACT deficiency	Palmitoylcarnitine↑	C16	456	C18:2, C18:1, C18
LCHADD	3-hydroxypalmitoyl-carnitine↑	C16-OH	472	C16:1-OH, C18:1-OH, C18-OH
MTP deficiency	3-hydroxypalmitoyl-carnitine↑	C16-OH	472	C16:1-OH, C18:1-OH, C18-OH

2.2.4. Confirmation of the diagnosis of FAOD

The diagnosis of FAOD relies largely on the above-mentioned biochemical investigations, which are informative for all disorders of the carnitine cycle and β-oxidation. If the biochemical changes are suggestive of the specific disorder, confirmation of the diagnosis at the enzymatic or molecular level is necessary (Derks *et al.,* 2008; Tyni and Pihko 1999).

2.2.4.1. Molecular analysis

Molecular studies in FAOD are performed using genomic DNA extracted from peripheral blood lymphocytes. In addition to the molecular confirmation of FAOD, genetic analysis makes it possible to establish genotype-phenotype relationships, which has implications for the prognosis and treatment options in some disorders (Sim *et al.,* 2002).

As all inherited FAOD are caused by mutations in different genes (table 3), the genetics of MCADD and LCHADD are discussed below, as these disorders are relevant from the viewpoint of this work.

2.2.4.1.1. LCHADD

LCHAD is one of the three enzymes of the MTP enzyme complex. The other enzymes of the same complex are long-chain 2,3-enoyl-CoA hydratase (LCEH) and long-chain 3-ketoacyl-CoA thiolase (LCKAT). MTP is a heterocomplex composed of four α-subunits that contain LCEH and LCHAD and four β-subunits that harbour LCKAT activity (Choi *et al.,* 2007; Tyni and Pihko 1999). LCEH activity resides in the N-terminal and LCHAD activity in the C-terminal part of the α-subunit. Therefore MTP deficiency is classified into two categories: the more common isolated LCHADD with the defects of α-subunits encoded by the *HADHA* gene and the less common pattern of complete MTP deficiency involving deficiencies of all three enzymes with defects of the α- and β-subunits encoded by the *HADHB* gene (table 3). Like all disorders affecting β-oxidation, MTP deficiency and LCHADD are inherited autosomal recessively.

The *HADHA* gene responsible for isolated LCHADD is located in the short arm of chromosome 2 (2p23.3). It contains 20 exons spanning over 52 kb (Sims *et al.,* 1995). Over 30 gene variations in the *HADHA* gene have been described so far (Gregersen and Olsen 2010), among them c.1528G>C (p.E510Q; NM_000182.4), which is by far the most common mutation, accounting for 87% all LCHAD alleles (Jlst *et al.,* 1994). This missense mutation results in the substitution of glutamate to glutamine at amino acid position 510. The base substitution creates a PstI restriction site (Jlst *et al.,* 1994). As this change is located in a catalytically active region, it deteriorates the dehydrogenase activity of α-subunit protein (Ijlst *et al.,* 1996; Sims *et al.,* 1995).

The carrier frequencies of this common mutation have been evaluated in a few populations, including some neighbours of Estonia (den Boer *et al.,* 2000; Ibdah *et al.,* 1999; Piekutowska-Abramczuk *et al.,* 2010; Zhu *et al.,* 2005; Tyni and Pihko 1999) (table 6).

Table 6. The carrier frequencies of mutation c.1528G>C in *HADHA* gene in different populations.

Population	No. of investigated individuals/ heterozygotes identified	Frequency of c.1528G>C	Reference
Finland	1200/5	1:240	Tyni and Pihko 1999
The Netherlands	2047/3	1:680	den Boer *et al.,* 2000
Poland	2161/10	1:216	Piekutowska-Abramczuk *et al.,* 2010
China (Han)	1200/0	ND	Zhu *et al.,* 2005
USA	351/2	1:175	Ibdah *et al.,* 1999
Estonia	**1040/6**	**1:173**	**Current study**

2.2.4.1.2. MCADD

The human *ACADM* gene encoding MCAD is located on chromosome 1p31 (Matsubara *et al.,* 1986) and spans 44 kb. It contains 12 exons encoding a mature protein of 396 amino acids (Zhang *et al.,* 1992). More than 30 mutations of the *ACADM* gene have been identified; most of these are missense mutations (Kennedy *et al.,* 2010).

The most prevalent mutation, c.985A>C (p.K304E), has been detected in 91% of mutant alleles in Japanese MCAD patients (Matsubara *et al.,* 1990). Andresen *et al* evaluated the genetics of MCADD in patients diagnosed prospectively by MS/MS based screening from a cohort of >900,000 newborns. In this cohort 80% of MCADD patients were homozygous for the common mutation, whereas a further 18% have this mutation in only one disease allele (Andresen *et al.,* 2001).

It has previously been shown that the carrier frequency for the common mutation in the *ACADM* gene in the Estonian population is 1:220 in a series of 1,098 newborns born during one month in Estonia (Lilleväli *et al.,* 2000).

2.2.4.2. Enzyme analysis

Assays of the enzyme activities of the FAO pathway may be performed in cultured skin fibroblasts, tissue biopsies or lymphocytes (Roe 1995; Wanders and Ijlst 1992). The determination of FAO enzyme activities usually involves measurement of enzyme activity with substrates of different chain lengths and activity ratio studies (Sim *et al.,* 2002). The specific measurement of acyl-CoA dehydrogenases (ACAD) requires: (a) a specific substrate, preferably reactive with only one particular ACAD (b) a specific detection system allowing the activity of each ACAD to be measured. The specific substrates used in β-oxidation defects are butyryl-CoA, 3-phenylproprionyl-CoA and palmitoyl-CoA in SCAD, MCAD and VLCAD respectively. The ACAD activities are usually measured spectrometrically – HPLC coupled to ultraviolet-defection or ultra-performance liquid chromatography coupled to MS/MS detection (Wanders *et al.,* 2010).

2.2.5. The clinical picture of FAOD

In general, FAOD have three different clinical presentations. The first is Reye-like syndrome associated with hypoketotic hypoglycaemia, which is triggered by a catabolic state and often manifests in association with intercurrent disease. This is the most severe clinical manifestation, lethal in a significant proportion of patients and presenting often in the neonatal period or in infancy. Patients may also present with cardiac symptoms such as dilatated or hypertrophic cardiomyopathy and cardiac arrhythmias. The milder form of the disease manifests in later life and is characterised by exercise-induced myopathy and rhabdomyolysis. This group of IEMs has also been associated with SIDS due to hypoglycaemia or cardiac disease (Roe 1995; Yamamoto *et al* 2010; Pryce *et al* 2011).

2.2.5.1. The clinical picture of LCHADD

In a series of 50 LCHADD patients, Den Boer *et al* (2002) observed that the majority (78%) of patients present in infancy with an acute metabolic derangement manifesting with hepatic dysfunction, coma and seizures or cardiac symptoms. Primary manifestation with SIDS was observed in 9% of patients. Twenty-two percent of patients presented with a more chronic disorder, consisting of liver disease, failure to thrive, feeding difficulties and hypotonia. The mortality in this series was high (38%).

Mental development in LCHADD patients is usually normal despite delayed motor development in infancy. Psychomotor retardation and epilepsy have only occasionally been found (Tyni and Pihko 1999).

All symptoms are reversible with sufficient energy supply. Disease-specific symptoms occur in some long-chain FAODs. Only in disorders of the TFP complex, including LCHADD, do progressive and irreversible neuropathy and retinopathy develop despite current treatment measures (Spiekerkoetter 2010a).

Peripheral neuropathy is an important clinical symptom of LCHADD and MTP deficiency and distinguishes these disorders from other FAOD (Tyni and Pihko 1999). Peripheral neuropathy has been described in 10% of LCHADD patients (den Boer *et al.,* 2002).

Ophthalmological findings are frequent features of LCHADD found in 63% of patients (Tyni *et al.,* 1998) and may be apparent from the age of 4 months. In the preliminary stages of eye disease, pale fundus is observed, which progresses to the retinal pigmentary chorioretinopathy. The changes in eye fundi have been associated with the accumulation of 3-hydroxylated compounds (Roomets *et al.,* 2008). In addition to above-mentioned symptoms affecting the peripheral nervous system and eyes, complications of pregnancy such as hemolysis, elevated liver enzymes, low platelets (HELLP) syndrome, acute fatty liver of pregnancy (AFLP) have been described in relationship to LCHADD in the fetus (den Boer *et al.,* 2000; Tyni and Pihko 1999).

In general, LCHADD and TFP deficiency could not be clinically distinguished in their acute clinical presentations and long-term complications. However, significantly lower survival has been observed in patients with TFP deficiency (Primassin *et al.,* 2010; Spiekerkoetter 2010b).

2.2.5.2. The clinical picture of MCADD

The clinical picture of MCADD is not complex compared to LCHADD. MCADD usually presents in childhood with an episode of vomiting and lethargy following a period of fasting. Often this episode is preceded by viral infection associated with decreased oral intake. Some patients have a history of previous similar episodes. On presentation to the hospital, a child may be comatose with hypoglycaemia and often there are low to moderate levels of ketones in urine. Intravenous infusion with 10% glucose yields a rapid improvement. The patients are usually asymptomatic between episodes (Roe 1995).

Adult presentations (age 16–45 years) with sudden clinical deterioration precipitated by fasting or alcohol consumption have been described (Lang 2009). MCADD deficiency in mother in relationship with AFLP have been described in one woman (Santos *et al.,* 2007).

Although episodes resolve rapidly with adequate treatment, the mortality of infantile patients is approximately 25% and is even higher in acutely presenting adult patients (50%) (Lang 2009).

2.2.6. The epidemiology of FAOD

The era of expanded NBS has significantly changed the understanding of the prevalence of FAO defects. According to the preliminary data from NBS from Australia, Germany and USA, the combined incidence of FAO defects is 1:9300 (Lindner *et al.,* 2010). NBS have demonstrated that disorders such as MCADD and VLCADD are more prevalent than previously suspected. Carnitine cycle defects are still extremely rare disorders, with CPT1 deficiency less than 50 known cases worldwide. The prevalence of CPT2 deficiency is slightly higher, with less than 400 published cases.

2.2.6.1. The epidemiology of LCHADD

Before expanded NBS, the estimated incidence of LCHADD was >1:250,000 (Chace *et al.,* 2003). The NBS screening data from Australia, Germany and USA indicate that in those countries LCHADD incidence remains low, but data from selective screening from Poland, where LCHADD prevalence is 118,336 (Piekutowska-Abramczuk *et al.,* 2010), make it possible to estimate higher incidence, at least in some populations. This observation is supported by the relatively high carrier frequencies observed in some populations (table 6).

2.2.6.2. The epidemiology of MCADD

The incidence of MCADD in the "pre-screening era" was estimated at about 1: 25,000 (Chace *et al.,* 2003). Expanded NBS has revealed that the overall frequency of this disorder generally ranges from 1:4900 to 1:17,000 and demonstrates significant variations among different populations. The incidence of MCADD based on available data from expanded NBS is given in table 7.

Table 7. The incidence of MCADD in different countries.

Population	Incidence/newborns	Reference
Northern Germany	1:4900	Sander *et al.,* 2001
Southern Germany	1:8500	Maier *et al.,* 2005
England	1:10,000	Oerton *et al.,* 2011
The Netherlands	1:6,600	Derks *et al.,* 2008
Ontario (USA)	1:14,000	Kennedy *et al.,*2010
Taiwan	1:700,000 live births	Niu *et al.,* 2010
Japan	1:51,000	Shigematsu *et al.,* 2002

2.2.7. The treatment of FAOD

The clinical symptoms of FAOD arise due to inadequacy of energy and the accumulation of metabolites secondary to enzymatic block. Therefore therapeutic approaches have two goals: to prevent the accumulation of potentially toxic metabolites such as acyl-CoA esters and to circumvent the metabolic block providing alternative energetic substrates.

In order to counteract the accumulation of toxic metabolites, dietary reduction of those metabolites (particularly long-chain fatty acids (LC-FA)) and the promotion of detoxification through carnitine supplementation are applied. Adequate energy consumption is ensured with high amounts of carbohydrates and by substituting LC-FA with medium-chain fatty acids (MC-FA). Although the restriction of dietary fat is no longer a mainstay in VLCADD, MCT seems to prevent episodic myopathic symptoms triggered by exercise. In LCHADD, however, the strict restriction of LC-FA is necessary to avoid the accumulation of toxic long-chain 3-hydroxyacylcarnitines and CoA esters (Spiekerkoetter *et al.,* 2009a; Spiekerkoetter *et al.,* 2009b).

Of course MC-FAs are not used in the treatment of MCADD – avoidance of fasting is the principal treatment measure for this defect. Carnitine supplementation is applied if free carnitine is low (Zschocke and Hoffmann 2011).

The avoidance of fasting in all FAOD is undoubtedly beneficial in terms of disease outcome, can be life-saving in many cases and is the main reason why early diagnosis of FAOD is vital. The preliminary data from expanded NBS, which includes FAOD, permit one to conclude that the outcome of FAOD and other metabolic diseases included in the screening panel in presymptomatic diagnosis is as good as in PKU (Lindner *et al.,* 2011).

2.3. Mitochondrial disorders

2.3.1. An introduction to MD

MDs are disorders of the enzymes or enzyme complexes directly involved in the generation of chemical energy through the oxidative phosphorylation system (OXPHOS). Mitochondriopathies arise due to mutations in genes of mitochondrial DNA (mtDNA) or nuclear DNA (nDNA) encoding for mitochondrial proteins with enzymatic, structural, signalling, channelling, transport, receptor or assembling functions (Finsterer 2004). A defect in OXPHOS can be clinically suspected in every patient with an unexplained combination of neuromuscular and/or non-neuromuscular symptoms with progressive disease course (Munnich and Rustin 2001). The clinical spectrum is extremely wide and has overlapping features. The single mutation in mtDNA or mutations in the same gene may present with different clinical manifestations, or the same phenotype may be caused by different mutations (Finsterer 2004). Mitochondrial dysfunction contributes to pathophysiological mechanisms underlying common diseases such as neurodegenerative diseases (Alzheimer's disease, amyotrophic

lateral sclerosis, Huntington's disease), cardiovascular disease, diabetes and cancer (DiMauro and Schon 2003; Koene 2011).

Mitochondrial pathology was first described in 1962 by Luft *el al* in a hypermetabolic patient (Luft *et al.,* 1962). The first patient was a 35-year-old woman who presented with profuse perspiration, polydipsia without polyuria and progressive asthenia despite polyphagia since the age of 7. Biochemical studies revealed loosely coupled state of the oxidative phosphorylation in the patient's mitochondria and electron microscopic study showed an increased amount of mitochondria with aberrant structure. An increased amount of total mitochondrial protein was also observed with increased cytochrome oxidase (COX) activity (Luft *et al.,* 1962).

In 1963 W. King Engel introduced a simple histochemical assay – a modification of a Gomori trichrome stain that allowed the detection of abnormal mitochondrial proliferation in muscle – ragged red fibres (RRF) (Dimauro 2011).

Although mtDNA has been known since 1963 (Nass and Nass 1963a; Nass and Nass 1963b), the sequence and organisation was described in 1981 by Anderson *et al* (1981). The first mtDNA mutations were described in patients with mitochondrial myopathy (Holt *et al.,* 1988) and Leber hereditary optic neuropathy (LHON) (Wallace *et al.,* 1988). In 1989 Zeviani described multiple mtDNA deletions in the muscular tissue of patients with progressive external ophthalmoplegia (PEO) and related these to faulty communication between nuclear and mitochondrial genomes (Zeviani *et al.,* 1989). Subsequently, in 1991 the quantitative defect of mtDNA, mtDNA depletion was described in patients with autosomal recessive diseases affecting the muscles or liver (Moraes *et al.,* 1991).

In 1992 the first nuclear mutation was described in two sisters with Leigh syndrome (Burgeois *et al.,* 1992). The first molecular causes for mtDNA depletion were identified in 2001. Saada *et al* identified mutations in the tymidine kinase-2 (*TK2*) gene in patients with mitochondrial depletion myopathy (Saada *et al.,* 2001) and the mutations in the deguanosine kinase (*DGUOK*) gene in depleted hepatocerebral mtDNA were first described by Mandel *et al* (Mandel *et al.,* 2001). To date > 100 point mutations in mtDNA are known (Finsterer 2004), and > 100 genes in nuclear DNA are known to affect energy production by OXPHOS.

2.3.2. The metabolism of MD

Mitochondria are the double-membrane organelles that are present in the cytosol of virtually all eukaryotic cells and play a crucial role in energy production. The mitochondrial inner membrane surrounds the mitochondrial matrix and is not smooth, but displays many protrusions. This serves to enhance the mitochondrial surface area, which is beneficial for the metabolic reactions occurring in the inner membrane and in the mitochondrial matrix (e.g. FAO,

pyruvate oxidation, tricarboxylic acid (TCA) cycle, UC, and heme biosynthesis) (Valsecchi *et al.,* 2010).

MD affects enzymes or enzyme complexes directly involved in the generation of chemical energy via oxidative phosphorylation. These include disorders of pyruvate metabolism, the TCA cycle, the mitochondrial respiratory chain (RC) and ATP synthase (Zschocke and Hoffmann 2011). A schematic overview of those pathways is offered in figure 2.

The central substrate for energy production is acetyl-CoA, which is produced by β-oxidation (figure 1) and pyruvate metabolism. Pyruvate is mostly formed via glycolysis in the cytosol and can be reversibly converted to lactate by lactate dehydrogenase (LDH) and to alanine by alanine aminotransferase (ALT). Pyruvate enters the mitochondrion and is subsequently decarboxylated into acetyl-CoA by the PDH complex. The PDH complex consists of 96 subunits organised into three functional proteins: pyruvate decarboxylase (E1), dihydrolipoyl transacetylase (E2) and dihydrolipoyl dehydrogenase (E3) (Robinson 1995).

The resulting acetyl-CoA enters the TCA cycle and is bound to oxaloacetate to form citrate. The cycle consists of nine enzymatic conversions (figure 2) and produces NADH and succinate for OXPHOS complexes I and II respectively. For each acetyl group entering the TCA cycle, three molecules of NADH are produced. At the end of each cycle, the four-carbon oxaloacetate has been regenerated and the cycle continues (Wallace *et al.,* 2010).

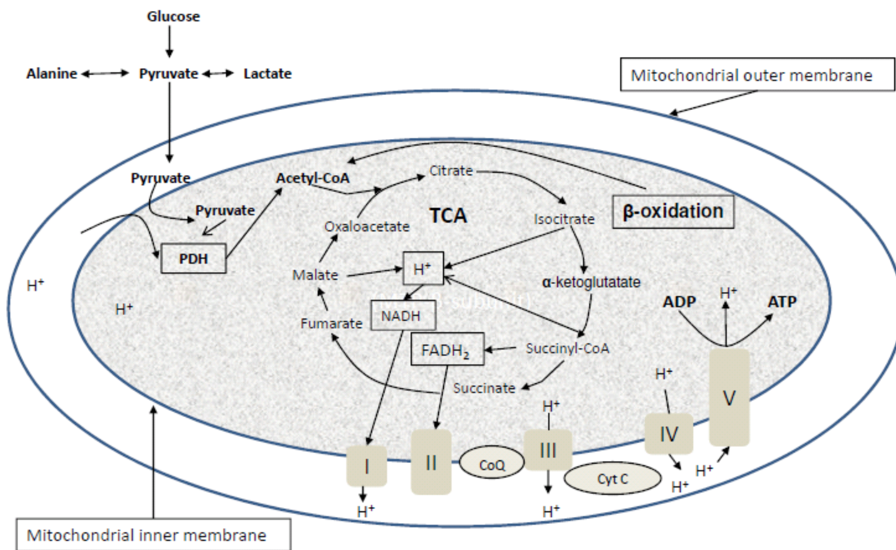


Figure 2. Principal pathways of the mitochondrial energy metabolism.

The RC is composed of five enzyme complexes that contain flavins (FMN, FAD), quinoid complexes (CoQ_{10}) and transition metal compounds (iron-sulfur clusters, hemes and protein-bound copper). These complexes are located in the mitochondrial inner membrane and are designated as complex I (NADH:ubiquinone oxidoreductase), complex II (succinate:ubiquinone oxidoreductase), complex III (ubiquinol:ferrocytochtome c oxidoreductase), complex IV (cytochrome c oxidase) and complex V (ATP synthase).

Complex I is the largest complex of the electron transport chain and has a major function in oxidative phosphorylation, as its main task is the electron transfer from NADH to ubiquinone, a lipid-soluble carrier of the inner mitochondrial membrane. The energy that originates from this process is used to move protons across the inner membrane, creating an inside negative membrane potential (Distelmaier *et al.,* 2009). Complex II catalyses the oxidation of succinate to fumarate, coupled to the reduction of ubiquinone (CoQ) to ubiquinol. The electrons extracted from fumarate are transferred to FADH, which passes them to the three Fe-S clusters and cytochrome b to CoQ. Complex II has no proton pumping activity (Koene 2011).

Complex III passes electrons from CoQ to cytochrome c and pumps protons across the mitochondrial inner membrane (Koene 2011). Complex IV is the final protein complex in the electron transport chain and donates electrons to oxygen, leading to the formation of water (Koene 2011). Finally, complex V, which is the final enzyme of the OXPHOS pathway, uses the energy stored in a proton gradient across the inner mitochondrial membrane to phosphorylate ADP to ATP (Jonckheere *et al.,* 2011).

In this sense, mitochondria are the key players in cell energy, redox balance and survival, but mitochondria are also crucial for apoptosis induction and Ca^{2+} homeostasis (Valsecchi *et al.,* 2010).

2.3.3. The genetics of MD

2.3.3.1. Mitochondrial DNA

MtDNA (figure 3) is a 16.6 kb circular double-stranded molecule, which consists of two strands differentiated by their nucleotide content. The guanine-rich strand is referred to as the heavy strand, and it encodes 28 genes. The cytosine-rich strand is referred to as the light strand, and it encodes 9 genes (Finsterer 2004). The genes encoded by mtDNA include: 2 mitochondrial ribosomal RNAs (rRNA) and 22 transfer RNAs (tRNA), 7 subunits of complex I, 1 cytochrome b (complex III) and 1 cytochrome c oxidase (complex IV) and 2 ATP synthase subunits (Koene 2011).

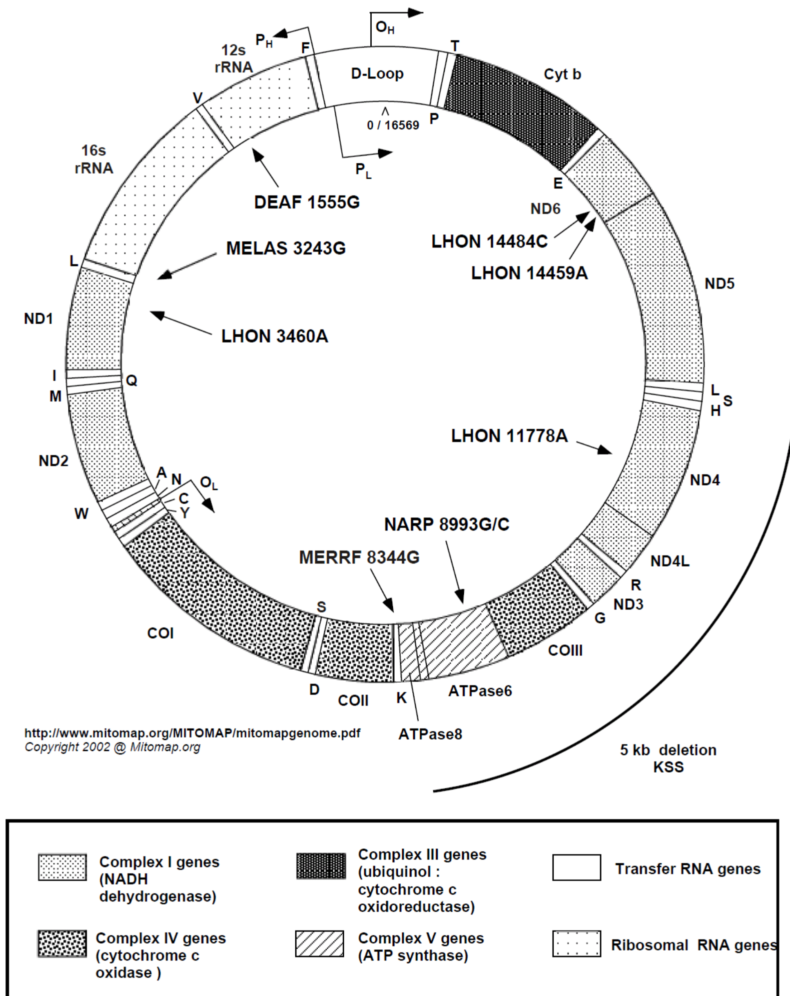


Figure 3. The structure of the mitochondrial genome. Adapted from MITOMAP (http://www.mitomap.org/MITOMAP/MitomapFigures). The location of genes responsible for OXPHOS function and more frequent mutations is shown. The D-loop region controls the initiation of the replication of mtDNA.

MtDNA is different from nDNA in many senses: it has no introns and 93% of it is responsible for coding; its structure is circular and it is not interwoven with histones. MtDNA has a mutation rate 10–20 times higher than nDNA and no effective repair system (Finsterer 2004).

Each cell contains thousands of mtDNA molecules and in general pathogenic mutations are not present in all of them. The situation in which both normal (wild-type) and mutant mtDNA is present in the cell and/or tissue is known as heteroplasmy. This phenomenon is known to exist at the organelle

level as well: a single mitochondrion can harbour both normal and mutant mtDNAs. Individuals with all identical mtDNAs are homoplasmic (DiMauro and Schon 2003).

The clinical symptoms of MD due to mtDNA mutations are dependent on the ratio between mutant and wild-type DNA – the threshold effect. The threshold for disease is lower in tissues that are highly dependent on oxidative metabolism, such as the brain, heart, skeletal muscle, retina, renal tubules, and endocrine glands (DiMauro and Schon 2003).

Generally all mitochondria and mtDNA are inherited from the mother, and therefore MD follow maternal inheritance. Since mitochondria from spermatozoid are labelled for destruction by ubiquination after fertilisation, mtDNA mutations from the mother are transmitted to all offspring (Koene 2011). Paternal inheritance of mitochondrial haplotype has been reported only once, in a family where the index patient had a deleterious mitochondrial mutation causing myopathy (Schwartz and Vissing 2002).

The clinical outcome of mitochondrial disease due to mtDNA mutation is mitotic segregation. This is the random redistribution of cell organelles during the cell division that can change the proportion of mutant mtDNA received by daughter cells (Figure 4). Age- and tissue-related variabilities arise from this (DiMauro and Schon 2003).

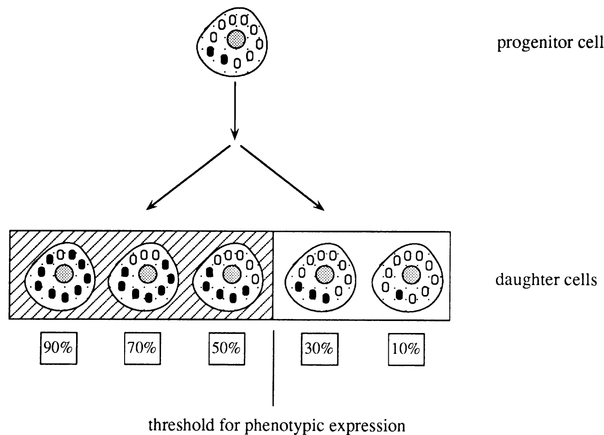


Figure 4. Mitotic segregation: the variable distribution of wild-type and mutated mtDNA (from Zeviani and Antozzi 1997).

2.3.3.2. Nuclear DNA

Nuclear-encoded factors are related to mitochondrial biogenesis, complex assembly, mtDNA replication, transcription and mitochondrial protein biosynthesis. It is estimated that about 1500 mitochondrial proteins are influenced by nuclear genes (Wong 2010). An overview of nuclear genes targeted to mitochondria is given in table 8.

Table 8. An overview of nuclear genes targeted to mitochondria, adapted from Wong 2010. *MRP – mitochondrial ribosomal proteins **ROS – reactive oxygen species.

Function	**No. of genes**	**Function**	**No. of genes**	**Function**	**No. of genes**	**Function**	**No. of genes**
Apoptosis	50	Metabolism	350	Proteases	25	Immune	2
Chaperone	20	Dynamics	10	Nucleases	5	Protein import	30
Cytochromes	15	Replication	10	RNA mod	5	ROS**	15
Cytoskeletal	10	Transcription	30	Translation	15	RNA modification and processing	10
DNA repair	12	Regulation	5	MRP*	91	Other	175
Fe-S cluster	3	Porins	4	tRNA synthase	22	Unknown	150
Protein folding and modification	12	RC complex and assembly	150	RNA modification and processing	10		

Nuclear genes associated with the development of MD are responsible for a) mtDNA maintenance; b) the encoding of OXPHOS complex subunits and the proteins involved in their assembly; c) biosynthesis of CoQ.

The defects in genes involved in mtDNA synthesis and mitochondrial dynamics lead to a reduction in cellular mtDNA content. To date 12 genes related to DNA repair are known (table 8), and majority of them (table 9) have been found to result in DNA depletion with tissue-specific involvement (Wong 2010). Although *TK2* and *DGUOK* were the first genes to be related to mtDNA depletion (Dimauro 2011), the mutations in the *POLG1* gene are the most common nuclear gene defects causing MD, including diseases arising from DNA depletion. *POLG1* encodes the catalytic subunit of the polymerase gamma, which is the only mtDNA polymerase and one of the three essential factors required for mtDNA replication (Suomalainen and Isohanni 2010).

There are over 100 nuclear genes coding for proteins involved in OXPHOS complex subunits and their assembly (table 8). Molecular defects of OXPHOS subunit genes or genes essential for complex assembly may be associated with isolated complex deficiency depending on the defective gene (Wong 2010). The most common of these disorders are defects in complex IV (cytochrome *c* oxidase) assembly factors caused by mutations in 8 genes (table 9). Complex I, the largest RC complex, is regulated by a number of genes (table 9). Mutations in these genes generally have a dramatic effect on neurodevelopment and overall patient survival (Distelmaier *et al.,* 2009). The *BCS* gene, located at 2q33, is the leading cause of isolated complex III deficiency (Wong 2010). Finally, complex V deficiency is often related to mutations in *TMEM70* (Jonckheere *et al.,* 2011).

Nuclear genes also affect the CoQ pathway, which is essential for RC function. Genes involved in CoQ biosynthesis (*PDSS1*, *PDSS2*, *COQ2*, *CABC1*, *COQ9)* cause primary CoQ deficiency, leading to deficiency of complexes I, II+III (Zschocke and Hoffmann 2011), clinically manifesting as mitochondrial encephalomyopathy (Wong 2010).

Additionally, there are genes that have an indirect influence on mitochondrial function. These are the genes involved in mitochondrial protein importation, such as *TIMM8A* encoding for the deafness-dystonia protein, a component of the mitochondrial-protein-import machinery in the intermembrane space. Mutations in *TIMM8A* are responsible for the deafness-dystonia syndrome (Mohr-Tranebjaerg syndrome), an X-linked recessive disorder characterised by progressive neurosensory deafness, dystonia, cortical blindness and psychiatric symptoms (DiMauro and Schon 2003). In addition, genes influencing fusion and fission dynamics, such as *OPA1* (located 3q29), which encodes mitochondrial dynamin-related guanosine triphosphatase, belong to this group. Mutations in this gene cause autosomal dominant optic atrophy, which causes blindness in young adults (DiMauro and Schon 2003). The indirect involvement of mitochondria has been related to many "common" conditions as normal aging, late-onset neurodegenerative diseases and cancer (DiMauro and Schon 2003).

The majority of nuclear-encoded MD are inherited autosomal recessively (table 9).

Table 9. The classification of nuclear genes causing MD according to the function and mode of inheritance.

Function/disorder		**AR inheritance**	**AD inheritance**	**X-linked inheritance**
mtDNA maintenance		*POLG, DGUOK, MPV17, TK2, SUCLA2, SUCLG1, RRM2B*	*C10ORF2*	
Isolated complex I deficiency	Subunit mutations	*NDUFS1-4, NDUFS6-8, NDUFV1-2, NDUFA2, NDUFA11*		*NDUFA1*
	Assembly factor mutations	*NDUFAF1-4, C20ORF7, CbORF38, FOXRED1, NUBpl,ACAD9*		
Isolated complex II deficiency	Subunit mutations	*SDHA*	*SDHB*	
	Assembly factor mutations	*SDHAF1*	*SDHAF2*	
Isolated complex III deficiency	Subunit mutations	*UQCRB, UQCRQ*		
	Assembly factor mutations	*BCS1, HCCS, TTC19*		

Function/disorder		AR inheritance	AD inheritance	X-linked inheritance
Isolated complex IV deficiency	Subunit mutations	*COX4I2, COX6B1*		
	Assembly factor mutations	*SURF1, COX10, COX15, SCO1, SCO2, LRPPRC, FASTKD2, TACO1*		
Isolated complex V deficiency	Subunit mutations	*ATP5E*		
	Assembly factor mutations	*ATPAF2, TMEM70*		
Mitochondrial translation		*PUS1,MRPS16,MRPS22,GFM1, TSFM, TUFM, DARS2, RARS2, SARS2, YARS2, TRMU, C12ORF65, MTPAP, SPG7, AFG3L2*		
CoQ biosynthesis		*COQ2, PDSS1, PDSS2, ADCK3, COQ9*		
Mitochondrial import		*SLC25A3, SLC25A12, SLC25A19, SLC25A38, DNAJC19,*		*TIMM8A*
Mitochondrial dynamics		*MFN2*	*OPA1, MFN2, DLP1*	*AIFM1*
Transporters across the mitochondrial membrane		*SLC25A22, SLC25A3, SLC25A20, SLC25A15, SLC25A13, SLC25A19*	*SLC25A4*	

2.3.4. The clinical picture of MD

Genetic disorders of the mitochondrial RC are a clinically heterogeneous group of disorders affecting the body's energy production. They may present at any age and affect any organ (Munnich and Rustin 2001). As cells with high-energy requirements such as neurons, skeletal and cardiac muscle are particularly vulnerable to limited ATP supply, encephalopathy and myopathy are often prominent features in the various mitochondrial phenotypes.

Mitochondrial energy production functions early in embryo-fetal development (Illsinger and Das 2010). Antenatal manifestations of RC disorders have been described infrequently, and they are probably related to the time course and tissue specificity of the underlying gene. The relationship of *TMEM70* mutations causing oligohydramnion in the third trimester of pregnancy was recently described (Spiegel *et al.,* 2010). Patients with PDH deficiency may present antenatally with brain anomalies including agenesis of the corpus callosum, cystic lesions of the brain and microcephaly (Illsinger and Das 2010).

OXPHOS disorders may manifest in neonates with a combination of nonspecific symptoms such as prematurity and intrauterine growth retardation (IUGR) with early postnatal decompensation, poor feeding, or vomiting and persisting lactic acidosis. Distinct clinical forms may also be observed: neonatal encephalopathy with seizures, intestinal dysmotility, liver disease and cardiomyopathy (Illsinger and Das 2010).

In the series of 129 patients with neonatal onset of MD Honzik *et al* (2010), muscular hypotonia was the most frequent clinical manifestation, presenting in 90% of patients. Thirty percent of patients needed ventilatory support due to muscular hypotonia. The other clinical presentations of MD in neonatal age were cardiomyopathy, seizures and LS, occurring in 40%, 16% and 15% of patients respectively. The neonatal origin of MD was associated with *TMEM70* mutations in 22 out of 129 patients. The other genes associated with the neonatal origin of the disease were: *PDHA1, SCO2, DLD, SCO1, POLG, TAZ, NDUFS4, ATP5E* and *SUCLG1*. Mutations in mtDNA were detected in 14 patients (Honzik *et al.,* 2012).

The most common childhood-onset mitochondrial disease is LS (Kisler *et al.,* 2010). LS or subacute necrotising encephalopathy is characterised by psychomotor retardation usually occurring between 3–12 months of age. Additional neurological, endocrine, renal or cardiac abnormalities may be present. LS has a specific finding on brain magnetic resonance imaging (MRI), which reveals symmetric hyperintensities on T2-weighted images in the basal ganglia, brainstem, thalamus, diencephalon and cerebellum (figure 9). LS is a progressive disease and has a poor prognosis (Koene 2011).

Myopathy is the most prominent symptom of MD in all ages. Patients may have normal or slightly elevated serum creatine kinase activity, and the results of electromyography are usually normal. Therefore it is reasonable to suspect a MD in patients with significant muscular hypotonia accompanied by normal electromyography and creatine kinase (Koenig 2008).

Seizures are a frequent symptom in MD. They may occur as a presenting symptom or may be part of a multisystem disease. The explosive onset of focal epilepsy, *epilepsia partialis continua* or status epilepticus, should arouse suspicion of Alpers syndrome, but otherwise mitochondrial epilepsies are genetically heterogeneous. The more common genetic causes for seizures associated with mitochondrial dysfunction are mtDNA and *POLG* mutations (Rahman 2012). Patients with MD are particularly sensitive to valproate-induced liver failure, and therefore MD should be considered in any child presenting with valproate-induced liver dysfunction (Koenig 2008).

Growth failure is present in about 20% of mitochondrial patients, and neurosensory hearing loss, progressive external ophtalmoplegia, axonal neuropathy, diabetes mellitus, and renal tubular acidosis are also important symptoms of RC dysfunction (Koenig 2008).

In some cases MD presents as clinical syndromes with the involvement of different organ systems. Syndromic presentation is more characteristic of mtDNA mutations, but sometimes points to certain nuclear genes. An overview of mitochondrial syndromes is offered in table 10.

Table 10. An overview of the mitochondrial clinical syndromes * DIDMOAD- *diabetes insipidus, diabetes mellitus*, optic atrophy and deafness.

Syndrome	Clinical feature	Age of onset	Genetic defect
LHON	Leber hereditary optic neuropathy, subacute painless visual loss	12–30 years	m.11778G>A (*MT-ND4*) m.3460G>A (*MT-ND1*) m.14484T>C (*MT-ND6*)
NARP	Neuropathy, ataxia, retinitis pigmentosa	5–30 years	m.8993T>G/C (*MT-ATP6*)
MELAS	Mitochondial encephalomyopathy, lactic acidosis, stroke-like episodes	5–15 years	m.3243A>G (*MT-TL1*)
KSS	Kearns-Sayre syndrome: PEO, pigmentary retinopathy, cardiac conduction block, cerebellar ataxia, CSF protein >0,1 g/l	<20 years	mtDNA deletions ± duplications
PEO	Bilateral ptosis, limited eye movement	20–30 years	Single mtDNA deletion; *POLG, POLG2, RRM2B, C10ORF2, SLC25A2*
MERFF	Myoclonic epilepsy with ragged red fibres	5–15 years	m.8344G>A (*MT-TK*)
MIDD	Maternally inherited diabetes and deafness	20–30 years	m.3243A>G (*MT-TL1*)
Alpers	Progressive neuronal degeneration of childhood (seizures+ liver involvement)	Early childhood	*POLG*, rarely *C10ORF2*
Barth	Cardiomyopathy, cyclical neutropenia, myopathy, short stature	Neonate/infancy	*TAZ*
DCMA	Dilatated cardiomyopathy with ataxia, neutropenia, myopathy	Neonate/infancy	*DNAJC19*
GRACILE	Growth retardation, aminoaciduria, cholestasis, iron overload, lactic acidosis, early death	Neonate/infancy	*BCS1L*
Wolfram	DIDMOAD*, bladder instability, ataxia, peripheral neuropathy, dementia, psychiatric disorders, seizures	3–40 years	*WFS1, WFS2*
MLASA	Myopathy, lactic acidosis, sideroplastic anaemia	Infancy-childhood	*PUS1, YARS2*
SANDO	Sensory ataxia, neuropathy, dysartria, ophtalmoplegia	20–30 years	*POLG*

In conclusion, due to extremely variable clinical presentation, oxidative phosphorylation defects are suspected in patients with a) an unexplained combination of neuromuscular symptoms and/or non-neuromuscular symptoms; b) a progressive disease course, and c) the involvement of seemingly unrelated organs or tissues (Munnich and Rustin 2001).

2.3.5. The diagnostic criteria of MD

Due to the extremely non-specific clinical picture, additional indications suggestive of MD including characteristic laboratory, metabolic and morphological abnormalities are helpful in diagnosis. Screening based on the measurement of lactate levels in serum and cerebrospinal fluid is used for first-line diagnostis, but lactic acidosis may be present in only 50% of patients (Gropman 2001; Koenig 2008). In patients with neurologic symptoms, cerebrospinal fluid lactate is considered to be more reliable than venous lactate (Koenig 2008). Elevated Krebs cycle intermediates observed in organic acid analysis and elevated alanine are the most widely observed metabolic changes. Lactate to pyruvate ratio is as important as each component individually, as a ratio greater than 20 is suggestive of a defect of oxydative phosporylation, whereas a ratio below 20 suggests a defect in the Krebs cycle (Gropman 2001). Suomalainen *et al* (2011) recently reported that the measurement of fibroblast growth factor-21 (FGF-21) concentrations in serum identified primary muscle-manifesting RC deficiencies in adults and children and might be feasible as a first-line diagnostic test for these disorders in order to reduce the need for muscle biopsy.

The most important instrumental investigations in patients with a suspected mitochondrial disease are brain MRI and magnet resonance spetroscopy (MRS). MRI is especially useful in children with nonspecific neurologic symptoms and subtle biochemical, biochemical and morphological abnormalities. Symmetric grey matter nuclei involvement may be the most predominant finding. In the brainstem, the periaqueductal grey matter, pons, and mesencephalon are the common sites of involvement. LS caused by necrosis and cavitations in the basal ganglia, midbrain, pons, and posterior column of the spinal cord is strongly suggestive of the presence of a defect in the energy-producing pathway. Putaminal involvement is reported to be a constant feature in LS. Other abnormalities where MD may be suspected are abnormal myelination and infarct-like lesions (Gropman 2001). The accumulation of lactate and N-acetyl-aspartate are the most common findings detected using MRS (Kisler *et al.,* 2010).

The diagnostic information for MD is often obtained on examinations performed on muscle tissue. Biopsy specimens should be examined with routine light microscopy for structural changes, evaluated histochemically or with an electron microscope. One pathomorphological hallmark observed from muscle tissue is abnormal mitochondrial proliferation, which is seen as RRF. These fibres also stain strongly for succinate dehydrogenase and negatively for COX, which is a sign of the accumulation of mitochondria in response to a defect in OXPHOS. Electron microscopic study reveals intramitochondrial paracristalline inclusions or disrupted cristae (Gropman 2001).

Biochemical analysis of RC enzymes can be performed from muscle biopsies, cultured skin fibroblasts and blood lymphocytes if necessary. Individual activities of the complexes I–V and the combined activities of the complexes I+III and II+III are measured (Finsterer 2004; Gropman 2001; Kisler *et al.,* 2010).

Different consensus diagnostic criteria for MD in childhood are available – for instance Wolfson (Nissenkorn *et al.,* 1999), modified Walker (Bernier *et al.,* 2002) and Nijmegen criteria (Morava *et al.,* 2006; Wolf and Smeitink 2002). These usually score major and minor criteria based on clinical, biochemical and molecular findings. The Wolfson criteria are based on a classic mitochondrial phenotype, which is considered to be the major criterion. For definite diagnosis of MD, at least one of the minor criteria should be present. These minor criteria consist of family history and characteristic metabolic, biochemical, genetic (mtDNA) or pathomorphological changes observed from muscle tissue (Nissenkorn *et al.,* 1999).

The modified Walker criteria involve the adaptation of the adult diagnostic criteria to children, allowing better sensitivity for the identification of MD in children (Bernier *et al.,* 2002). The major criteria in this scoring system consist of clinical phenotype (classic syndrome or unexplained newborn, infant death) and the histochemical, biochemical and genetic findings in muscle tissue at a certain level. Minor criteria consist of the same criteria, but at a lower level (for example – RRF <2% of fibres) and also metabolic abnormalities. Finally patients are classified as definite, probable and possible MD (Bernier *et al.,* 2002).

The Nijmegen criteria for MD (table 11) are more complex and involve detailed clinical symptoms, the results of instrumental investigations and metabolic findings. All general criteria are evaluated by points – each criterion gives 1–2 points.

Table 11. Nijmegen criteria for mitochondrial disease (Wolf and Smeitink 2002).

General criteria (GC)

1. Muscle presentation
   a. PEO
   b. Ptosis
   c. Excercise intolerance
   d. Muscle weakness
   e. Rhabdomyolysis
   f. Abnormal EMG
2. CNS+ other system involvement
   a. CNS alone
   b. Any other isolated organ system
   c. Two or more organ systems
3. Metabolic and Imaging studies
   a. Elevated blood lactate on 3 occasions
   b. Elevated CSF lactate
   c. Elevated blood alanine
   d. Elevated CSF alanine
   e. Elevated urine TCA cycle intermediates)
   f. Elevated urine EMA, 3-methylglutaconic or dicarboxylic acids
   g. Abnormal P-MRS in muscle with reduced phosphocreatine/P ratio
   h. Abnormal T2 signal in basal ganglia
   i. Elevated brain H-MRS lactate
4. Tissue morphology
   a. RRF
   b. Diffuse reduction of COX staining
   c. Strongly SDH-positive vessels
   d. Abnormal mitochondria by EM

Biochemical criteria

1. ^{14}C oxidation below lowest control
2. ATP+ Phosphocreatine synthesis below lowest control
3. Single RC Enzyme Deficiency

Evaluation: MD is unlikely / possible / probable / definite

2.3.6. The epidemiology of MD

The first epidemiological study evaluating mitochondrial abnormalities in children with unexplained psychomotor retardation was performed in Finland, where a biochemical defect of oxidative phosphorylation was found in one out of 5634 children (Uusimaa *et al.,* 2000). Subsequent epidemiological investigations have not succeeded in showing such high prevalence in Sweden (Darin *et al.,* 2001) and Australia (Skladal *et al.,* 2003). A comparison of epidemiological data is given in table 12.

Table 12. The prevalence of MD in childhood in different populations

Study population	Prevalence	95% confidence interval: per 100,000	References
Northern Finland Children <18 years	1:5634	Not calculated	Uusimaa et al 2000
Western Sweden Children <16 years	1:21,000	2.8–7.6	Darin *et al.,* 2001
Victoria, Australia Children <16 years	1:20,000	4.0–6.2	Skladal *et al.,* 2003
Estonia Life-birth prevalence	1:20,764	1.5–11.2	**Current study**

With the improving diagnostic possibilities and new phenotypes and genetic associations discovered, the number of MDs is rapidly growing, and it is estimated that the prevalence of MD in the general population is as high as one in 5000 (Elliott *et al.,* 2008; Schaefer *et al.,* 2004; Skladal *et al.,* 2003).

2.4. Urea cycle disorders

2.4.1. An overview of UCD

Ammonia (NH_4^+) arising from amino acid metabolism is mainly detoxified in the liver through its conversion to urea (Zschocke and Hoffmann 2011). UC (figure 5) comprises six different enzymes, three of which function in the mitochondrial matrix: N-acetylglutamate synthase (NAGS), carbamoylphosphate synthetase 1 (CPS 1), ornithine transcarbamoylase (OTC). The other enzymes are found in cytosol – argninosuccinate synthetase (ASS), argininosuccinate lyase (ASL) and arginase (Nassogne *et al.,* 2005). CPS1 catalyses the initial condensation of ammonia and bicarbonate. This requires activation by N-acetylglutamate, which is itself formed by NAGS. Subsequently, carbamoylphosphate is bound to ornithine by OTC. This results in the formation of citrulline, which is transported out of the mitochondrion and bound to aspartate by ASS. The resulting argininosuccinate is split by ASL into fumarate and

arginine, which is in turn hydrolysed by arginase into ornithine and urea. Urea is excreted and ornithine is transported back into the mitochondrion via the ornithine transporter (Zschocke and Hoffmann 2011).

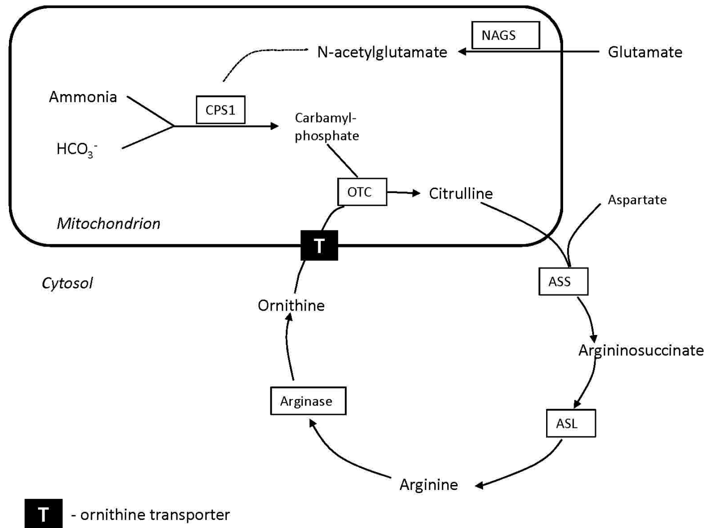


Figure 5. Urea cycle, adapted from *Vademecum Metabolicum (Zschocke and Hoffmann 2011).*

The list of UCDs is given in table 13.

Table 13. Overview of UCD, related genes and modes of inheritance.
*HHH syndrome– hyperammonaemia, hyperornithinaemia, homocitrullinaemia syndrome

Enzyme	Disease	Gene	Inheritance	Phenotype OMIM
CPS1	CPS1 deficiency	*CPS1*	AR	237300
NAGS	NAGS deficiency	*NAGS*	AR	237310
OTC	OTC deficiency	*OTC*	X-linked	311250
ASS	Citrullinaemia	*ASS*	AR	215700
ASL	Argininosuccinic aciduria	*ASL*	AR	207900
Arginase	Argininaemia	*ARG1*	AR	207800
Mitochondrial ornithine transporter	HHH syndrome*	*SLC25A15*	AR	603861

Clinically, UCD may present at any age with hyperammonaemia and its neurologic sequelae. The two most proximal defects (OTC and CPS1 deficiency) that present often with severe hyperammonaemia in the neonate have the highest risk for acute neurological injury (Gropman *et al.,* 2007). The developing brain is more susceptible than the adult brain to the deleterious effects of ammonia. Hyperammonaemia causes irreversible damage to the developing brain, causing cognitive impairment, seizures and cerebral palsy. Children develop cortical atrophy and demyelination of the grey and white matter. The extent of the irreversible damage depends on the maturation of the brain and on the magnitude and duration of hyperammonaemia. Irreversibility mainly occurs in the case of prolonged hyperammonaemic crises, when the blood level reaches levels between 200 and 500µM during the first two years of life (Braissant 2010).

The clinical picture of arginase deficiency is different. Those patients usually present with spastic diplegia or quadriplegia and there is rarely hyperammonaemia to the extent experienced in other UCDs. A similar clinical picture with evidence of pyramidal tract involvement and moderate hyperammonaemia is also observed in HHH syndrome (Gropman *et al.,* 2007).

The prevalence of UCDs as a group in the USA is estimated to be 1:8200 (Braissant 2010). Keskinen *et al* evaluated the incidence of this group of diseases in Finland (Keskinen *et al.,* 2008). In the period 1998–2007 the total incidence of UCDs in Finland was 1:39,000, with OTC deficiency being the most frequent of this group of disorders, having a frequency of 1:62,000. The incidence of UCDs in Estonia has not been systematically evaluated (Keskinen *et al.,* 2008).

2.4.2. Ornithine transcarbamylase deficiency

2.4.3. The clinical picture of OTC

The clinical manifestations in patients with CPS, OTC, ASS and ASL deficiencies are virtually identical, but demonstrate great variability within and among these diseases (Brusilow 2001). Diseases can manifest in the neonatal period and be fatal, or they may appear any time thereafter with varying degrees of severity. Hyperammonemia is the common clinical presentation of all of these diseases (Brusilow 2001). Clinically, children with OTC deficiency are almost always born from a normal full-term pregnancy and normal labour, and generally there are no known prenatal or perinatal risk factors. Some days after birth (often 24 to 72 hours, occasionally longer), the child becomes lethargic. Within hours additional symptoms may develop, including vomiting, increasing lethargy, hypothermia and hyperventilation. The clinical symptoms are often misinterpreted as pulmonary disease, notwithstanding radiological findings and respiratory alcalosis. A workup for sepsis in such patients is unrevealing. If the plasma ammonium level is not measured, the infant's death will be ascribed to sepsis, intracranial haemorrhage or some other disease (Brusilow 2001). If hyperammonaemia is detected, prompt metabolic investigations should follow. The biochemical hallmarks of OTC deficiency are elevated plasma ammonia,

glutamine and alanine, low levels of arginine and citrulline and increased excretion of urinary orotic acid (Bachmann 2003; Msall *et al.,* 1984).

Apart from acute neonatal presentation, a late-onset beginning has been described in up to 44% of patients (Nassogne *et al.,* 2005). This form of the disease can present from infancy up to the fourth decade (Brusilow 2001). The late-onset form has four different clinical presentations: hepatogastric with average onset at the age of 14 months; neurological disease in the form of chronic encephalopathy or relapsing neurological episodes (average age of onset 4 years); episodic psychiatric disease with average onset at the age of 8 years (Nassogne *et al.,* 2005).

Symptoms in carrier females are variable – some are totally asymptomatic, while approximately 15% of others have episodes of severe hyperammonemia, which can lead to brain damage or even death (Yorifuji *et al.,* 1998). More than 80% of heterozygous females are asymptomatic, while others present symptoms including vomiting, lethargy, coma, ataxia, cognitive disability and death due to hyperammonaemia. Protein aversion is common (Balasubramaniam *et al.,* 2009).

The principles for the treatment of OTC deficiency are strict protein restriction and arginine supplementation to overcome the enzymatic block. Hyperammonaemic episodes are treated aggressively with drugs, promoting the removal of ammonia (Na-benzoate, Na-phenylbutyrate) and hemodialysis if necessary (Zschocke and Hoffmann 2011).

Precise prognosis and neurologic outcome is not straightforward, as there is no direct correlation between genotype, age of onset, peak ammonia level, imaging and/or phenotype. Normal intelligence is possible after a hyperammonaemic event and appears to depend on the duration of the coma (Gropman *et al.,* 2007; Msall *et al.,* 1984).

2.4.4. The genetics of OTC

OTC deficiency is inherited as an X-linked semidominant trait. The *OTC* gene maps to the locus Xp21, spans 73 kb and contains 10 exons (Hata *et al.,* 1988). Nearly 350 different mutations, predominantly missense mutations and small deletions or insertions, have been reported. Mutations in the *OTC* gene are found in about 80% of patients. It has been hypothesised that the remaining 20% of cases may be caused by mutations in the promoter region or introns of the *OTC* gene and locus heterogeneity or copy number variants, such as microdeletions (Yamaguchi *et al.,* 2006). Schelochkov *et al* (2009) have shown that 15% of OTC deficiency cases have deletions identifiable by array comparative genomic hybridisation (aCGH) involving the *OTC* gene (Shchelochkov *et al.,* 2009).

Like the other urea cycle enzymes, *OTC* gene is expressed in the liver and gut (Brusilow 2001).

3. AIMS OF THE STUDY

The aims of this study were:

1. To introduce MS/MS method for acylcarnitine analysis into clinical practice in Estonia.
2. To evaluate the effectiveness of biochemical diagnosis of FAOD and to establish the prevalence of FAOD in Estonia.
3. To investigate the genotype and phenotype of Estonian LCHADD patients.
4. To evaluate the effectiveness of a diagnostic algorithm for mitochondrial diseases in Estonia.
5. To establish the live-birth prevalence of childhood-onset mitochondrial diseases in Estonia.
6. To characterise complexity of genetic mechanisms contributing to the manifestation of IEM in rare occasions.

4. MATERIAL AND METHODS

4.1. Study subjects

4.1.1. Patients with suspicion of FAOD

4.1.2.1. The study group of anonymous newborns

4.1.2.1.1. The study group of anonymous newborns tested for the presence of the c.1528G>C mutation

For the NBS of PKU and hypothyreosis in Estonia, six bloodspots were obtained from every newborn between 3–5 days of age. The test cards were sent to the Department of Genetics of Tartu University Hospitals for analysis and were subsequently stored there.

We obtained DNA from anonymous samples collected from a cohort of 1040 anonymous newborn samples. These neonates were consecutively born in Estonia in January 2005. We screened these samples for the presence of the c.1528G>C mutation in the *HADHA* gene. About 90% of newborns born during this month throughout Estonia were covered.

4.1.2.1.2. The study group of anonymous newborns tested for the presence of the c.1690–2A>G mutation

Genomic DNA isolated from 59 anonymised dried bloodspots collected from the general Danish population was used to determine the frequency of the *HADHA* c.1690 -2A>G mutant allele.

4.1.2.2. The study group of symptomatic patients

4.1.2.2.1. The study group of symptomatic patients with suspicion of FAOD in the period 2004–2007

In the years 2004–2007, the blood samples of 102 individuals were sent from various hospitals in Estonia to a molecular laboratory of the Department of Genetics of Tartu University Hospitals for the determination of both LCHAD and MCAD deficiency. All were molecularly tested for the common c.1528G>C *HADHA* mutation that causes LCHADD and for the common c.985A>C *ACADM* mutation that causes MCADD. In some cases, in addition to the molecular analysis (including heterozygotes for the c.1528G>C mutation found among the general population), ACP by tandem MS analysis was performed using dried blood spots collected on a Guthrie card at the Charité-Virchow Klinikum (Berlin, Germany).

4.1.2.2.2. The study group of symptomatic patients with clinical suspicion of FAOD in the period 2008–2011

Since 2008 496 patients with clinical symptoms of FAOD were first investigated by tandem MS analysis of acylcarnitines from plasma. The indication list for the study of plasma acylcarnitine profiles was developed and adapted from Duran (Duran 2003) – table 14.

Table 14. List of indications for plasma ACP (Duran 2003).

Clinical indications	Presymptomatic indications
Respiratory distress	Family history
Lethargy	SIDS in family
Coma	Pregnancy complications (AFLP, HELLP)
Recurrent vomiting	
Failure to thrive	
Apnoe	
Muscular hypotonia	
Bradycardia	**Laboratory abnormalities**
Ventricular arythmia	Acidosis
Cardiomyopathy	Ketosis
Hepatopathy	Hypoglycaemia
Encephalopathy	Elevated transaminases
Seizures	Elevated creatine kinase activity
Dystonia	Dicarboxylic aciduria
Myopathy	Hydroxydicarboxylic aciduria
Rhabdomyolysis	
Reye or Reye-like syndrome	
Renal tubular acidosis	

If a child had at least one feature from the indication list, plasma acylcarnitine analysis was performed. All investigated patients were selected from two regional hospitals (Tartu University Hospital and Tallinn Children's Hospital), which serve as reference centres for the whole of Estonia (population of about 1.34 million).

4.1.2. Patients with suspicion of MD

All patients referred for the investigation to the departments of neonatology or pediatric neurology of two regional hospitals (Tartu University Hospital and Tallinn Children's Hospital) were screened for the possible symptoms of MD. The study was performed from 2003 to 2009. The study protocol is schematically illustrated in Figure 5.

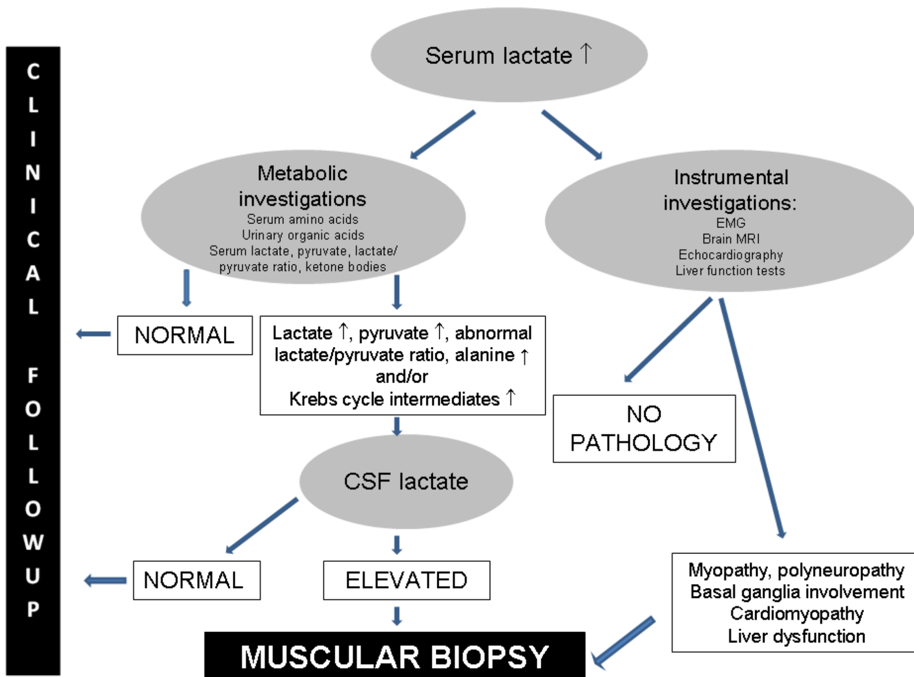


Figure 5. Diagnostic algorithm for patients referred to muscular biopsy due to suspicion of MD.

Serum lactate was routinely measured in patients presenting with symptoms suggestive to MD. If an increased concentration was detected, lactate level was checked repeatedly to avoid elevations due to technical factors. If the lactate level was constantly above the reference range, MD was suspected and metabolic and instrumental investigations were performed to establish the disease phenotype. If biochemical and instrumental investigations supported the diagnosis of MD, a muscular biopsy was performed.

Pathomorphological studies, including immunohistochemical and ultrastructural examinations, were performed on muscle biopsy using routine histological evaluation, immunohistochemistry (including cytochrome c oxidase activity, staining for nicotinamide adenine dinucleotide, ragged red fibres) and electron microscopy. If changes characteristic of MD were found, biochemical investigations were carried out on muscle tissue to evaluate the function of respiratory chain enzyme complexes. If the dysfunction of RC complexes was diagnosed in muscle tissue, their activity was also in some cases evaluated in skin fibroblasts. Finally, molecular studies were performed. The strategy for molecular investigations was based on the clinical presentation, pathomorphological findings and biochemical phenotype observed in muscle tissue, and the biochemical phenotype observed in skin fibroblasts.

4.1.3. Individual cases with rare metabolic diseases

Patients with unusual presentation of metabolic diseases referred to genetic consultation at Tartu University Hospital and Tallinn Children's Hospital were investigated and described in the framework of the current study.

4.2. Methods

4.2.1. MS/MS method for acylcarnitines

Acylcarnitines were analysed as butylesters. Sample preparation was performed using standard methods (Matern 2008b). 100 µl deuterated internal standards in methanol were added to 20 µl of plasma. 300 µl acetonitrile was used to sediment plasma proteins. Samples were centrifuged at 4°C for 3 minutes, and the supernatants were removed. After evaporation, the extracts were butylated using 60 µl butanol/HCl at 65°C for 15 min. The samples were dried again on a SpeedVac and finally dissolved in 100 µl of acetonitrile/H_2O/formic acid (80:20:0,025, respectively). 25 µl of the samples were injected into tandem MS (3200 Qtrap, Applied Biosystems MDS Sciex, Canada). Acylcarnitines were measured as a positive precursor ion scan for the 85 Da fragment. Quantification was performed using labeled carnitine standards set B from Cambridge Isotopes Inc (MA, USA).

4.2.2. Molecular analysis of *HADHA* gene

4.2.2.1. DNA extraction

Patients' DNA was extracted from peripheral blood using the standard salting out method or from newborn screening test cards. DNA was extracted from a 3 mm disc of the test card, which was soaked in 1 ml of distilled water for at least 2 hours, with constant vigorous swirling of the tubes. Then the supernatant was discarded, 100µl of methanol was added under a ventilation hood, and samples were incubated for 15 min at room temperature. In the next step, the methanol was discarded and 100µl of freshly-prepared 5mM NaOH and 20–50µl of mineral oil was added. Then samples were incubated at 100°C for 15 min and immediately placed on ice. The obtained DNA solution can be stored briefly at +4°C or at –20°C for years. This extraction method is cost-effective and also quite robust, as the DNA can easily be used for other PCR-based applications for at least 5 years (Laugesaar *et al.,* 2010; Teek *et al.,* 2010).

4.2.2.2. The detection of mutation in the *HADHA* gene

For c.1528G>C detection, a slight modification of the PCR-RFLP method and primers described by Den Boer *et al* (2002) were used. PCR was carried out in 96-well plates with a total volume of 10 µl. We used a higher Mg_2Cl concentration (2.5 mM) than that described in the article by den Boer *et al* (2002),

i.e. 1.5 μl of DNA solution and 0.65U HOT FIREPol® DNA Polymerase (Solis BioDyne, Estonia) per reaction. In addition, for test card DNAs the number of PCR cycles was increased from 30 to 35. The amplified 224 bp PCR products were directly digested in the 96-well plate after the addition of 1.2 μl of Buffer O and 8U of restriction enzyme *Pst*I (Fermentas; Lithuania). The restriction fragments of 175 bp and 49 bp in wild-type alleles and 117 bp, 58 bp and 49 bp in mutant alleles were analysed on a 2% (wt/vol) agarose gel with ethidium bromide staining.

a) HADHA gene sequencing

The 20 exons that make up the *HADHA* gene and nearby intronic elements were sequenced as described by Olpin *et al* (2005).

b) Detection of the HADHA c.1690 -2A>G mutant allele

For detection of the *HADHA* c.1690 -2A>G mutant allele, the sequence analysis of a 355 bp PCR fragment covering exon 17 and nearby intronic elements was used.

4.2.3. Methods used in diagnostic algorithm for MD

Our diagnostic algorithm of MD comprised of different investigation methods: serum and CSF lactate concentration was measured by photometric enzymatic method, amino acids in bodily fluids by HPLC and organic acids by GC/MS. Routine instrumental investigation methods (EMG, MRI, MRS) were used. Also pathomorphological studies from muscle tissue and biochemical studies from muscle tissue and fibroblasts were performed using routine methods used in clinical practice. As the author of the dissertation has not participated in those investigations, these methods are not described.

4.2.4. Establishment of the prevalence of FAOD in Estonia

The estimated prevalence of FAOD is calculated on the basis of the heterozygote frequency using the Hardy-Weinberg equilibrium and taking into account the genotyping data of Estonian patients.

The live-birth prevalence of FAOD was defined as the total number of cases with an FAOD born from 2007 to 2011, divided by the total number of live births in the same period. Annual live-birth data was obtained from the Statistical Database of Statistics Estonia, an agency of the Ministry of Finance of Estonia (www.stat.ee).

4.2.5. Establishment of the live-birth prevalence of MD presenting in childhood in Estonia

The live-birth prevalence of MD was defined as the total number of cases with a mitochondrial defect born from 2003 to 2009, divided by the total number of live births in the same period. Annual live-birth data was obtained from the

Statistical Database of Statistics Estonia, an agency of the Ministry of Finance of Estonia (www.stat.ee).

4.2.6. Ethics

This study was approved by the Research Ethics Committee of the University of Tartu.

The evaluation of the mutation c.1690–2A>C in *HADHA* gene from DNA isolated from anonymised dried bloodspots collected from the general Danish population study was approved by the Central Denmark Regional Committee on Biomedical Research Ethics.

5. RESULTS AND DISCUSSION

5.1. Fatty acid oxidation defects (Publication I)

5.1.1. Results of the investigation of newborn children

The c.1528G>C mutation is the most common LCHADD mutation in other European countries (Finland, Poland, The Netherlands) (den Boer *et al.,* 2000; Piekutowska-Abramczuk *et al.,* 2010; Tyni and Pihko 1999). Therefore we decided to study the frequency of the c.1528G>C *HADHA* mutation in the general Estonian population in order to establish a carrier frequency of the common LCHADD mutation and to estimate disease prevalence in Estonia.

For this purpose we tested 1040 anonymous Estonian neonates for the presence of the above-mentioned mutation. Six heterozygotes for the c.1528G>C mutation, and no c.1528G>C homozygotes, were found. To exclude the possible LCHADD in the patients due to a different mutation on the other allele, the acylcarnitine profiling of all heterozygous individuals from a newborn Guthrie card was performed at the Charité-Virchow Klinikum (Berlin, Germany). None of the c.1528G>C heterozygotes had biochemical abnormalities suggestive of LCHADD. Our results showed that the frequency of heterozygotes for the c.1528G>C mutation in Estonia is high – 1:173. This is similar to the frequency observed in Finland and Poland (Piekutowska-Abramczuk *et al.,* 2010; Tyni and Pihko 1999). The comparison is given in Table 6.

Based on this data, the theoretical prevalence of c.1528G>C homozygotes calculated using the Hardy-Weinberg Equilibrium in Estonia is 1:119,700.

5.1.2. Results of selective screening for FAOD

5.1.2.1. The study group of symptomatic patients with suspicion of FAOD in the period 2004–2007

During the period 2004–2007, biochemical investigations of FAO defects were not available in Estonia; therefore mutation screening for the common mutation in the *HADHA* gene was used for LCHADD diagnostics.

During this period, 102 individuals were molecularly tested for the c.1528G>C *HADHA* mutation. One patient was found to be homozygous and one heterozygous for the c.1528G>C mutation (Table 15; case 3). The homozygous child presented at the age of 6 months with fatal hypoglycemia during intercurrent illness, and FAOD was suspected due to the characteristic clinical picture. The heterozygous individual was referred due to the complicated family history, as two of his children had died at an early age (Table 15; cases 1 and 2).

In retrospective molecular study we were able to specify the diagnosis in one family many years after the death of the sick children. Nevertheless, mutational analysis has some shortcomings for usage in the selective screening of LCHADD, as it is relatively time-consuming and expensive, and is not particularly informative. Therefore its clinical applications are limited and it is

generally used in case of a high suspicion of LCHADD, for instance in patients presenting with hypoglycemia during an intercurrent illness, Reye or Reye-like syndrome, SIDS or near-SIDS syndrome and complicated family history (SIDS in siblings or maternal HELLP syndrome).

5.1.2.2. The study group of symptomatic patients with clinical suspicion of FAOD in the period 2008–2011

During the period 2008–2011, a group of 496 patients were analysed by ACP according to the indication list given in table 14. Acylcarnitines were analysed from plasma in the majority of patients (426); in 70 patients ACP was performed from a dried blood spot. Generally, heparinised plasma is the preferred material for the diagnosis of FAOD in symptomatic patients (Matern 2008a), and a dried blood spot is used in NBS. It is also recommended that the positive cases detected from the dried blood spot sample are confirmed from the plasma sample (Zschocke and Hoffmann 2011).

Out of this group, we identified two patients (table 15; cases 4 and 5) who had an abnormal acylcarnitine profile typical of LCHADD. The reasons for the referral of these patients were: recurrent vomiting, lethargy, hypoglycemia, liver dysfunction (table 15; case 4) and lethargy, failure to thrive, muscular hypotonia and slight liver dysfunction (table 15; case 5). Molecular analysis showed that both patients were homozygous for the c.1528G>C mutation in the *HADHA* gene.

Additionally, we identified one case of MCADD in a patient with borderline hypoglycemia, which was easily corrected by the administration of a 40% oral glucose solution.

The two LCHADD patients are the first living patients in Estonia who are also currently undergoing treatment.

5.1.3. Patients with LCHADD

5.1.3.1. The clinical picture of LCHADD patients

The clinical presentation of patients is shown in table 15.

Table 15. The clinical presentation of LCHAD patients identified during the study.

	Case 1	**Case 2**	**Case 3**	**Case 4**	**Case 5**
Born (year)	1987	1995	2007	2009	2010
Age at first presentation	3.5 months	9 months	6 months	12 months	5 months
Muscular hypotonia	ND	+*	ND	+ (observed in infancy)	+ (since newborn period)
Liver disease	ND	+*	ND	+	+
Cardiomyopathy	+	+*	ND	–	+
Hypoglycemia	ND	+**	+	+	–
Lactic acidosis	ND	+**	ND	+	+
Family history	–	SIDS in sibling (case 1)	HELLP syndrome during the pregnancy; antenatal death +HELLP syndrome during previous pregnancy	–	–
Outcome	Died at the age of 3.5 months	Died at the age of 2 years 7 months	Died at the age of 6 months	Alive	Alive
Genotype	c.1528G>C/ c.1690–2A>G***	c.1528G>C/ c.1690–2A>G***	c.1528G>C/ c.1528G>C	c.1528G>C/ c.1528G>C	c.1528G>C/ c.1528G>C

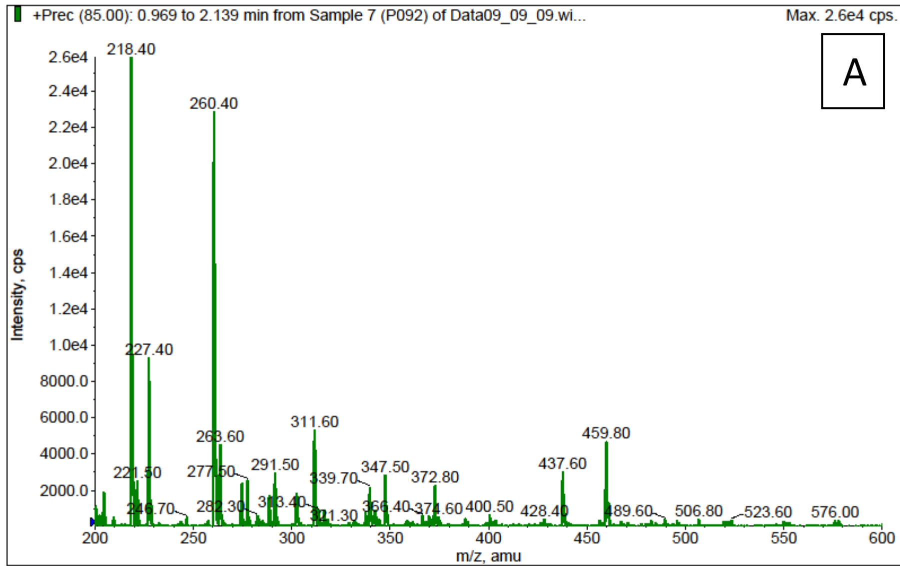
ND- not detected/observed
* diagnosed during a disease episode at the age of 14 months
** observed during the fatal episode at the age of 2 years 7 months
*** based on the results of the *HADHA* gene analysis performed on both parents

The clinical presentations of our patients represent the severe LCHADD phenotype that is characteristic of patients who are homozygous for the c.1528G>C mutation (Olpin *et al.,* 2005; Tyni *et al.,* 1997). The medium age at first presentation was 7.1 months (3.5–14 months). At least three patients had

hypoglycemia in initial presentation, and in at least two cases hypoglycemic episodes prior to diagnosis were fatal (cases 2 and 3). Retrospective analysis of the disease histories of those two cases revealed that both patients had symptoms indicating LCHADD prior to the fatal episode of the disease. These children might not have died if early specific diagnostic possibilities and treatments had been available. The family history was complicated in families 1 and 2 (Case 1 died at the age of 3.5 months due to cardiomyopathy; Case 3 had antenatal death and HELLP syndrome in the family history). Therefore we suggest that a complicated family history is an important symptom of possible LCHADD, and this disease should be excluded in all patients born to such families. We observed muscular hypotonia causing a delay in infantile motor development in two patients (cases 4 and 5). Therefore we assume that this is a very important clinical sign of LCHADD, and acylcarnitine profiling should be considered in every such patient in order to prevent life-threatening metabolic crisis.

During our retrospective molecular study we were able to specify the diagnosis in one family many years after the deaths of the sick children. As no material from cases 1 and 2 were available for genetic studies, we performed molecular investigations of the *HADHA* gene in the DNA of both parents. We detected the c.1528G>C mutation in the father and a novel c.1690–2A>G mutation in the mother, which is expected to cause abnormal *HADHA* splicing. We assume that the *HADHA* genotype c.[1528G>C]+[1690–2A>G] is responsible for the death of the two siblings in this family. Establishing the exact molecular genotype in this family enabled us to offer genetic consultation for the other two siblings, who were both heterozygous for the c.1528G>A mutation.

Specific biochemical investigations were only performed in cases 4 and 5. Urinary organic acid GC/MS analysis revealed markedly increased excretion of 3-hydroxy dicarboxylic acids (3-OH adipic, 3-OH suberic, 3-OH sebacic, 3-OH dodecanedioic, 3-OH tetradecanedioic acid), with some dicarboxylic aciduria in both patients. Plasma acylcarnitine analysis revealed elevated C14–18 and their hydroxy-compounds characteristic of LCHADD (figure 6).



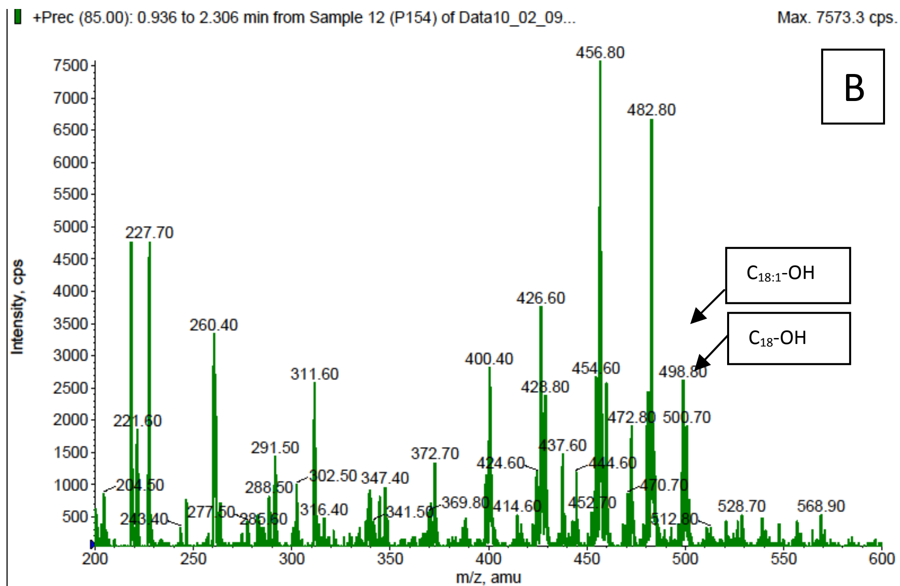


Figure 6. The normal acylcarnitine profile from plasma (A) in comparison with that of the LCHADD patient (B). Diagnostic peaks of C_{18}-OH and $C_{18:1}$-OH are marked. C_{16}-OH is seen at 472 and C_{14}-OH at 444 m/z.

Although the presence of 3-hydroxy-dicarboxylic acids with a chain length of 10 to 14 carbon atoms in urine by GC/MS have been demonstrated in several patients (Choi *et al.,* 2007; Hintz *et al.,* 2002), urinary organic acid analysis is not completely reliable in the diagnosis of FAOD, as abnormal results can only be obtained during acute symptoms (Olpin *et al.,* 2005; Roe and Coates 1995; Tyni and Pihko 1999).

Acylcarnitine profiling by tandem MS is considered to be the "cornerstone" in the diagnosis of FAO defects, as it is a very sensitive test for β-oxidation defects, and also gives information about the type of defect (Tyni and Pihko 1999). This method has been available in Estonia since 2008. In the last 3 years, two patients (cases 4 and 5) were identified using tandem MS analysis of plasma acylcarnitines by elevated 3-hydroxyacylcarnitines. This profile has been demonstrated in LCHADD patients (Choi *et al.,* 2007), (Hintz *et al.,* 2002), but has been observed in patients with MTP and isolated LCKAT deficiency (Sander *et al.,* 2005). Therefore the confirmation of enzymatic or molecular analysis is necessary after a characteristic profile is detected (Hintz *et al.,* 2002; Tyni and Pihko 1999).

Early identification and treatment of FAOD have the potential to improve outcome and may be lifesaving in some cases, as 5% of SIDS incidents are attributable to undiagnosed FAOD (den Boer *et al.,* 2002; Hintz *et al.,* 2002). This observation is also true in our small cohort of LCHADD patients – two patients (cases 4 and 5) identified by acylcarnitine analysis are the first living patients in Estonia who are also currently undergoing treatment. Although mutational screening was available a few years earlier, the introduction of tandem MS-based acylcarnitine analysis allowed much broader recruitment of patients with symptoms suggestive of FAO disorders. As plasma acylcarnitine profiling is an informative, relatively inexpensive and rapid method that enables simultaneous screening for many different FAOD, the clinical indications for its application are much broader than the mutational analysis of the *HADHA* gene. According to our experience, the indications given by Duran (2003) are very practical and suitable for the selective screening of FAOD. Our experience shows that muscular hypotonia causing a delay in infantile motor development is a very important clinical sign of LCHADD, and acylcarnitine profiling should be considered in every such patient in order to prevent a life-threatening metabolic crisis. Our main goal for the future is to introduce acylcarnitine analysis to all newborns as a part of our national newborn screening program.

5.1.3.2. The genotype of LCHADD patients

In our relatively small patient group, we identified eight independent LCHADD alleles from four families. Seven of them (87.5 %) carried the c.1528G>C mutation, and in one allele the c.1690–2A>G mutation was found after *HADHA* gene sequencing. In conclusion, the common c.1528G>C mutation is present on 87.5% of disease alleles in Estonian LCHADD patients. This corresponds to the previous observation by Ijlst *et al*, who detected the common mutation in 87%

of the *HADHA* gene in the cohort of 34 patients (Ijlst *et al.,* 1996). The c.1528G>C mutation is the main cause of LCHADD in Finland, as all patients in the cohort of 28 patients were homozygous for this mutation (Tyni and Pihko 1999).

The c.1690–2A>G mutation in the *HADHA* gene is a novel mutation. It most likely causes abnormal *HADHA* splicing, because according to the splice site prediction program (http://www.fruitfly.org/seq_tools/splice.html), the c.1690 –2A>G mutation changes the splice score of the IVS16 3' splice site from 0.97 to 0.0 (the maximum splice score is 1.0). Unfortunately the *in vivo* consequences of the splice site mutation could not be investigated, as no cDNA was available from the mother and the two deceased children. The c.1690 –2A>G mutation was not found in the 59 DNA samples collected from the general population (study group 4.1.2.1.2).

5.1.4. The prevalence of FAOD

5.1.4.1. LCHADD

Based on heterozygote frequency and taking into account that this common mutation is present on 87.5% of disease alleles in our small cohort of patients, we calculated that the prevalence of LCHADD in Estonia is 1:91,670. LCHADD frequency in Estonia is comparable to that in Poland, where its prevalence is 1:118,336 (Piekutowska-Abramczuk *et al.,* 2010), and higher than the 1:250,000 observed in Australia, Germany and the USA (Lindner *et al.,* 2010). In addition, the data about carrier frequency allow one to assume that LCHADD frequencies in Estonia and Finland are similar.

However, three out of five known patients were born in the period 2007–2010, when the patient's recruitment was wider. Therefore, we found it justified to calculate the live-birth prevalence for the last period 2007–2011. As there were 78,891 births in this period, the prevalence of LCHADD from 2007–2011 was 1 in 26,297. As the annual birth rate in Estonia in recent years is about 16,000, then according to live-birth prevalence, we can expect one LCHADD patient every 2–6 years.

5.1.4.2. MCADD

Using ACP, we evaluated the prevalence of MCADD in Estonia among patients in the study group with clinical symptoms suggestive of FAOD (study group 4.1.2.2.2). We identified only one patient with the characteristic profile of MCADD (figure 7).

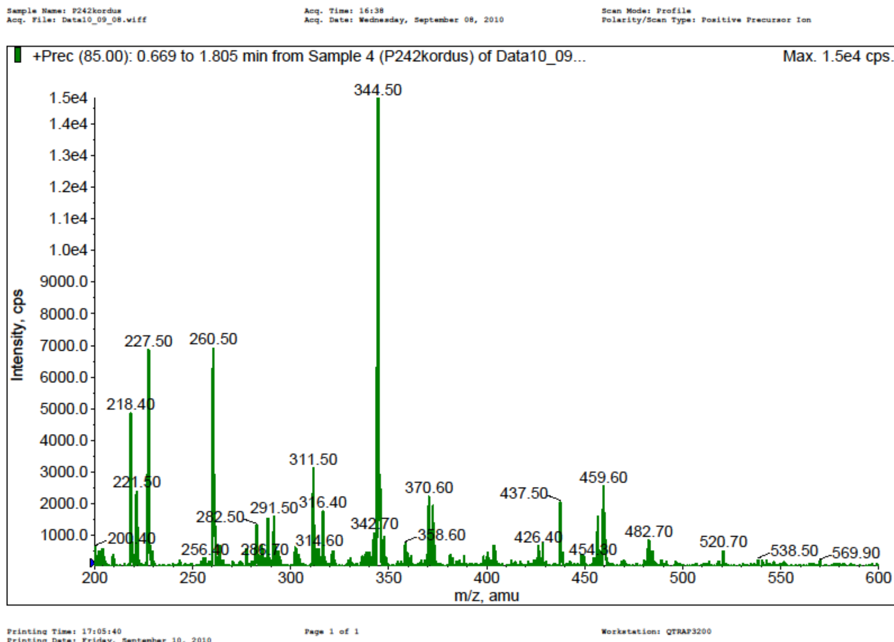


Figure 7. Acylcarnitine profile characteristic of MCADD. The prominent peak at 344 m/z corresponds to octanoylcarnitine (C8).

This patient presented at the age of 2 years and 11 months due to lethargy after fasting for 11 hours. The child had experienced a similar episode of lethargy 6 months before, which resolved spontaneously. Upon hospitalisation, muscular hypotonia, tremors, subfebrile temperature and pharyngitis were observed. As the laboratory analyses revealed hypoglycaemia (1.6 mmol/l), the patient was included in our study group. The hypoglycaemia resolved with oral administration of a 40% glucose solution. Due to the acylcarnitine profile characteristic of MCADD, molecular analysis of the *ACADM* gene was performed. The patient was homozygous for the c.985A>C mutation in the *ACADM* gene.

As the only MCAD patient identified during this study was living in Russia and decompensation occurred while visiting relatives in Estonia, this does not influence the prevalence of MCADD among Estonian patients. Lilleväli *et al* have previously shown that the carrier frequency of the c.985A>C mutation in the *ACADM* gene in Estonia is 1:220, and the frequency of possibly affected homozygotes is 1 in 193,000 (Lilleväli *et al.,* 2000). As during the selective screening of FAOD no patients of MCADD were identified, our data support the genetic data and imply that MCADD is uncommon compared to LCHADD in Estonia. MCADD may, however, still be underdiagnosed, as patients may present with very mild clinical symptoms, and this was also the case with our patient. Therefore it is also necessary to evaluate the incidence of MCADD in Estonia in NBS.

Although MCADD is the most frequent FAOD in several populations (table 7) and a remarkable north-south trend is evident in the Netherlands (Derks *et al.,* 2005) and in Germany (Maier *et al.,* 2005; Sander *et al.,* 2001), with higher frequency in the north, our data are not consistent with these observations. Controversially, a low carrier frequency for the common mutation of the *ACADM* gene has been reported in Finland. In the cohort of random DNA samples from 1908 subjects, a carrier frequency of 1:191 was reported, and the calculated frequency for c.985A>C homozygotes was 1:147,000 (Romppanen *et al.,* 1998). Therefore we can conclude that the prevalence of MCADD in Estonia is lower than in countries of Central and Western Europe, but similar to that of Finland.

5.1.4.3. Other FAOD

Although ACP is informative in diagnosing several disorders of FAO, no other FAOD or carnitine metabolism defects were detected during our study. This is probably related to the small population of Estonia, the limited duration of the study and the rare prevalence of the other disorders. The known prevalence of the other enzymatic defects affecting FAO is given in table 16. The combined incidence of the disorders that can be diagnosed using ACP is between 1:5000 and 1:10,000 (Rinaldo *et al.,* 2008).

Table 16. The prevalence of enzymatic defects of FAO and carnitine metabolism.

Disorder	Incidence	Population/Region	Reference
Carnitine uptake defect	67,000 (CI 31,600–512,000)	Japan	Lee *et al.,* 2010
CPT1 deficiency	>1:250,000	United States	Chace *et al.,* 2003
CPT2 deficiency	>1:250,000	United States	Chace *et al.,* 2003
VLCAD deficiency	1:177,000	California	Feuchtbaum *et al.,* 2006
SCAD deficiency	1:20,000	California	Feuchtbaum *et al.,* 2006
MAD deficiency	1:177,000	California	Feuchtbaum *et al.,* 2006

5.1.5. Follow-up of LCHADD patients

Due to the lack of systematic studies, current treatment recommendations are based on expert opinion (Spiekerkoetter *et al.,* 2010). The management guide-lines of LCHADD patients in Estonia were adapted from the Physician's Guide to the Treatment and Follow-up of Metabolic Diseases (Ogier de Baulny and Superti-Furga 2006).

The restriction of dietary LC-FA is necessary in order to avoid long-term complications as peripheral neuropathy and retinopathy due to the accumulation of toxic acylcarnitines (Gillingham *et al.,* 2003; Spiekerkoetter *et al.,* 2010). Two patients identified during this study (case 4 and 5) started a low-fat high carbohydrate diet promptly after the diagnosis. Eighty percent of their energy intake consisted of carbohydrates, and fat, mostly given as medium chain triglycerides, provided 20% of their energy. It has previously been shown that the strict LC-FA restriction (<10%) of caloric intake is associated with near normal plasma long-chain hydroxyacylcarnitines (Gillingham *et al.,* 2003). Slight elevation of long-chain hydroxycarnitines was still observed during follow-up, probably related with the not very strict LC-FA restriction allowed by our guidelines.

There is much ongoing debate regarding carnitine supplementation, with little data supporting either its beneficial or harmful effects (Gillingham *et al.,* 2003). About half of the patients treated in different European centres receive L-carnitine supplementation (Spiekerkoetter *et al.,* 2009a), but carnitine should be avoided at a time of severe metabolic derangement (Spiekerkoetter *et al.,* 2009b). No deleterious effects have been recognized while using L-carnitine at a median dose of 75 mg/kg daily (Ogier de Baulny and Superti-Furga 2006). As both patients had significantly reduced free carnitine at diagnosis, we chose to start L-carnitine supplementation at the dosage of 50 mg/kg/day at the beginning of treatment, under clinical and metabolic supervision. Low free carnitine levels have been observed during follow-up in both of our patients and oral L-carnitine supplementation was administered.

Clinical improvement was observed shortly after treatment was started. The first signs of clinical improvement were increased physical activity and improved muscle tone. Patient 5, who had cardiomyopathy at the moment of diagnosis, had no signs of cardiac involvement detected through ultrasound at the repeat check performed two months later.

Emergency regimens for acute illness were made for both of the patients. These regimens consisted of the guidelines for home treatment, where a 25% oral glucose solution is provided during the acute illness in order to avoid metabolic decompensation. Additionally, emergency regimens provide guidelines for hospitalisation and hospital treatment, where a glucose infusion of 10–12 mg/kg/min is necessary for the maintenance of normoglycaemia, and hypoglycemia is corrected with a glucose infusion dosage of 0.5–1 g/kg/dosi. Patient 4 has been followed up for about two years and patient 5 for one year – they have been treated during an intercurrent illness according to this schedule and both of them have had no episodes of decompensation during the follow-up.

As there is no possibility for the measurement of total and free carnitine in Estonia, ACP is used for laboratory follow up in LCHADD patients. Minor changes characteristic of LCHADD observed during the follow-up allow us to conclude that ACP is informative in the vast majority of LCHADD cases and can also be used in the follow-up of LCHADD patients.

5.2. Mitochondrial disorders

5.2.1. Results of the application of the diagnostic algorithm for MD (Publication II)

Based on the preliminary biochemical, metabolic and instrumental investigations, 22 children were referred to a muscular biopsy due to suspicion of MD.

Pathomorphological changes characteristic or suggestive of MD were found in five patients (table 17). Biochemical analysis performed from the muscle tissue confirmed mitochondrial disease in three of them: two patients (cases 1–2) had combined deficiency of complexes I and IV in muscle and one (case 3) had a complex IV deficiency caused by mutations in the *SCO2* gene [synthesis of cytochrome c oxidase, subunit 2 formerly known as SCO cytochrome oxidase deficient homolog 2 (yeast)] (**publication III**). Two patients (cases 4–5) had characteristic changes in muscle pathomorphology, but neither enzymatic analysis revealed normal activity of RC complexes, and no changes were identified in the mtDNA of both patients (table 17). Additionally, biochemical investigations performed on muscle tissue confirmed PDH deficiency in two patients (cases 6–7), who had no pathomorphological changes suggestive of MD, but whose metabolic investigations were characteristic of PDH deficiency.

During the study period (2003–2009), 103,821 live births were recorded in Estonia. Based on the data of this study, the live-birth prevalence for mitochondrial defects is 1 per 20,764 live births.

The live-birth prevalence observed in our cohort (1:20,764) correlates closely with the Swedish and Australian data, supporting the conclusion that MD has similar prevalence in different populations (Schaefer *et al.,* 2004), but is lower than in Finland (Table 12). One possible explanation for the difference between our cohort and the Finnish cohort may be the strict pre-selection criteria in our cohort – in the Finnish study a muscular biopsy was performed routinely in patients with unexplained encephalomyopathy and myopathy. This strategy is, however, unavailable to us in daily clinical practice, and therefore the algorithm for clinical practice was developed.

Most of the available diagnostic schemes for mitochondrial disorders (Bernier *et al.,* 2002; Morava *et al.,* 2006; Nissenkorn *et al.,* 1999; Wolf and Smeitink 2002) are useful for the evaluation of the probability of MD based on the pathomorphological and biochemical findings in the muscular tissue. Although most authors acknowledge the broad clinical spectrum of the diseases (Darin *et al.,* 2001; Gropman 2001; Kisler *et al.,* 2010), there is a shortage of published data about clinical criteria in patients in whom a muscular biopsy should be performed. Kisler *et al* (2010) have published a review about the management of MD in childhood, covering all known clinical presentations, diagnostic strategy and management (Kisler *et al.,* 2010). However, since the measurement of respiratory chain enzymatic activity in tissue samples is not available in Estonia, careful pre-selection becomes crucially important for our clinical practice.

Table 17. Patomorphological findings, enzymatic activity of respiratory chain complexes and PDH complex, and molecular findings in the patients.

	Age at investigation	**Ragged red fibres**	**Cytochrome c oxidase activity**	**Pathological ultrastucture of mitochondria**	**Lipid storages**	**Enzymatic activity of respiratory chain complexes**	**Molecular analysis**
Case 1 (R.-M. L.)	1 month	+	++	+++	+++	Decreased activity of complexes I and IV	**Mutation m.14674T>C in mt-RNA^{Glu}**
Case 2 (T. K.)	1 month	–	Not done	–	+++	Decreased activity of complexes I and IV	No mutations in *POLG* and *TK2* gene, and in mitochondrial DNA
Case 3 (M. P.) (Joost *et al.*, 2010)	1 month	–	+++	+	–	Decreased complex IV activity	**c.418G>A/c.17INS19bp mutations in *SCO2* gene**
Case 4 (K. T.)	15 years	–	–	+++	–	Normal	No deletions/duplications and point mutations (m.3243A>G, m.8344A>G, m.8993T>G/C) in mitochondrial DNA
Case 5 (A. O.)	15 years	++	–	++	–	Normal	No deletions/duplications and point mutations (m.3243A>G, m.8344A>G, m.8993T>G/C) in mitochondrial DNA
Case 6 (J. M.)	1 month	–	–	–	–	Decreased activity of PDH complex	**c.904C>T mutation in the *PDHA1* gene**
Case 7 (D. O.)	1 month	–	–	–	–	Decreased activity of PDH complex	**c.904C>T mutation in the *PDHA1* gene**

As our algorithm (figure 5) is based on the measurement of serum lactate level, the actual incidence of MD in Estonia may be higher, since the normal lactate level may be present in 30–70% of patients (Koenig 2008). In future more attention should be paid to the subtle biochemical changes (elevated excretion of Krebs cycle metabolites and alanine in case of normal lactate level etc.) and clinical presentation, including the results of instrumental investigations. Measurement of FGF21 (Suomalainen *et al.,* 2011) may also be considered in future. All of our patients with enzymatically confirmed mitochondrial disease presented in the newborn period, and thus it is possible to conclude that screening for mitochondrial disorders based on serum lactic acid measurement is informative in the case of the early onset of the disease. It has recently been shown that an elevated level of lactate was found in 87% of patients with neonatal onset of MD (Honzik *et al.,* 2012).

5.2.2. Individual cases with rare MD detected during the study

5.2.2.1. Patients with a defect in RC

5.2.2.1.1. Case 1 (Publication II)

The girl was born from the mother's third pregnancy and first delivery. Her birth weight was 4190 g (+1.5 SD), length 52 cm (+1.5 SD) and Apgar score 7/8. During the first days of life transient hypoglycemia and tachypnoe were observed. At the age of 1 month, following a vaccination against tuberculosis and hepatitis B, progressive sluggishness, muscular hypotonia, feeding and respiratory difficulties were observed, and the child was hospitalised. Clinically severe muscular hypotonia and myopathic phenotype were observed. She had no active movements and needed supportive ventilation with continuous positive nasal airway pressure. Serum lactate level was constantly elevated, with a maximum of 9.1 mmol/l (ref. <2.6mmol/l). Electromyographic investigation (EMG) showed myopathic findings, whereas brain MRI showed no pathology. CSF lactate was 3.67 mmol/l (ref. 1.1–2.4 mmol/l). Amino acid analysis revealed an increased concentration of alanine – 519.6 µmol/l (ref. 130–442) and elevated alanine/lysine ratio ~10 (ref. <3). The urinary organic acid profile showed increased excretion of succinic, fumaric, glutaric and ketoglutaric acid. In both muscular biopsies (performed at the age of 2 months and again at the age of 5 years), the pathological ultrastructure of mitochondria (whorled mitochondria) was the most prominent finding. At the age of 2 months ca 50% of fibres had RRF phenotype and at the age of 5 years ca 5–6 % of fibres were RRF (figure 8). Many cytochrome c oxidase negative fibres were present in both biopsies. Biochemical investigations from muscle tissue revealed lowered activities of complexes I and IV (table 18). The enzymatic activities of respiratory chain complexes I-IV from fibroblasts were normal. The sequencing of *POLG* and *DGOUK* genes revealed no mutations. Mutation m.14674T>C mt-tRNA^{Glu} was present in mtDNA.

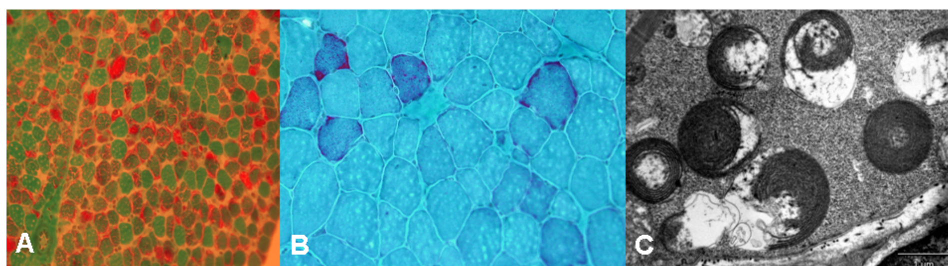


Figure 8: Case 1 muscle biopsy: (A) RRF at the age of 2 months and (B) at the age of 5 years; (C) the ultrastructure of pathological mitochondria (whorled mitochondria) in electron microscopic investigation.

Clinical course: Supportive treatment for RC was initiated at the age of 1.5 months. The treatment schedule consisted of ubiquinone 20 mg/kg/day, riboflavin 10 mg/kg/day (max 200 mg per day) and L-carnitine 100 mg/kg/day. 10–15% more calories were added to her daily diet, mostly by fat. The first active movements occurred at the age of 4 months. At the age of 7 months she was free of supportive ventilation and her physical activity gradually began to improve. She began to walk independently at the age of 1 year 10 months.

At the age of 7 years she is still on treatment supporting respiratory chain function (the child began to complain of muscle pain upon discontinuation). Clinically she has muscular hypotonia and a myopathic phenotype. She is slightly slower in physical activities than her age-matched mates, but her cognitive development is appropriate for her age.

Family history: elevated lactate level was detected during physical exercise in the patient's mother. Therefore we performed a muscular biopsy on her. Pathological mitochondria were also observed in the mother, but the changes were different to those observed in her daughter. Mitochondrial storages destroying their structure were present. No typical RRF were observed, but regions staining similarly to RRF in trichrome stain were present in some fibres. The enzymatic activities of respiratory chain complexes from the muscle tissue were within the normal range (table 18). Mutation m.14674T>C mt-tRNA^{Glu} was also present in mt DNA in the mother. The muscular biopsy of the mother was performed in 2005, and at the time the mother had no muscular complaints. In 2010 she began to complain of episodic muscle pain related to physical exercise, which showed a slow progression. These complaints resolved during CoQ10 supplementation.

Table 18. Enzymatic activities of RC complexes measured from the muscle tissue of patients (case 1 and 2) and the mother of case 1.

	Complex I mU/UCS*	**Complex II+III** mU/UCS	**Complex IV** mU/UCS	**Citrate synthase** mU/mg
Case 1	4	101	48	470
Case 2	28	51	58	118
Mother (case 1)	107	271	1355	113
Ref.	84–559	37–285	520–2080	45–187

* UCS – unit of citrate synthase

In the patient, RC deficiency was already diagnosed during the first months of life, but the molecular cause remained unknown for years. Although the m.14674T>C mt-tRNA^{Glu} mutation was already detected in the patient's mitochondrial DNA in 2008, it was considered to be a polymorphism at the time. As Horvath *et al* (2009) described, this mutation in patients with reversible COX deficiency myopathy is clinically similar to that of our patient; we related the clinical problems in the patient to this molecular change. Patients with homoplasmic m.14674T>C mt-tRNA^{Glu} mutations present with clinical deterioration shortly after birth or within the first months of life, clinically characterised clinically by lactic acidosis, profound hypotonia and feeding difficulties. Although a remarkable recovery is observed in these patients, mild myopathy is present up to adulthood (Uusimaa *et al.,* 2011). This is also the case with our patient – she showed a gradual improvement within the first three years of life, but at the age of 7 years she still has a myopathic phenotype, and her physical abilities are lower than those of her coevals. The treatment supporting mitochondrial RC function is still necessary for her, as she starts to complain muscle pain if treatment is discontinued.

Detection of molecular cause allowed genetic consultation to the mother – as this change is present in maternal mitochondrial DNA as well; the familial recurrence risk for mitochondrial myopathy is 100%.

5.2.2.1.2. Case 2 (Publication II)

The girl was born from third pregnancy and second delivery. Her birth weight was 3520 g (0 SD), length 50 cm (-1 SD) and Apgar score 8/9. She developed breathing difficulties and hypoglycaemia (s-glycose 2.4 mmol/l; ref. 2.8–4.4) on the third day of life. Additionally she developed lactic acidosis up to 9.7 mmol/l (ref. <2.6). Clinically she had muscular hypotonia and a slightly dysmorphic phenotype with a broad forehead, hypoplastic supraorbital ridges and downturned corners of the mouth. EMG showed myopathic findings. Urinary organic acid analysis revealed increased excretion of Krebs cycle intermediates (lactic, 2-OH-butyric, fumaric, ketoglutaric and 4-OH-phenyllactic acid). Amino acids from plasma were within the reference range, but urinary amino acid analysis

revealed increased excretion of alanine. Lactate from CSF was 4.8 mmol/l (Ref. 1.1–2.4). Pathomorphological examination of muscle tissue showed lipid and glycogen storages in muscle fibres. No RRF were present. Cytochrome c oxidase activity could not be evaluated due to technical reasons. Biochemical analysis of muscle tissue showed decreased activity of RC complexes I and IV (table 18). RC activities from fibroblasts were normal. Sequencing of *TK2* genes and mutation analysis in *POLG* gene revealed no mutations. Also, no mutations were found in the mtDNA using Affimetrix Human Mitochondrial Resequencing Array 2.0.

Treatment supporting RC function was initiated from the age 7 days. Her treatment schedule consisted of CoQ10 (dosage 10 mg/kg/die), L-carnitine (100 mg/kg/die) and creatine (200 mg/kg/die).

Clinical course: due to breathing difficulties the child was intubated at the age of 4 days and tracheostomy was established at the age of 2 weeks. Although her muscle strength improved gradually, she has remained dependent on artificial ventilation and therefore is treated in the intensive care unit of the local hospital. At the age of 1 year she had active movements in her extremities, she was able to grab toys, play with them and turn to the side.

Although clinical features in cases 1 and 2 are similar, their molecular genetic cause is different as patient 2 does not have mutation m.14674T>C mt-$tRNA^{Glu}$ in her mtDNA. Genetic cause of the combined deficiency of RC complexes I and IV is still unknown, but patient does not have mutations in *TK2* gene related to progressive mitochondrial myopathy due to mitochondrial DNA depletion. Also 3 common mutations in *POLG* gene are excluded. Patient 2 has 1 variant with unknown significance in non-coding region of mtDNA and 36 polymorphisms were detected by mitochondrial resequencing array.

Although the patient showed some clinical improvement during the first year: she had good growth, her muscle strength improved quickly and active movements developed, her improvement slowed down on the 2nd year of life. Whether it is related to the genetic cause of her disease, or to the limitations of her physical activity due to artificial ventilation, is still unclear.

5.2.2.1.3. Case 3 (publication III)

A boy was born in 2005 after uneventful pregnancy at 38 week of gestational age. His birth weight was 3040g (–1 SD), length 47 cm (–1.3 SD). At the age of 3 weeks he was hospitalised due to the failure to thrive. Clinically he presented with muscular hypotonia, feeding problems (weak sucking reflex), jaundice, hepatomegaly and extreme apathy. Also he had abnormal shape of the skull-flat forehead and slight turricephaly, slightly dysmorphic facial phenotype – high nasal bridge, high palate and microretrognathia. Patient's cardiac ultrasound showed hypertrophic cardiomyopathy. Brain MRI investigation revealed bilateral hyperintensity in putamen region with T2 signals consistent to the LS (figure 9).

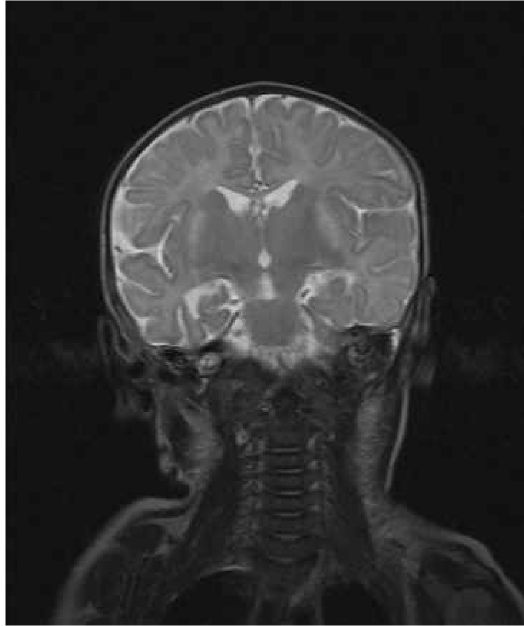


Figure 9: Brain MRI investigation of the patient at the age of 7 weeks. Note bilateral hyperintensity in the *putamen* region in T2 (TR 4000 ms/TE 96 ms) images consistent with LS.

EMG investigation revealed fibrillations in the muscles of the upper and lower limbs. No response was registered in the evaluation of motor and sensory nerves. Lactate in serum and cerebrospinal fluid was elevated up to 4.2 mmol/l and 3.8 mmol/l respectively (ref. <2.9 mmol/l). The serum amino acid profile (including alanine) was normal. Urinary organic acid analysis by GC/MS revealed elevated excretion of mitochondrial metabolites: pyruvate, malate, 2-hydroxy-glutaric acid, ketoglutaric acid and citric acid. These metabolites were not quantified, but their excretion is normally virtually undetectable.

Routine histological examination of this patient's muscle biopsies revealed a variation in muscle fibres. Muscle enzyme histochemistry showed normal checkerboard staining for NADH, while there was no detectable cytochrome c oxidase activity at all. No RRFs were found. Electron-microscopic evaluation showed enlarged and irregularly shaped mitochondria with an electron-dense matrix as well as hypertrophic mitochondria with a pale matrix and granular deposits.

RC enzyme activities were measured in the patient's muscle and fibroblasts. The studies showed severely reduced cytochrome c oxidase activity and mildly reduced activity for complex I in the muscle and reduced cytochrome c oxidase activity in this patient's fibroblasts. As both parents had mild mental retardation, we performed RC enzyme activity measurements on them too. Normal results were observed. All results are given in Table 19.

Table 19. Activities of cytochrome c oxidase and complex I of the patient (case 3) and his parents.

Investigated person	**Investigated material**	**COX** (mU/UCS*, control range)	**Complex I** (mU/UCS*, control range)
The patient (case 3)	Skeletal muscle	115 (520–2080)	42 (84–273)
	fibroblasts	477 (680–1190)	158 (100–310)
The mother	fibroblasts	721 (680–1190)	949 (262–973)
The father	fibroblasts	810 (680–1190)	797 (262–973)

* UCS – unit of citrate synthase

During molecular genetic studies, four genes associated with cytochrome c oxidase deficiency (*SURF1*, *SCO1*, *SCO2*, *COX10*) were sequenced in the patient. A c.418G>A heterozygous mutation (leading to a p.E140K) in exon 2 and heterozygous insertion of 19 basepairs at position 17 (c.17INS19bp, leading to a truncated protein) was detected in the coding region of the synthesis of the cytochrome c oxidase 2 (*SCO2*) gene by sequence analysis.

Treatment to support mitochondrial energy production (ubiquinone 5 mg/kg/day; thiamine 150 mg/day; L-carnitine 50 mg/kg/day) was initiated at the age of 6 weeks. Despite the treatment, the child's condition worsened rapidly: stridorous breathing was observed from the age of 8 weeks, and muscular hypotonia and feeding difficulties worsened. The patient died at the age of 13 weeks due to respiratory insufficiency.

Family history: both parents had mild mental retardation. His father was found to be a heterozygous carrier of the c.418G>A mutation and his mother was a heterozygous carrier of the c.17INS19bp mutation in the *SCO2* gene.

MD in patient 3 was caused by the compound heterozygosity of mutations in *SCO2* gene. This gene encodes a 266 amino acid protein, which is involved in copper supply for the assembly of cytochrome c oxidase. Mutations in this gene have been reported in patients with fatal infantile cardioencephalomyopathy. At least 26 patients, are described and all of them have been associated with 1541G>A (E140K), which is the missense mutation probably affecting protein stability (Bohm *et al.,* 2006; Jaksch *et al.,* 2001a; Jaksch *et al.,* 2000; Knuf *et al.,* 2007; Leary *et al.,* 2006; Papadopoulou *et al.,* 1999; Sacconi *et al.,* 2003; Salviati *et al.,* 2002; Tarnopolsky *et al.,* 2004; Verdijk *et al.,* 2008; Vesela *et al.,* 2004). The other eleven described mutations in the *SCO2* gene result in a premature stop codon, deletions or insertions with a resultant frameshift and therefore have more serious deleterious structural consequences for the protein product (Bohm *et al.,* 2006; Jaksch *et al.,* 2000; Knuf *et al.,* 2007; Leary *et al.,* 2006; Papadopoulou *et al.,* 1999; Salviati *et al.,* 2002; Tarnopolsky *et al.,* 2004; Verdijk *et al.,* 2008). Patients who are homozygous for the E140K mutation have a milder phenotype with delayed age of onset and a more prolonged course of disease compared to compound heterozygotes (Tay *et al.,* 2004; Verdijk *et al.,* 2008). The c.418G>A mutation in homozygous or heterozygous state is

associated with all previously published cases, but the c.17INS19bp mutation) has not previously been described. The novel c.17INS19bp mutation in exon 2 of this gene in our patient leads to the truncated protein, explaining our patient's severe clinical course.

In patients with *SCO2* gene mutations, skeletal and cardiac muscle shows a severe COX deficiency (Leary *et al.,* 2006; Verdijk *et al.,* 2008), whereas skin fibroblasts exhibit a relatively high residual enzyme activity, despite fact that this gene is ubiquitously expressed (Bohm *et al.,* 2006; Jaksch *et al.,* 2001b). COX activity in our patient's muscle was very low, and significantly higher in fibroblasts (see Table 19), supporting this observation.

Reduced complex I activity in muscle is unusual in patients with *SCO2* gene defects. It is likely that the complex I deficiency is secondary to the complex IV deficiency, as the secondary deficiencies have earlier been described for other complexes (Lenaz *et al.,* 2006). The mechanism behind this remains specula-0tive; perhaps it has something to do with the supercomplex formation that has been shown to occur (Lenaz *et al.,* 2006). Perhaps the lack of complex IV prevents the formation of supercomplexes, which destabilises complex I, resulting in reduced complex I activity in muscle.

5.2.2.2. Patients with PDH deficiency

5.2.2.2.1. Case 6

The girl was born preterm on the 36th week of pregnancy in 2003. Her birth weight was 2219 g (–1 SD), length 44 cm (–2 SD) and head circumference 32 cm (–1 SD). Her Apgar score was 7/7. Due to poor neonatal adaptation, the patient was hospitalised to the department of neonatology. At the age of 2 weeks, a congenital brain anomaly was diagnosed – she had periventricular cysts, agenesis of the corpus callosum and dysmyelinisation. Elevated lactate level (up to 6.6 mmol/l; ref. <2.9 mmol/l) was observed. Metabolic studies revealed a high blood level of alanine – 1672 μmol/l (ref. 150–600 μmol/l) – and elevated urinary excretion of lactate and pyruvate. PDH deficiency was suspected but not confirmed at the time, as the family moved away from Estonia.

At the next referral, at the age of 6 years, she presented with poor growth – weight 10 kg (–4.5 SD), height 100 cm (–4.5 SD) and head circumference 41.5 cm (–6.5 SD). She has spastic tetraplegia with multiple contractures, a convergent strabismus and dysmorphic phenotype with upward-slanting palpebral fissures, a high palate, a small mandible and dysmorphic ears. Her psychomotor development corresponded to the age of 1–2 months. Investigations to confirm PDH deficiency in the patient were performed. The PDH complex activity from fibroblasts was decreased – 4.5 mU/UCS (ref. 9.7–36). Molecular genetic analysis revealed heterozygous change c. 904C>T (p.R302C) in the *PDHA1* gene.

Due to the late diagnosis, specific treatment to overcome metabolic blockage was not indicated for the patient, but thiamine (50–500 mg/day) was advised as a cofactor of the PDH complex.

5.2.2.2.2. Case 7

The girl was born in 2005. Foetal hydrocephaly was diagnosed on the 33rd week of gestation. Intrauterine growth retardation and oligohydramnion were also observed at that stage of pregnancy. The child was born preterm on the 35th week of gestation. Her birth weight was 1782 (–2SD), length 39 cm (<–2 SD) and head circumference 29.5 cm (<–2SD). A congenital brain anomaly was confirmed post-natally – in addition to the hydrocephaly, the patient also had a hypoplastic corpus callosum. She had a dysmorphic phenotype at birth with dysmorphic ears, downward-slanting palpebral fissures, exophthalmus, a long smooth philtrum and microretrognathia. On the day 5 rapid deterioration was observed, with lactic acidemia up to 10.6 mmol/l (ref. <2.4) and elevated lactate concentration in CSF up to 13 mmol/l (ref. <1.6). The amino acid profile from serum, CSF and urine revealed elevated proline and alanine in all samples. Organic acid analysis by GC/MS from serum and urine revealed elevated lactate and pyruvate in both samples. The lactate/pyruvate ratio in serum was low (3.5; ref.10–20).

The enzymatic study from muscle revealed lowered PDH complex activity – 4 mU/UCS (ref. 28–89). The PDH complex activity from skin fibroblasts was in the lower reference range – 9.6 mU/UCS (ref. 9.7). Pathogenic mutation c.904C>T (p.R302C) was heterozygously detected in exon 10 of the *PDHA1* gene through sequence analysis.

Treatment with thiamine as the cofactor for PHD complex was initiated at the age of 2 weeks, with a dosage of 500 mg daily. In addition, a ketogenic diet was introduced to overcome metabolic block and provide energy. The patient's ketogenic diet consisted of fat, which provides up to 80% of daily calories. The administration of carbohydrates was restricted.

Clinical course: the implemented treatment gave good control over biochemical pathology in the patient – the serum lactate level dropped promptly after the ketogenic diet was started. Despite the good metabolic control, the patient's clinical condition gradually worsened: at the age of 2 months seizures developed, and optic atrophy was diagnosed. The patient had severely retarded physical and psychomotor development, and she died at the age of 2.5 years. Autopsy revealed generalised brain atrophy.

Both patients with PDH complex deficiency had the same *PDHA1* genotype. Although the clinical course of case 7 was more severe, the cardinal clinical features such as congenital brain anomalies and poor growth overlapped.

Codon 302 is a hot spot for mutations in the *PDHA1* gene, as it contains the highly mutagenic GpG dinucleotide (Otero *et al.,* 1998). The mutations in this codon are responsible for a large number of cases in females (Lissens *et al.,* 2000). It has previously been estimated that mutation c.904C>T may result in complete inactivation of the E1α enzyme (Otero *et al.,* 1998). Our case 7 is rare and represents a unique clinical presentation, as antenatal onset of PDH complex deficiency has been reported infrequently (Otero *et al.,* 1998; Robinson *et al.,* 2001; Zand *et al.,* 2003; Tamaru *et al.,* 2012; Wada *et al.,* 2004). The c.904C>T mutation has previously been reported by Otero *et al* in a girl

who first presented with ventriculomegaly/hydrocephalus at 32 weeks of gestation (Otero *et al.,* 1998).

The brain involvement is a cardinal feature of PDH complex deficiency. Two mechanisms have been related to neurological damage. The first is the lack of thiamine during the early stages of embryonic development – this causes energy deficiency in early embryogenesis, when neuronal proliferation and migration require a great amount of energy (Barnerias *et al.,* 2010). Clastic brain lesions resulting from acute energy failure account for the second type of neuropathological findings.

5.3. Individual cases detected during selective metabolic screening in Estonia

During the study period (2007–2011), the author of this study participated in the management of patients with inherited metabolic diseases from other groups, and is the co-author of publications dealing with the clinical diagnosis and treatment of classical galactosemia (Krabbi *et al.,* 2011; Õunap *et al.,* 2010) and the establishment of the live-birth prevalence of mucopolysaccharidoses in Estonia (Krabbi et al 2012).

As a part of this study, an 8.5-year-old girl with mental retardation, epilepsy, and some evidence of OTC deficiency was studied in order to establish the mechanisms of disease manifestation and the role of a defective urea cycle pathway in the pathogenesis of the disease.

5.3.1. Case report – OTC deficiency (Publication IV)

The girl was born in 2000 from the mother's third pregnancy and third delivery. Her birth weight was 3128 g (–1 SD), length 48 cm (–1.5 SD), and her Apgar score was 8/8. On day 2 she was admitted to the intensive care unit due to breathing difficulties and hypoglycaemia, and meconium aspiration was diagnosed. Episodic hypoglycemia was present during the first 3 weeks of life. Specific metabolic investigations were not performed in the newborn period. At the age of 2 months, during an episode of acute bronchitis, hepatomegaly was observed. EEG showed nonspecific paroxysmal activity, and treatment with phenobarbital was started. Ammonia was first increased, i.e. 90 μmol/l (ref. <48 μmol/l), at the age of 3 months. The first signs of developmental problems were noticed at the age of 6 months, and at 9 months psychomotor retardation was diagnosed. At the age of 9 months a high level of blood ammonia was detected – 297 μmol/l (ref. <48). Amino acid analysis showed no detectable citrulline in serum, and in urinary organic acid GC/MS analysis a slightly elevated excretion of orotic acid (47.7 μmol/g creatinine; ref. 1.7–35) was observed. Citrulline in serum was constantly low during the first 2 years of life (between 0 and 18 μmol/l; ref. 8–52). The highest glutamine level in serum was

1951 µmol/l (ref. 350–720) at the age of 2 years. Thereafter, an allopurinol loading test was performed in the Charité-Virchow Klinikum (Berlin, Germany). This test showed a significant increase in the excretion of orotic acid (3–4 times over the upper normal limit 12–24 hours after loading). Based on the metabolic investigations, this girl was suspected to be affected with a mild form of OTC deficiency. The treatment with oral citrulline (5g/day) and a low protein diet was initiated at the age of 2 years. Since that age all metabolic characteristics (ammonia and amino acids) were in the normal range.

At the age of 12 months she had the first generalized seizures. Her EEG showed generalized complexes of spike waves, and her brain MRI showed bilateral white matter damage in the periventricular region. Treatment with *carbamazepine* was initiated (20mg/kg/day) and continued alongside a low protein diet and citrulline.

At the age of 7.5 years she was investigated at Tartu University Hospital, as despite the normal ammonia level and antiepileptic treatment, the patient's seizure control was poor. Our clinical evaluation revealed short stature – her height was 111 cm (–3 SD), weight 19 kg (–2 SD) and OFC 48.5 cm (–2 SD), and she had developmental delay. In addition, she had slightly upward-slanting eyes, widely spaced teeth, a high-arched palate, low set and posteriorly rotated dysmorphic ears, many pigmented naevi and a large (10x10x10cm) depigmented area in the upper spinal region. Anticonvulsive treatment was changed to *lamotrigine* (7 mg/kg/day), and in addition treatment with *sodium phenylbutyrate* (Ammonaps) was initiated (395mg/kg/day) for a period of one month, with a positive effect on seizure control.

One year later, at the age of 8.5 years, she was readmitted to hospital. Her height remained low (–3 SD), and moderate MR was diagnosed. Neuropsychological evaluation with a Kaufman ABC test revealed impaired abilities in all areas. She had a more pronounced attention deficit, difficulties in exercises demanding analysis and synthesis, and limited short-term memory. She could read simple words, but she did not know numbers. She was able to cope in daily activities (walking, dressing and eating). In addition to her facial dysmorphy and skin pigmentation anomalies, the brain MRI showed partial agenesis of the corpus callosum and bilateral white matter damage in the periventricular region. Additional investigations were performed to establish genetic etiology.

Molecular and cytogenetic investigation of the patient gave the following results:

(1) Bidirectional sequencing of all 10 coding exons of the *OTC* gene including intron-exon boundaries was performed using the ABI PRISM 3100 Genetic Analyzer, but no genetic variations within coding regions were identified.

(2) Standard G-banding cytogenetic analysis was performed on peripheral blood lymphocytes using the standard methods, and this showed an abnormal karyotype 46,X,del(X)(p11). Therefore, Turner syndrome was diagnosed in the patient due to the loss of the short arm of the X chromosome (figure 10).

Figure 10. X chromosome of the patient with karyotype 46,X,del(X)(p11).

(3) Whole genome karyotyping analysis using Human CytoSNP-12 BeadChips (Illumina Inc., San Diego, USA) confirmed the deletion of the whole short arm of the X chromosome. The deletion involved the Xp22.33-p11.1 region, and the borders of the deletion were found to be the following: 1–56,975,659 bp (NCBI build 36). The data was analysed using Illumina BeadStudio v3.1 and QuantiSNP v1.1 software (Colella *et al.,* 2007)

(4) For the evaluation of the X-inactivation status, the androgen receptor *(AR)* (CAG)n and fragile X mental retardation 1 *(FMR1)* (CGG)n variable repeat region was examined. X-chromosome inactivation (XCI) pattern was determined by comparative quantitative detection of fluorescent-labeled PCR products using intact and methylation-sensitive restriction enzyme *Hpa*II-digested (Bolduc *et al*., 2008) and *Hha*I-digested DNA templates respectively. *FMR1* PCR was performed using the *FMR1* sizing PCR Set (Abbot Molecular, Illinois, USA) in accordance with the manufacturer's protocol. Amplicon size and inactivation ratios were determined via electrophoresis on an ABI 3130XL Genetic Analyzer (PE Applied Biosystems) and analysed using GeneScan 4.0 software (PE Applied Biosystems). The XCI study showed that the aberrant X chromosome was completely skewed in our patient (figure 11).

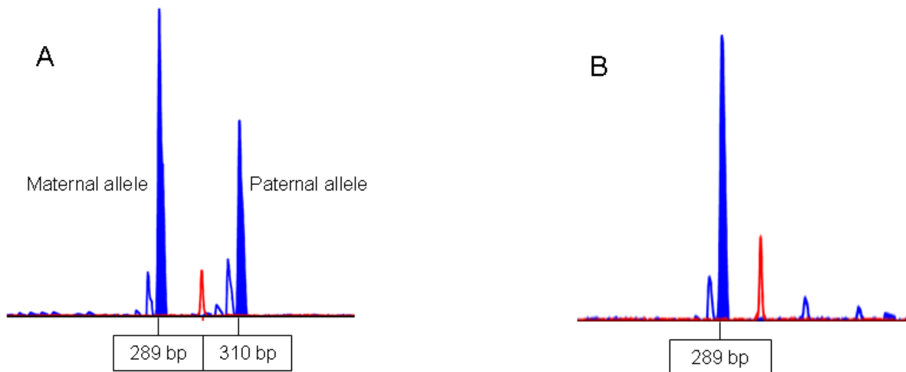


Figure 11. Results of the *FMR1* methylation assay. **A** Undigested DNA of the patient. **B** DNA of the patient predigested with *Hha*I, showing an extremely skewed X-inactivation pattern.

Our patient presented relatively non-specific symptoms in the newborn period, but later developed psychomotor retardation and seizures. Based on biochemical abnormalities, OTC deficiency was suspected, and corresponding treatment was started. Sequencing of the *OTC* gene was performed for final confirmation of the diagnosis in the patient. However, no pathogenic change was found in the *OTC* gene, and therefore further cytogenetic and molecular studies were indicated to explain the biochemical changes observed in the patient. As a result, deletion of the whole short arm of the X chromosome and completely skewed X-inactivation were detected.

Females with partial deletions of the short arm of the X chromosome have variable phenotypes, but MR rarely occurs (Lachlan *et al.,* 2006; Schinzel 2001). There are a few reported cases with small deletions located in Xp22 that had MR or developmental delay and/or autistic features (Chocholska *et al.,* 2006; Lachlan *et al.,* 2006; Puusepp *et al.,* 2008; Shinawi *et al.,* 2009; Thomas and Huson 2001). The haploinsufficiency of deleted genes that escape X-inactivation may explain the occurrence of autism and developmental delay in the previously described cases (Shinawi *et al.,* 2009).

Lachlan *et al* (2006) have shown that if the breakpoint of an X chromosome deletion is proximal to Xp.22.3, there is a non-random X-inactivation, with the deleted X being preferentially inactivated. XCI study of our patient also showed a completely skewed X-inactivation. The variability in the phenotype associated with Xp deletions can be explained by different mechanisms. A mosaicism for a normal cell line in other tissues that are not routinely tested by cytogenetic methods can be present (Thomas and Huson 2001). Unfortunately, it was not possible to perform karyotyping of skin fibroblasts (normal skin and depigmented areas) from our patient. The degree of expression of genes that escape inactivation from the inactive X chromosome is variable among different females (Carrel and Willard 2005), and the degree of skewed X-inactivation can vary in different tissues (Sharp *et al.,* 2000).

The first cytological demonstration of cellular mosaicism in an obligate heterozygote of OTC was provided by Ricciuti *et al* (1976). The authors stated that the patchy distribution of OTC-positive cells within the liver provided an explanation both for the marked variation in OTC activity observed in different heterozygous females and for variable OTC activities in repeated biopsies from the same patient. The X-inactivation of a manifesting female with OTC deficiency has previously been studied by Yorifuji *et al* (1998), who performed an *AR* assay using genomic DNA extracted from liver samples to study the skewing of the X chromosome. The results were compared with residual enzyme activity. A close correlation was observed, and the authors stated that residual enzyme activity is actually determined by the X-inactivation status in the liver. Unfortunately, no liver sample was available from our patient. We realise that our studies are insufficient to draw a correlation between X-inactivation and the severity of clinical manifestations, but they shed some light on the complexity of the genetic mechanisms that are sometimes involved in the manifestation of genetic disorders.

As our patient has MR and epilepsy, the question arises whether the patient has any other symptoms of haploinsufficiency in the genes located in the deleted region. At least 22 genes are related to MR, and some of these are also connected to seizures, according to the OMIM database (http://www.ncbi.nlm.nih.gov/omim/). These genes may contribute to the cognitive problems and epilepsy observed in our patient. Another possible cause for her symptoms is an encephalopathy due to hyperammonemic crises in infancy and childhood.

The characterisation of the precise nature of the genetic mechanisms involved in the presentation of the X-linked disorder in the patient not only enabled us to offer the family an accurate genetic consultation, but also made possible better future surveillance. Due to the presence of Turner syndrome, treatment with growth hormone was considered.

In conclusion, we hope that this case report will contribute to the understanding of the underlying genetic factors of the manifestation of X-linked disorders in female patients.

6. CONCLUSIONS

1. We have successfully introduced acylcarnitine analysis, which is a rapid, informative method for the diagnosis of FAOD that enables the simultaneous diagnosis of different disorders affecting FAO from the same sample, into the clinical practice in Estonia. This has been potentially lifesaving for 3 patients identified during this study.
2. The clinical criteria developed and used in our study are effective, practical and suitable for the selective screening of FAOD in Estonia.
   a) LCHADD is the most prevalent FAOD in Estonia, with a prevalence of 1:91,670. The frequency of heterozygotes for the common mutation causing LCHADD in Estonia is 1:173, which is comparable to the prevalence in the other countries in the Baltic Sea region.
   b) The results of our selective screening of MCADD correspond to the genetic data from Lilleväli *et al* (2000) that MCADD in Estonia is rare compared to other European countries.
   c) No other FAOD or carnitine metabolism defects were detected during our study. This is probably related to the small population of Estonia, the limited duration of the study and the rare prevalence of the other disorders.
3. During our study five patients with LCHADD from four families were diagnosed.
   a) Seven out of eight independent LCHADD alleles (87.5 %) carried the most common c.1528G>C mutation in the *HADHA* gene.
   b) In one allele a novel c.1690–2A>G mutation was found. The mutation causes abnormal HADHA splicing and is associated with the classical disease phenotype.
   c) The clinical presentations of our LCHADD patients represent the severe phenotype characteristic of patients who are homozygous for the c.1528G>C mutation. The medium age at first presentation was 7.1 months (3.5–14 months). At least three patients had hypoglycemia in initial presentation, and in two of them hypoglycemic episodes prior to diagnosis were fatal.
4. Our algorithm for identifying patients with MD is informative in diagnosing the cases presenting with elevated lactate. The vast majority of neonatal and infantile onset cases are identified using this algorithm.
5. Based on the data using in this study, the live-birth prevalence of MD is 1 in 20,764 live births. The live-birth prevalence observed in our cohort correlates well with the Swedish and Australian data, supporting the conclusion that MD has a similar prevalence in different populations.

6. The detailed description of three patients with RC defects, two patients with PDH deficiency and one patient with OTC deficiency are given. We would like to point out the following:
   a) The novel c.17INS19bp mutation in exon 2 of the *SCO2* gene was identified in one patient with reduced activity of IV of the RC. This mutation leads to truncated protein and is associated with the clinical course that is fatal at an early age.
   b) Patients with homoplasmic mutation m.14674T>C mt-tRNA^{Glu} in mtDNA present in infancy with severe myopathy, which improves with age, but these children and adults may still need treatment supporting muscular energy metabolism.
   c) Both patients with PDH complex deficiency had the same *PDHA1* genotype – heterozygous mutation c.904C>T. Although the clinical course of case 7 was more severe, the cardinal clinical features, such as congenital brain anomalies and poor growth, overlapped.
   d) We described a patient with mental retardation, epilepsy, and some evidence of reduced OTC activity, but with no mutation identified in *OTC* gene sequencing. Cytogenetic analysis and molecular karyotyping using SNP array revealed a deletion of the whole short arm of the X chromosome (Xp22.33-p11.1). Inactivation studies also revealed completely skewed X-inactivation. Complex mechanisms such as large deletions on the X-chromosome, skewing of X-inactivation in different tissues and at different ages contribute to the manifestation of X-linked disease in females.

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SUMMARY IN ESTONIAN

Pärilike ainevahetushaiguste haiguste valikskriining Eestis: uute diagnostikameetodite rakendamine

Pärilikud ainevahetushaigused on organismi biokeemilise tasakaalu häired, mida põhjustavad geenimutatsioonid. Paljud nendest haigustest avalduvad vastsündinu- või imikueas, kuid kiiresti suureneb hilisemas vanuses diagnoositavate pärilike ainevahetushaiguste hulk, kaasaarvatud täiskasvanul avalduvate haigusvormide hulk. Hetkel on teada üle 700 päriliku ainevahetushaiguse. Vaatamata sellele, et iga üksiku haiguse esinemine on üliharuldane, on nende haiguste summaarne esinemissagedus märkimisväärne – hinnanguliselt 1:2500 vastsündinu kohta.

Patofüsioloogia alusel jaotatakse pärilikud ainevahetushaigused kolme gruppi: a) ladestushaigused, mille korral on häiritud kompleksmolekulide katabolism, põhjustades laguproduktide ladestumise rakusiseselt; b) intoksikatsiooni tüüpi haigused, mille korral kuhjub puuduliku ensüümreaktsiooni substraat, põhjustades intoksikatsiooni; c) energia defitsiidi tüüpi haigused, mille korral on häire organismi energiatootmises osalevates protsessides. Viimasesse gruppi kuuluvad teiste hulgas rasvhapete oksüdatsioonidefektid, häired trikarboksüülhapete tsüklis ja mitokondriaalse hingamisahela ensüümdefektid.

Rasvhapped on organismi olulisim energiaallikas, tagades kuni 80% organismi energiavajadusest. Rasvhapete metabolismi põhirada organismis on β-oksüdatsioon, mille käigus lühendatakse triglütseriidis olev pika ahelaga rasvhape 2-süsinikuliste jääkide võrra järk-järgult keskmise ja lühikese ahelaga rasvhapeteks. Vabanev atsetüül-koensüüm A siseneb trikarboksüülhapete tsüklisse. Ensüümdefektid võivad mõjutada rasvhapete metabolismi kõigil tasemetel – teada on pika, keskmise ja lühikese ahelaga rasvhapete oksüdatsiooni defektid.

Rasvhapete oksüdatsiooni defektid põhjustavad patsientidel Reye' sündroomi sarnast kliinilist pilti, mis avaldub hüpoketootilise hüpoglükeemiana, maksa funktsioonihäire ja lihaskahjustusena. Haigushoog vallandub kataboolses seisundis, sageli kaasuva üldhaigestumise foonil, ning avaldub tihti vastsündinu- või imikueas. Esmane haigusepisood on sageli letaalne.

Igale β-oksüdatsiooni defektile on iseloomulik spetsiifilise süsinikuahelapikkusega atsüül-CoA akumuleerumine. Need metaboliidid moodustavad karnitiinestrid, mida on võimalik määrata atsüülkarnitiinide tandemmass-spektromeetrilisel analüüsil. Sellel metoodikal teostatud analüüs on korraga informatiivne kõigi rasvhapete oksüdatsiooni defektide ja karnitiini ainevahetushäirete suhtes. Atsüülkarnitiinide analüüsil tuvastatud diagnoos kinnitatakse vastaval ensüümanalüüsil ja/või vastavat ensüümi kodeeriva geeni analüüsil.

Mitokondriaalsed haigused on haigused, mis haaravad ensüüme või ensüümkomplekse, mis osalevad otseselt keemilise energia tootmises oksüdatiivse fosforüülimise protsessis. Neid haigusi põhjustavad mutatsioonid mitokondriaalses DNAs (mtDNA) või rakutuumas asuvates geenides. Oksüdatiivse

fosforüülimise defekt võib esineda igal patsiendil, kellel on ebaselge etioloogiaga neuromuskulaarse haiguse ja/või mitte neuromuskulaarse haiguse sümptomid. Nende haiguste kliiniline pilt on erakordselt mitmekülgne ning erinevate haigusvormide kliiniline pilt võib kattuda. Mitokondriaalne düsfunktsioon mängib rolli ka mõnede „tavaliste“ haiguste patogeneesis – näiteks neurodegeneratiivsete haigusete, kardiovaskulaarsete haigusete, diabeedi ja vähi patogeneesis.

Kuna mitokondriaalsete haiguste kliiniline pilt on äärmiselt mittespetsiifiline, põhineb nende haiguste diagnostika suures osas iseloomulikele laboratoorsetele, metaboolsetele ja morfoloogilistele muutustele. Esmaseks biokeemiliseks markeriks on laktaaditaseme tõus veres, kuid märkimisväärsel osal patsientidest (kuni 50%) võib vere laktaadisisaldus olla normikohane. Mitokondriaalse energia tootmise defitsiidile viitavad veel Krebsi tsükli vahemetaboliitide erituse suurenemine uriiniga ning alaniini sisalduse suurenemine veres. Iseloomulike morfoloogiliste muutuste hindamine on kõige olulisem aju- ja lihaskoes. Ajukoes esinevate muutuste tuvastamine on võimalik aju magnetresonantstomograafial ning spektroskoopial, kus tüüpilisimaks muutuseks on hallaine tuumade sümmeetriline kahjustus ning laktaadi ja N-atsetüülaspartaadi akumuleerumine ajukoes. Lihaskoes esinevate patomorfoloogiliste muutuste olemasolu on mitokondriaalse haiguse diagnostikas määrava tähtsusega. Sagedasimateks muutusteks on mitokondrite patoloogilisele proliferatsioonile viitavad muutused ning elektronmikroskoopiliselt määratavad mitokondrite struktuuri muutused. Reeglina toimub lihasbiopsia materjalil ka hingamisahela ensüümkomplekside aktiivsuse määramine ning sageli teostatakse ka molekulaargeneetilised analüüsid diagnoosi lõplikuks kinnitamiseks.

Uurimistöö eesmärgid:

1) atsüülkarnitiinide tandemmass-spektromeertilise metoodika rakendamine kliinilisse praktikasse Eestis;
2) rasvhapete β-oksüdatsioonidefektide biokeemilise diagnostika efektiivsuse hindamine ning nende haiguste esinemissageduse välja selgitamine Eestis;
3) pika ahelaga hüdroksüatsüül-CoA dehüdrogenaasi (LCHAD) defitsiidi genotüübi ja fenotüübi kirjeldamine Eesti patsientidel;
4) mitokondriaalsete haiguste diagnostilise algoritmi efektiivsuse hindamine;
5) mitokondriaalsete haiguste esinemissageduse kindlaks tegemine Eestis;
6) harvaesinevate pärilike ainevahetushaiguste kirjeldamine.

Materjal ja meetodid

Rasvhapete oksüdatsiooni defektide esinemissagedust hinnati kahes grupis, millest kumbki jagunes kaheks alagrupiks. Esimene grupp oli asümptomaatiliste vastsündinute grupp, kellel hinnati LCHAD defitsiidiga seotud geenimuutuste esinemist *HADHA* geenil. Selle grupi esimese alagrupi moodustasid 1040 anonüümset Eesti vastsündinut, kellel analüüsiti *HADHA* geeni põhimutatsiooni

esinemist ja teise alagrupi moodustasid 59 anonüümset Taani vastsündinut, kellel uuriti antud töö käigus kirjeldatud uusmutatsiooni c.1690 -2A>G esinemist. Teise grupi moodustasid patsiendid, kellel esines rasvhapete oksüdatsiooni defektidele viitavaid kliinilisi sümptomeid. Selles grupis moodustus 2 alagruppi vastavalt rakendatud uuringumetoodikale. Esimese alagrupi moodustasid rasvhapete oksüdatsiooni defektide kahtlusega patsiendid perioodil 2004–2007, keda testiti *HADHA* geeni põhimutatsiooni esinemise suhtes. Sellesse alagruppi kuulus 102 patsienti, kes olid suunatud kahest regionaalhaiglast Eestis (SA TÜK Lastekliinik ja SA Tallinna Lastehaigla). Teise alagrupi moodustasid sümptomaatilised patsiendid perioodil 2008–2011, kellel teostati plasma atsüülkarnitiinide analüüs. Uuringusse suunamise näidustused olid ühtsed ja välja töötatud kirjanduse alusel – Duran jt., 2003. Sellesse alagruppi kuulus 496 patsienti, kes olid samuti suunatud kahest regionaalhaiglast.

2003.–2009. aastal hinnati mitokondriaalsete haiguste esinemist hinnati kõigil patsientidel, kes olid hospitaliseeritud uuringuteks kahe regionaalhaigla vastsündinute ja lasteneuroloogia osakondadesse. Rutiinselt määrati kõigil mitokondriaalsele haigusele viitavate sümptomitega patsientidel seerumi laktaadi sisaldus. Referentsväärtusest kõrgema laktaadisisalduse esinemisel teostati täiendavad metaboolsed ja instrumentaalsed uuringud. Juhul, kui biokeemilised ja instrumentaalsed uuringud toetasid mitokondriaalse haiguse võimalikku esinemist patsiendil, teostati diagnoosi täpsustamiseks lihasbiopsia iseloomulike patomorfoloogiliste muutuste tuvastamiseks ning vajadusel biokeemilisteks ja molekulaargeneetilisteks uuringuteks lihaskoest.

HADHA geeni molekulaargeneetilisel analüüsil rakendati 1) põhimutatsiooni otsest detekteerimist ja 2) geeni sekveneerimist. Plasma atsüülkarnitiine analüüsiti butüülestritena ning mõõdeti positiivse eellasiooni analüüsil.

Tulemused ja arutelu

1) Käesolevas töös rakendati kliinilisse praktikasse atsüülkarnitiinide analüüs, mis on kiire ja informatiivne meetod rasvhapete oksüdatsiooni defektide diagnostikas, võimaldades ühel analüüsil mitmete β-oksüdatsiooni mõjutavate pärilike haiguste diagnostika. Selle meetodi kasutusele võtmine on olnud potensiaalselt elupäästev vähemalt 3 patsiendi jaoks, kes tuvastati antud uurimistöö käigus.
2) Uuringus kasutatud kliinilised kriteeriumid atsüülkarnitiinide analüüsiks on praktilised ning sobivad rasvhapete oksüdatsiooni defektide selektiivseks skriininguks Eestis.
   a) LCHAD defitsiit on kõige sagedamini esinev rasvhapete oksüdatsiooni defekt Eestis, esinemissagedusega 1:91,670 vastsündinu kohta. Heterosügootne mutatsioon c.1528G>C *HADHA* geenil esineb sagedusega 1:173 vastsündinu kohta – seega geenikandluse esinemine on võrreldav teiste Läänemere regiooni riikidega.

b) Selektiivse skriiningu tulemused toetavad Lilleväli jt., (2000) uuringu varasemat järeldust, et MCAD defitsiidi esinemissagedus Eestis on väiksem võrreldes teiste Euroopa riikidega.
c) Antud uuringu käigus ei tuvastatud ühtegi teist rasvhapete oksüdatsiooni defekti ega karnitiini ainevahetushäiret. See on tõenäoliselt seotud Eesti väikesearvulise populatsiooni ja nende haiguste väga väikese esinemissagedusega.

3) Uuringu käigus tuvastati LCHAD defitsiidi esinemine kolmes perekonnas.
a) Seitsmel *HADHA* alleelil kaheksast (87,5%) esines põhimutatsioon c.1528G>C.
b) Ühel alleelil tuvastati ka üks varem kirjeldamata mutatsioon c.1690–2A>G, mis põhjustab *HADHA* geeni ebanormaalse splaissingu ning on seotud LCHAD defitsiidi klassikalise kliinilise avaldumisega.
c) LCHAD defitsiidi kliiniline avaldumine vastas haiguse raskeimatele avaldumisvormidele. See on iseloomulik mutatsiooni c.1528G>C homosügootidele. Keskmine vanus esmasel sümptomite avaldumisel oli 7,1 kuud (3,5–14 elukuud). Vähemalt kolmel patsiendil esines esmase episoodi ajal hüpoglükeemia ning kahel neist oli hüpoglükeemiline episood fataalne.

4) Kasutatud mitokondriaalsete haiguste diagnostika algoritm on informatiivne kõrgenenud vereseerumi laktaadi sisaldusega patsientidel. Kasutatud algoritm võimaldab diagnoosida enamiku vastsündinu- ja imikueas avalduvaid haigusjuhte.

5) Mitokondriaalsete haiguste esinemissagedus Eestis on 1:20,746 elussünni kohta. See korreleerub hästi mitokondriaalsete haiguste esinemissagedusega Rootsis ja Austraalias, toetades järeldust, et nende haiguste esinemine on sarnane erinevates populatsioonides.

6) Töös on kirjeldatud haiguse kliinilist pilti kolmel hingamisahela kompleksi defitsiidiga patsiendil, kahel PDH defitsiidiga patsiendil ja ühel OTC defitsiidiga patsiendil. Nende haigusjuhtude juures tahame rõhutada järgmist:
a) Varem kirjeldamata c.17INS19bp mutatsioon *SCO2* geeni teises eksonis tuvastati patsiendil, kellel esines hingamisahela IV kompleksi defitsiit. See mutatsioon põhjustab valgu sünteesi katkemise ning on seotud haigusvormidega, mis lõpevad surmaga varases eas.
b) Patsientidel, kellel esineb homoplasmiline mutatsioon m.14674T>C mt-$tRNA^{Glu}$ mitokondriaalses DNAs, esineb imikueas avalduv raske müopaatia, mis paraneb lapse kasvades. Siiski vajavad need patsiendid mitokondriaalset energia tootmist toetavat ravi.
c) Mõlemal PDH defitsiidiga patsiendil tuvastati sarnane *PDHA1* genotüüp – heterosügootne mutatsioon c.904C>T. Kuigi ühel patsiendil (patsient 7) oli kliiniline kulg raskem, olid peamised kliinilised sümptoomid nagu ajuanomaaliad ja kasvupeetus kattuvad.
d) Me kirjeldasime patsienti, kellel esines vaimne alaareng, epilepsia ja OTC aktiivsuse langusele viitavad sümptomid, kuid *OTC* geeni sekveneerimisel muutusi ei tuvastatud. Tsütogeneetilisel analüüsil ja moleku-

laarsel karüotüpiseerimisel ilmnes X-kromosoomi lühikese õla deletsioon (Xp22.33-p.11.1). Inaktivatsiooni uuringutel tuvastati täielikult kallutatud X-kromosoomi inaktivatsioon. Tulemused võimaldavad järeldada, et X-liitelise haiguse avaldumisel naissoost indiviidil mängivad rolli erinevad mehhanismid nagu suured deletsioonid X-kromosoomil, kallutatud X-kromosoomi inaktivatsioon erinevates kudedes ning selle muutumine elu jooksul.

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PUBLICATIONS

CURRICULUM VITAE

Kairit Joost

Date and place of birth: 18.12.1967 Jõgeva, Estonia
Citizenship: Estonia
Family status: single
Address: Department of Biochemistry
University of Tartu
Ravila street 19
Tartu 50411
Phone: +372 731 9172
+372 566 26204
E-mail: kairit.joost@kliinikum.ee

Education

1975–1986 Jõgeva Secondary School
1986–1992 University of Tartu, Faculty of Medicine, Department of Pediatrics
2000–2004 University of Tartu, Residency in Medical Genetics
2007–... University of Tartu, School of Neurosciences, PhD studies

Professional employement

1992–1993 Tallinn Children's Hospital, internship
1993–1994 Tallinn Children's Hospital, epidemiological study ISAAC (Incidence of Symptoms of Asthma and Allergy in Childhood), researcher
1994–1996 Tallinn Children's Hospital, pediatrician
1996–1997 Krka Slovenia, medical representative
1997–2000 Glaxo Wellcome, medical representative
2000–2002 Estonian Genome Project Foundation, Project manager
2004–2011 Tallinn Children's Hospital, Clinical Geneticist
2009–... Asper Biotech Ltd., Medical Consultant
2011–... Tartu University Hospital, United Laboratories, Department of Genetics, Clinical Geneticist

Special courses

1994 Stockholm Huddinge Hospital/ Perinatal Health Care
1995 Rotary International/ GSE USA Oklahoma
2001 Good Clinical Practice in Clinical Trials
2002 4th Course of Genetic Councelling in Practice, European School of Genetic Medicine, Italy

2004 4th European Metabolic Course
2003 Training in syndrome diagnostics and genetic counselling in the Medical Genetics Clinic at the Family Federation, Helsinki, Finland
2004 15th ENTIS meeting- Prague
2004 Training course „Clinical and Molecular Dysmorphology", Warsaw, Poland
2004 44th European Metabolic Course, Paris
2005 International workshop on Advances in Paediatrics-Metabolomics, Tartu,
2005 Advances in paediatrics- Mitochondrial Medicine 2nd workshop, Poland
2006 Advanced Metabolic Course, Paris
2008 The training on tandem-mass spectrometry in Amsterdam Medical Centre
2009 The training on purine-pyrimidine metabolism defects in Maastricht University Hospital
2010 ERNDNIM Course, Manchester

Scientific work

A total of 7 publications and 15 presentations at international scientific conferences.

In 2010 recieved The Science Award of the Tartu University Hospital (together with T. Kahre, K. Kilk, M.-L. Uudelepp, U. Soomets, K. Õunap) for the study „Long-chain hydroxyacylCoA dehydrogenase deficiency in Estonia".

The scientific work has been focused on medical genetics and inborn errors of metabolism.

Member of the Estonian Society of Human Genetics, Society for the Study of Inborn Errors of Metabolism, Baltic Metabolic Group.

ELULOOKIRJELDUS

Kairit Joost

Sünniaeg ja -koht: 18.12.1967 Jõgeva, Eesti
Kodakondsus: Eesti
Perekonnaseis: Vallaline
Aadress: Biokeemia Instituut
Tartu Ülikool
Ravila 19
Tartu 50411
Phone: +372 731 9172
+372 566 26204
E-mail: kairit.joost@kliinikum.ee

Haridus

1975–1986	Jõgeva Keskkool
1986–1992	TÜ arstiteaduskond pediaatria eriala
2000–2004	TÜ, arstiteaduskond, meditsiinigeneetika residentuur
2007–...	TÜ, neuroteaduste kool, doktorantuur

Teenistuskäik

1992–1993	Tallinna Lastehaigla, internatuur
1993–1994	Tallinna Lastehaigla, epidemioloogiline uuring ISAAC (Incidence of Symptoms of Asthma and Allergy in Childhood), andmekoguja
1994–1996	Tallinna Lastehaigla, pediaater
1996–1997	Krka Slovenia, ravimiesitleja
1997–2000	Glaxo Wellcome, ravimiesitleja
2000–2002	Eesti Geenivaramu, projektijuht
2004–2011	Tallinna Lastehaigla, meditsiinigeneetik
2009–...	Asper Biotech Ltd., meditsiinikonsultant
2011–...	TÜ Kliinikum, Ühendlabor, Geneetikakeskus, meditsiinigeneetik

Erialane täiendus

1994	Stockholmi Huddinge Haigla/ Perinataalmeditsiin
1995	Rotary International/ GSE USA Oklahoma
2001	Kursus „Head kliinilised tavad kliinilistes katsetes“
2002	4th Course of Genetic Councelling in Practice, European School of Genetic Medicine, Itaalia

2004	4th European Metabolic Course
2003	meditsiinigeneetilise konsultatsiooni alane koolitus Soome Perekeskuse Meditsiinigeneetika osakond, Helsingi, Soome
2004	15th ENTIS meeting- Praha
2004	Training course „Clinical and Molecular Dysmorphology”, Varssavi
2004	44th European Metabolic Course, Pariis
2005	International workshop on Advances in Paediatrics-Metabolomics, Tartu,
2005	Advances in paediatrics-mitochondrial medicine 2nd workshop, Poola
2006	Advanced Metabolic Course, Pariis
2008	TandemMS-alane väljaõpe Amsterdami Akadeemilises Meditsiinikeskuses
2009	Puriinide-pürimidiinide diagnostika-alane väljaõpe Maastrichti Ülikooli Haiglas
2010	ERNDNIM Course, Manchester

Teadustegevus

Kokku on ilmunud 7 publikatsiooni ja 15 ettekannet rahvusvahelistel konverentsidel.

2010. aastal pälvis TÜ Kliinikumi teadustöö preemia (koos T. Kahre, K. Kilgi, M.-L. Uudelepa, U. Soometsa, K. Õunapiga) uuringu „Pika ahelaga hüdroksü-atsetüül-CoA dehüdrogenaasi defitsiidi esinemine Eestis“ eest.

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