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MITOCHONDRIAL DISEASE IN CHILDREN – FROM CLINICAL PRESENTATION TO GENETIC BACKGROUND

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Mitochondrial Disease in Children – from Clinical
Presentation to Genetic Background
THESIS FOR DOCTORAL DEGREE (Ph.D.)

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Till Mor

Ju mer man tänker, desto mer inser man att det inte finns något enkelt svar

Nalle Puh

ABSTRACT

Mitochondrial disorders are amongst the most common groups of inborn errors of metabolism. They are caused by deficiencies in the final pathway of the cellular energy production, the mitochondrial respiratory chain. The disorders are clinically and genetically heterogeneous and the aetiology can be found in the mitochondrial, or in the nuclear genome.

This thesis describes children with mitochondrial disorders, with focus on clinical symptoms, disease courses, biochemical abnormalities and genetic causes of disease. The research aimed to increase the understanding of the clinical phenotypes and pathophysiological mechanisms. We also aimed to identify novel disease-causing variants in mitochondrial (mtDNA), as well as nuclear, DNA in order to generate better tools for genetic counselling.

In a study of patients with deficiencies of complex I of the mitochondrial respiratory chain, we observed a variety of clinical presentations. Early-onset of disease and muscle weakness were features in common. Developmental retardation and failure to thrive were seen in a majority of the patients. Causative variants in mtDNA were identified in six of the 11 patients.

Leigh syndrome (LS) is a severe, neurodegenerative disease of early childhood. The genetic aetiology is heterogeneous. In a study of 25 children with LS, we observed early onset of disease, in 80% before six months of age. A subset of patients had a rapidly progressive disease and early death, 60% survived beyond the age of five years. Eight of the patients had a disease causing variant in mtDNA. The age of onset, clinical symptoms or prognosis did not differ significantly between patients with mitochondrial and nuclear mutations in this cohort.

A defect in the *POLG* gene was detected in a patient with Alpers syndrome. He had a heterozygous variant on one allele, the other allele being entirely deleted. The patient had rapid disease progression and died in a valproate induced liver failure.

Massively parallel sequencing of the entire human genome and its implementation in clinical use is a diagnostic leap in the field of mitochondrial disorders. In a cohort of patients with combined deficiencies of the mitochondrial respiratory chain, 31 patients were subjected to whole genome/exome sequencing. A genetic diagnosis was established in 16 of these (52%), so far. Two novel gene defects were identified; *SLC25A26* and *COQ7*. The latter gene encodes an enzyme of the Coenzyme Q (CoQ) biosynthesis. These disorders are responsive to CoQ10 treatment. We demonstrated a new mechanism of treatment using 2,4-dihydroxybenzoic acid in order to bypass the deficient step.

In conclusion, paediatric mitochondrial disorders are severe, progressive and usually multi-systemic. The most common symptoms are often non-specific and the diagnostic procedure is a challenge. The genetic aetiology is heterogeneous, a substantial proportion of the causative variants are found in mtDNA. The phenotype-genotype correlation is poor, making whole genome sequencing an excellent diagnostic tool.

LIST OF SCIENTIFIC PAPERS

- I. Esteitie N, Hinttala R, Wibom R, Nilsson H, Hance N, **Naess K**, Teär-Fahnehjelm K, von Döbeln U, Majamaa K, Larsson NG. Secondary metabolic effects in complex I deficiency. *Ann Neurol* 2005 Oct;58(4):544-52
- II. **Naess K**, Freyer C, Bruhn H, Wibom R, Malm G, Nennesmo I, von Döbeln U, Larsson NG. MtDNA mutations are a common cause of severe disease phenotypes in children with Leigh syndrome. *Biochim Biophys Acta* 2009 May;178 (5):484-90
- III. **Naess K**, Barbaro M, Bruhn H, Wibom R, Nennesmo I, von döbeln U, Larsson NG, Nemeth A, Lesko N. Complete deletion of a *POLG1* allele in a patient with Alpers syndrome. *J Inherit Metab Dis Rep* 2012;4:67-73
- IV. **Naess K**, Bruhn H, Stranneheim H, Freyer C, Wibom R, Engvall M, Nennesmo I, Lesko N, Wredenberg A, Wedell A, von Döbeln U. Combined defects of the mitochondrial respiratory chain complexes – a diversity of clinical presentations and genetic causes. In manuscript.
- V. Lesko N, **Naess K**, Wibom R, Solaroli N, Nennesmo I, von Döbeln U, Karlsson A, Larsson NG. Two novel mutations in thymidine kinase-2 cause early onset fatal encephalomyopathy and severe mtDNA depletion. *Neuromuscul Disord* 2010 Mar;20(3):198-203
- VI. Freyer C, Stranneheim H, **Naess K**, Mourier A, Felser A, Maffezzini C, Lesko N, Bruhn H, Engvall M, Wibom R, Barbaro M, Hinze Y, Magnusson M, Andeer R, Zetterström RH, von Döbeln U, Wredenberg A, Wedell A. Rescue of primary ubiquinone deficiency due to a novel *COQ7* defect using 2,4-dihydroxybenzoic acid. *Med Genet* 2015 Nov;52(11):779-83

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LIST OF ABBREVIATIONS

ADP	Adenosine diphosphate
AS	Alpers syndrome
ATP	Adenosine triphosphate
CK	Creatine kinase
CMMS	Centre for inherited metabolic diseases
CNS	Central nervous system
CoQ	Coenzyme Q
COX	Cytochrome c oxidase
CPEO	Chronic progressive external ophthalmoplegia
CSF	Cerebrospinal fluid
CT	Computed tomography
CVS	Chorionic villus sample
2,4-dHB	2,4-Dihydroxybenzoic acid
DNA	Deoxyribonucleic acid
DPYS	Dihydropyrimidinase
EEG	Electroencephalography
dNTP	Deoxyribonucleoside triphosphate
GABA	Gamma aminobutyric acid
GI	Gastrointestinal
GRACILE	Growth retardation, aminoaciduria, cholestasis, iron overload, lactic acidosis and early death
FADH ₂	Flavin adenine dinucleotide
LHON	Leber's hereditary optic neuropathy
LS	Leigh syndrome
MAPR	Mitochondrial ATP production rate
MCRN	Mitochondrial clinical research network
MEGDEL	3-Methylglutaconic aciduria, deafness and Leigh-like encephalopathy
MELAS	Mitochondrial encephalopathy with lactic acidosis and stroke like episodes

MERRF	Miochondrial encephalomyopathy with ragged red fibres
MIP	Mutation identification pipeline
MLPA	Multiplex ligation-dependent probe amplification
MMA	Methylmalonic acid
MNGIE	Mitochondrial neuro-gastro-intestinal encephalomyopathy
MRI	Magnetic resonance imaging
mtDNA	Mitochondrial DNA
nDNA	Nuclear DNA
NADH	Nicotinamide adenosine dinucleotide
NGS	Next generation sequencing
OXPHOS	Oxidative phosphorylation
PDH	Pyruvate dehydrogenase
PGD	Preimplantation genetic diagnosis
RC	Respiratory chain
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RRF	Ragged red fibres
TP	Thymidine phosphorylase
rRNA	Ribosomal RNA
SAM	S-adenosylmethionine
SDH	Succinate dehydrogenase
SNV	Single nucleotide variants
TK-2	Thymidine kinase 2
tRNA	Transfer RNA
VPA	Valproic acid
WES	Whole exome sequencing
WGS	Whole genome sequencing

1 BACKGROUND

1.1 INTRODUCTION

The first patient with a mitochondrial disease was described in 1962 by the Swedish endocrinologist Rolf Luft at Karolinska Institutet and the biochemist Lars Ernster at Stockholm University (1). The patient was a woman with hypermetabolism. Symptoms had already started in childhood and consisted of profuse perspiration, polydipsia, polyphagia, decreased body weight, progressive asthenia and muscle weakness. Biochemical and morphological studies clearly indicated a mitochondrial disorder. Professor Luft and colleagues were able to demonstrate an uncoupling of the respiratory chain from the final step of adenosine diphosphate (ADP) phosphorylation to adenosine triphosphate (ATP). The genetic cause of this first mitochondrial disease has never been established.

Since then, there has been a remarkable expansion of knowledge in the field of mitochondrial medicine and many patients have been diagnosed. Today, defects in the mitochondrial respiratory chain (RC) are considered to be amongst the most common groups of inborn errors of metabolism, with an estimated lifetime risk of developing disease of approximately 1/5000 live births (2).

Mitochondrial disorders are highly heterogeneous with regard to the clinical phenotype, as well as the genotype. The clinical spectrum is extremely broad, from multi-organ, life-threatening disease at birth to single symptoms with onset in middle age.

The genetic cause of a mitochondrial disease can be found either in the mitochondrial or in the nuclear genome. We expect approximately one third of the paediatric patients to have disease-causing variants in mitochondrial DNA (mtDNA) (3) and the rest to carry pathogenic variants in nuclear genes. To date, more than 250 nuclear genes have been linked to mitochondrial disease (4).

Once a mitochondrial disorder is suspected, the diagnostic procedure is a challenge. There is no specific test to exclude or confirm the diagnosis.

Nevertheless, it is of great importance for these patients and their families to establish a definite diagnosis on the genetic level.

This thesis illustrates the exceptional evolution in the possibilities of settling the exact genetic diagnosis, the most important step being the introduction of next-generation sequencing in clinical use. The techniques for investigating all genes in parallel have not only facilitated the diagnostic work-up, but have also revealed novel genes and new disease mechanisms (4).

‘Respiratory chain deficiency can theoretically give rise to any symptom, in any organ or tissue, at any age, with any mode of inheritance’

A. Munnich

1.2 STRUCTURE AND FUNCTION OF THE MITOCHONDRIA

Mitochondria are organelles that are present in the cytoplasm of all human cells, except the mature erythrocyte. They are structures enclosed in a double membrane consisting of phospholipids. The inner membrane is highly convoluted, which increases the membrane surface and allows a higher capacity for ATP generation (*Figure 1*).

The outer membrane is permeable to most ions and small molecules. The inner membrane is, in contrast, impermeable to most charged and hydrophilic substances, such as ADP, ATP and pyruvate. Specific carriers are required to transport metabolites that are essential for the intramitochondrial processes across the inner membrane (5).

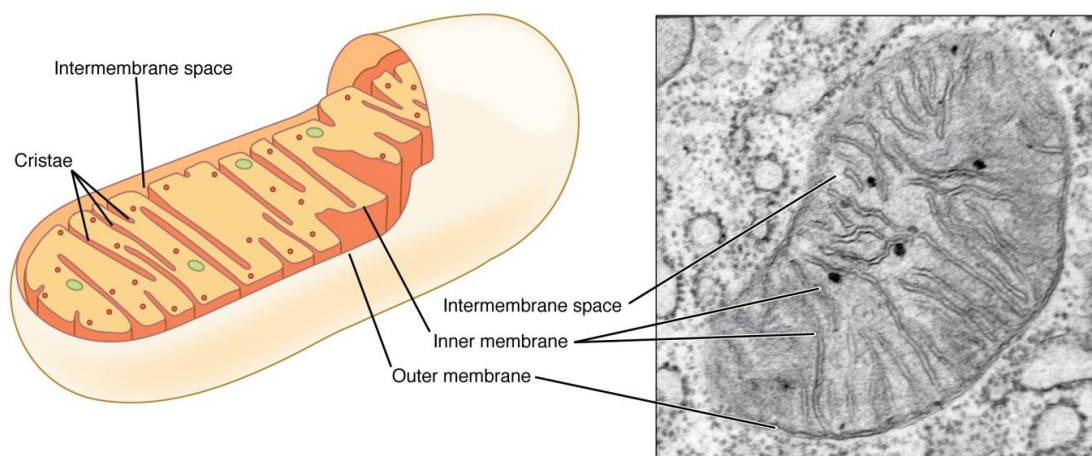


Figure 1. The mitochondrion. The mitochondrial matrix is enveloped in a double membrane, the inner part of which is highly convoluted into so called cristae. The name mitochondrion originates from the Greek ‘mitos’ (thread) and ‘chondros’ (granule or grain-like) (6).

The mitochondria are not separated structures, but a dynamic network, continuously dividing and fusing into new units. The mechanism of mitochondrial fission and fusion is complicated and several proteins are required for the process to work smoothly (7). The fission-fusion machinery is essential for generating new mitochondria, eliminating the old or damaged ones and for distributing mitochondria throughout the entire cell. It also enables an exchange of substrates and energy between mitochondria in the cell.

The crucial function of the mitochondria is to produce energy (ATP) by oxidative phosphorylation. Mitochondria are also highly involved in other cellular processes, such as intracellular calcium homeostasis (8), regulation of programmed cell death (apoptosis) (9), production of reactive oxygen species (ROS) (10), cellular growth (11) and cell signalling (12).

The term mitochondrial disorder usually refers to deficiencies in the final common pathway of aerobic energy production, the oxidative phosphorylation (OXPHOS) process, which takes place in the mitochondrial RC. The five enzyme complexes of the RC are embedded in the inner mitochondrial membrane (*Figure 2*).

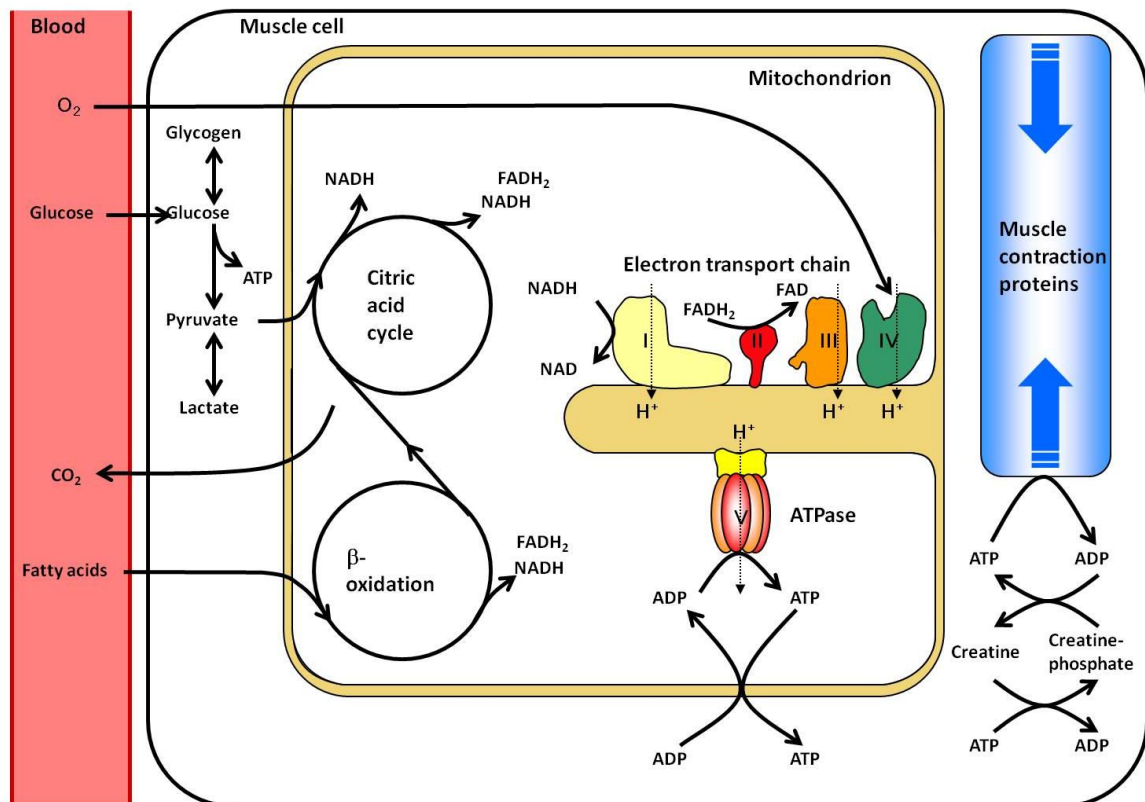


Figure 2. The mitochondrion houses several metabolic processes, such as the pyruvate dehydrogenase complex, the tricarboxylic acid cycle (Krebs cycle), the β -oxidation of fatty acids, parts of the urea cycle, haeme synthesis, the biosynthesis of steroid hormones and the RC. Illustration: Rolf Wibom

1.2.1 The oxidative phosphorylation and generation of ATP

In the cytosolic process of glycolysis, glucose is converted to pyruvate, which can be either converted to lactate or transported into the mitochondrial matrix. Inside the mitochondria, pyruvate is oxidised by the pyruvate dehydrogenase (PDH) complex to form acetyl-CoA, which enters the tricarboxylic acid cycle, which, in turn, generates nicotinamide adenine dinucleotide (NADH) and flavine adenine dinucleotide ($FADH_2$). Acetyl-CoA, NADH and $FADH_2$ are also provided by the β -oxidation of fatty acids (13).

NADH and $FADH_2$ each donate a pair of electrons to the respiratory chain, when oxidised by NADH dehydrogenase (complex I) and succinate dehydrogenase (complex II), respectively. The electrons are then transported to cytochrome b (complex III) by the mobile carrier coenzyme Q (CoQ) and, further on, to cytochrome c oxidase (complex IV) by the other mobile carrier, cytochrome c. The electrons are finally accepted by oxygen to form water. Concomitant with the electron transport, protons are pumped into the intermembrane space, creating a proton gradient across the mitochondrial inner membrane which, in turn, is used by ATP synthase (complex V) to generate ATP from ADP and inorganic phosphate (14).

1.3 MITOCHONDRIAL GENETICS

The OXPHOS process is under dual genetic control and the genetic cause of a mitochondrial disorder can therefore be found in either the nuclear DNA (nDNA) or in the mitochondrial DNA (mtDNA).

1.3.1 Mitochondrial DNA

The mitochondrial genome is double stranded, circular and consists of 16 569 base pairs (*Figure 3*). The sequence was determined throughout its entire length in 1981 by Anderson et al.(15).

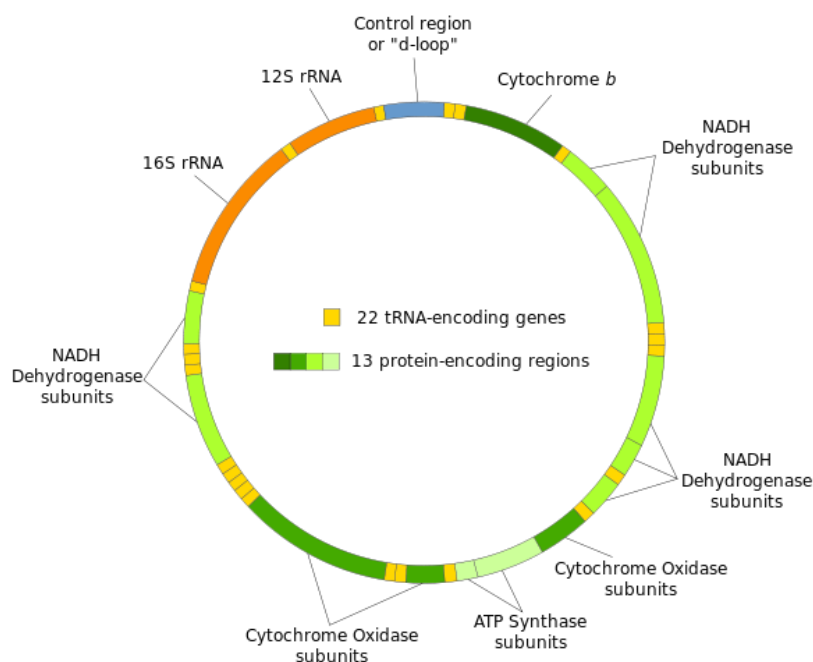


Figure 3. The mitochondrial genome. The included 37 genes encode 13 polypeptides, 22 transfer RNAs (tRNAs) and two ribosomal RNAs (rRNAs). Unlike the nDNA, no introns intervene with the coding parts of mtDNA. The proteins encoded from mtDNA are subunits of the complexes I, III, IV and V of the respiratory chain.

The mitochondrial genome is present in multiple copies in each cell, varying from approximately 100 in the sperm cell to more than 100 000 in the mature oocyte (16). The DNA circles are compacted into small protein-DNA clusters called nucleoids (17).

The replication of mtDNA is independent of the cell cycle and also occurs in post-mitotic tissues. The process is tightly regulated by a number of factors. Essential in this regulation are not only the proteins of the replication fork, but also enzymes involved in the supply of nucleotides (deoxynucleoside triphosphates, dNTPs) and proteins for the structural stabilisation of mtDNA (18).

A certain number of mtDNA copies are needed for survival of the cell. A defect in any part of the replication machinery can result in depletion of mtDNA, which is linked to a number of severe mitochondrial disorders of infancy and childhood (19).

The mtDNA transcription, likewise the replication, relies on nuclear encoded proteins. The translation is partly autonomous, using mtDNA encoded ribosomal and transfer RNAs (20). An increasing number of disorders are caused by defects in mtDNA transcription, translation or posttranslational modifications (21).

Normally, all mtDNA copies within a cell have identical sequences, a situation called homoplasmy. When a mutation occurs in one copy of mtDNA, it can eventually result in heteroplasmy, which means that mixed populations of mutant and wild type DNA coexist in the same cell (22). During mitosis, these populations are randomly segregated to each of the daughter cells. This phenomenon affects both disease expression and inheritance of the disease.

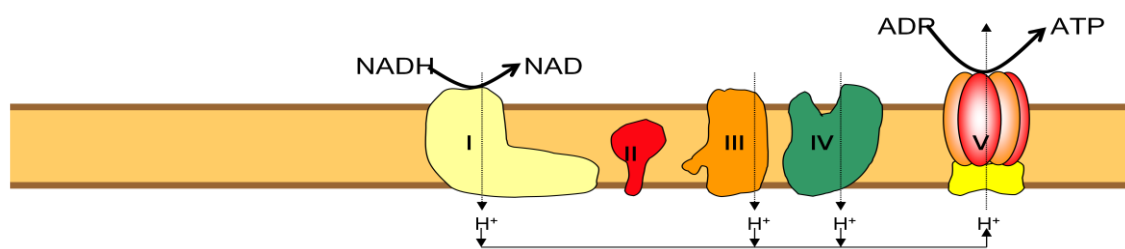
Disease from an mtDNA mutation occurs when a certain fraction of mutant mtDNA is present in the cell. This threshold represents a level when the amount of remaining wild type mtDNA is not enough to maintain the OXPHOS process, resulting in cellular dysfunction. Commonly, the threshold is reached at a level of 60-90% mutated mtDNA, but it varies with the mutation, the tissue involved and probably also between individuals (23).

MtDNA has a higher mutation rate than nuclear DNA, possibly due to its proximity to the RC complexes and the mutagenic free radicals they generate, the lack of non-coding regions and protective histones or a less efficient repair system (24).

Pathogenic variants in mtDNA can be point mutations or rearrangements (deletions or insertions). Mitochondrial tRNA gene mutations account for a major portion of mtDNA-linked disease (25).

From a biochemical perspective, mutations in genes encoding subunits of the RC complexes give rise to isolated enzyme deficiencies, whereas mutations in tRNA genes result in combined enzyme deficiencies. The enzyme deficiencies in cases of tRNA mutations usually include the complexes I, III, IV and V, since they contain subunits encoded from mtDNA (*Figure 4*).

The genotype-phenotype correlation for a certain mtDNA mutation is generally poor, but there are exceptions. Large scale deletions of mtDNA usually present with clinical features of Pearson syndrome (26), Kearns-Sayre syndrome (27) or chronic progressive external ophthalmoplegia (CPEO) (28) Clinical features will be described later.



Complex	I	II	III	IV	V	Total
Subunits encoded from mtDNA	7	0	1	3	2	13
Subunits encoded from nDNA	37	4	10	11	17	79
Total	~44	4	11	14	19	~92

Figure 4. The enzyme complexes of the mitochondrial RC consist of several subunits encoded from specific genes in mtDNA, as well as nDNA. To date, ~92 subunits have been identified (29). Illustration: Rolf Wibom.

The majority of patients with mitochondrial encephalomyopathy, lactic acidosis and stroke-like episodes (MELAS) carry the, above all, most common point mutation in mtDNA: the tRNA mutation 3243A>G in the *MT-TL1* gene. A syndrome involving myoclonic epilepsy with ragged red fibres (MERRF) is commonly caused by an A>G transition at m.8344 in *MT-TK*. Leber's hereditary optic neuropathy (LHON) is caused, in at least 90% of cases, by one of three different point mutations in three genes encoding subunits of complex I (m.3460G>A in *MT-ND1*, m.11778G>A in *MT-ND4* or m.14484T>C in *MT-ND6*) (30).

High mutation loads (95-100%) of a number of different mtDNA mutations may result in the clinical presentation of Leigh syndrome. This has been reported in mutations located in genes encoding RC subunits as well as tRNAs. The most frequently occurring ones are the m.8993T>G/C mutations in the *MT-ATP6* gene. Lower levels of heteroplasmy in these particular mutations often present as neuropathy, ataxia and retinitis pigmentosa (NARP) syndrome.

Mutations in mtDNA are maternally inherited. The paternal mtDNA in the sperm cell is labelled with a ubiquitin tag, which induces rapid targeted proteolysis on entering the oocyte (31).

Large scale deletions are mainly sporadic and usually are not transmitted to the offspring, although inherited deletions have been described (32).

Maternal mtDNA mutations are transmitted to an offspring through a genetic bottleneck, which occurs during the oogenesis. The copy number of mtDNA molecules is highly reduced in each primordial egg cell and consequently a small number of mtDNA molecules (wild-type and mutated) become founders of the entire population of mtDNA in the offspring. This

explains why the children of a mother carrying an mtDNA mutation, display a variety of different mutation loads (33).

1.3.2 Nuclear DNA

The majority of patients with mitochondrial disorders have a genetic defect in the nuclear genome. Approximately 1500 nuclear gene products are necessary for proper mitochondrial function and maintenance (34).

Nuclear encoded proteins essential for the mitochondrial function participate in several pathways, including: (i) subunits and assembly factors for the five RC enzyme complexes, (ii) mtDNA maintenance and expression, (iii) mitochondrial biogenesis and dynamics and (iv) import and export across the mitochondrial membrane (29). Examples of additional pathways are those for the biosynthesis of different factors that are necessary in the OXPHOS process, such as CoQ, haeme and iron-sulphur clusters (35-37).

The overall most frequently affected nuclear gene in mitochondrial disease is *POLG*, which encodes the catalytic subunit of polymerase γ , the sole polymerase replicating mtDNA. The first *POLG* variant associated with disease was described in a family with autosomal dominant CPEO in 2001(38). Since then, more than 150 disease-causing variants have been identified (<http://tools.niehs.nih.gov/polg>).

The mode of inheritance when the disease is caused by defects in a nuclear gene is usually autosomal recessive. Autosomal dominant or X-linked inheritance is also seen.

1.4 CLINICAL FEATURES OF MITOCHONDRIAL DISEASE

Mitochondrial disorders are clinically heterogeneous. Symptoms can emerge from any organ or tissue, although the central nervous system and skeletal muscles are the above all most frequently affected tissues, owing to their high energy demands.

In infancy and early childhood, the disease is often multi-systemic, with involvement of not only the central nervous system (CNS) and muscles, but also the liver, heart, kidneys and bone marrow, to mention the most frequently involved organs. Early onset of the disease indicates a severe defect in the mitochondrial respiratory chain, and this is related to a poorer prognosis (39). Mitochondrial disorders, with an onset in the adolescence or adulthood, are more often single-organ diseases, such as CPEO, or LHON.

1.4.1 Symptoms and signs from the central nervous system

Symptoms from the CNS are seen in the majority of children with mitochondrial diseases (39). The most frequent symptom is a developmental delay (40), which is usually global and affects cognitive, language and motor skills. The end-point cognitive level varies, from mild learning disabilities to severe mental retardation. There is probably no specific cognitive profile since mitochondrial disorders are, in all aspects, extremely heterogeneous. A study by

Turconi et al. indicated a greater impairment in the non-verbal area, particularly the visuo-spatial abilities. Impairment of the verbal short term memory (working memory) was also seen (41). Symptoms from the autism spectrum are seen as well, and one hypothesis is that mitochondrial dysfunction can be part of the disease mechanism in autism spectrum disorders in general (42).

Seizures are a frequent complication of mitochondrial disease at all ages. The exact prevalence is not known, but it is estimated to be approximately 40% (43). Various seizure types may occur and a substantial proportion of patients have mixed seizure-type epilepsy (44). Partial seizures, with or without secondary generalisation, were the most common seizure types in a study by Khurana et al., 2008. Recurrent status epilepticus was seen in as many as 60% of the patients. Also epileptic syndromes, such as West syndrome and Lennox-Gastaut syndrome have been reported (45). Alpers syndrome (AS), due to recessive mutations in the *POLG* gene, is one of the most common mitochondrial syndromes associated with epilepsy (46). Patients with AS often present with focal, myoclonic or complex seizures. Status epilepticus is common, sometimes starting with *epilepsia partialis continua*, followed by a generalised, therapy-resistant status. Electroencephalography (EEG) may initially show characteristic unilateral, occipital, high-amplitude, slow waves with superimposed polyspikes, evolving into a generalised pattern (47). Apart from what is seen in AS, EEG changes are not specific for certain mitochondrial syndromes.

The underlying pathomechanisms of mitochondrial epilepsy are not known. The energy failure is an important factor, but other aspects of a mitochondrial dysfunction, such as ROS production, disturbed calcium homeostasis and apoptosis are likely to contribute (48). It has also been hypothesised that GABA-ergic inhibitory interneurons are more vulnerable to respiratory chain dysfunction, thereby causing an imbalance of neuronal excitation and inhibition (49).

Movement disorders are seen in a substantial proportion of the patients with mitochondrial diseases. In the paediatric population, dystonias are the most frequent symptoms, and are seen particularly in Leigh syndrome (50). This is not surprising, as the syndrome includes lesions in the basal ganglia and other extrapyramidal structures, from which these types of symptoms arise. Ataxia is not classified as a movement disorder, but it is a common symptom in several mitochondrial phenotypes caused by mutations in either mtDNA or nDNA (51).

Neurological symptoms in mitochondrial disease are often progressive, and sometimes rapid, with developmental arrest and loss of skills. The progression can also be stepwise, with a preceding infection or other catabolic situation. Some patients have a very slow progression, appearing like a static condition.

A considerable proportion of patients suffer from acute neurological events, such as strokelike episodes, status epilepticus, coma, vomiting or lethargy.

1.4.2 Symptoms from skeletal muscle

Myopathy is the above all most common single symptom in mitochondrial disease. It is often part of an encephalomyopathy with additional symptoms from other organs, but pure myopathic presentations are seen in the adult, as well as the paediatric population.

The isolated mitochondrial myopathy typically presents with axial and proximal muscle weakness. Distal weakness has been reported in sporadic cases in the myopathic group and occur regularly in the group of patients with mitochondrial polyneuropathies and neurogenic muscle weakness (52). Exercise intolerance and a general fatigue are other hallmarks of the mitochondrial myopathy.

Infantile-onset mitochondrial myopathies are usually severe disorders with pronounced weakness, hypotonia and a need for ventilation support and intensive care. It is important to be aware of a subset of patients with this severe phenotype and a cytochrome c oxidase (COX)-deficiency, who turn out to have a reversible disease. This phenotype was reported by Di Mauro et al in 1981 (53) and was recently shown to be caused by the mtDNA mutation m.14674T>C in the *MT-TE* gene (54).

1.4.3 Ophthalmological manifestations

Ophthalmological findings in mitochondrial disease are common, although the frequency remains unclear. The prevalence reported in three different studies was 81%, 53% and 35% , respectively (40, 55, 56). Grönlund et al included visual impairment due to refraction defects, which is prevalent in the healthy population, and which might explain the high prevalence (81%) in their study.

The extraocular muscles are strongly dependent on a sufficient energy supply, with mitochondria occupying approximately 60% of the cell volume (56). It is therefore not surprising, that external ophthalmoplegia is a common finding in patients with mitochondrial disorders. CPEO may constitute the presentation of a late-onset mtDNA deletion disease and is usually seen in autosomal dominant disorders of mtDNA maintenance.

Optic atrophy is often part of a systemic disease with CNS involvement, as in Leigh syndrome. It can also appear in isolation, such as in patients with LHON. In this disease, the function of the retinal ganglion cells is specifically affected, which results in subacute, painless, bilateral visual failure (57). Occasionally, additional symptoms can be seen, preferentially from the nervous system (58). The onset of disease usually occurs in young adulthood, but childhood onset is also seen.

Pigmentary retinopathy is another rather common finding and was seen in 16% of the patients in a recent study by Zhu et al.(55). It is a non-specific sign of retinal dysfunction which has been associated with a variety of mtDNA and nDNA mutations.

Other abnormalities of the eye and/or vision to be mentioned are cataract, cortical blindness and homonymous hemianopsia.

1.4.4 Hepatopathy and gastrointestinal symptoms

Gastrointestinal and hepatic symptoms are frequently seen, but they are rarely the sole symptom of disease.

Hepatic disease is estimated to occur in 10-20% of patients with mitochondrial disease and usually presents in early childhood (59, 60). The spectrum of severity ranges from transient elevated liver transaminases to acute, fatal liver failure early in life.

The causes of mitochondrial hepatopathies are mainly: (i) disorders of mtDNA maintenance, (ii) defects in mitochondrial protein synthesis, (iii) defects of RC complex assembly and (iv) disorders of the mitochondrial lipid membranes (61).

The first group includes the so-called hepatocerebral mtDNA depletion syndromes, which are characterised by early onset liver failure, hepatomegaly, hypoglycaemia and jaundice. The syndromes are also associated with a spectrum of neurological symptoms, such as seizures, developmental delay or regress, nystagmus and other abnormal eye movements. Among the genes linked to these syndromes are: *POLG*, *DGUOK*, *PEO1*, *MPV17* and *SUCLG1* (62). In *POLG*-associated disease, the acute liver failure is sometimes triggered by antiepileptic medication with valproic acid (63).

The second group includes gene defects in mtDNA (the tRNA or rRNA genes), as well as in nDNA. The nuclear gene *TRMU* encodes the enzyme mitochondrial tRNA 5-methylaminomethyl-2-thiouridylate-methyltransferase, which is essential for the posttranscriptional modification of mitochondrial tRNAs. Mutations in *TRMU* have been linked to infantile onset liver failure, with the unique feature of spontaneous recovery in a substantial proportion of the patients (64).

Patients with intrauterine growth retardation, aminoaciduria, cholestasis, iron overload, lactic acidosis and early death (GRACILE syndrome) (65) belong to the third category of mitochondrial disorders with hepatic involvement. The syndrome was linked to mutations in *BCSIL*, encoding an assembly factor for complex III (66).

The last group is the most recently defined one and consists of defects in the biosynthesis and remodelling of mitochondrial phospholipids. Examples from this group are the patients with 3-methylglutaconic aciduria, deafness and Leigh-like encephalopathy (MEGDEL), due to mutations in the *SERAC1* gene (67). Some of these patients exhibit early-onset liver disease with hepatomegaly, elevated liver transaminases and hypoglycaemia.

Gastro-intestinal symptoms are common in mitochondrial disorders, regardless of the genetic backgrounds, although they are more prominent in association with certain defects. The mechanism behind the symptoms varies and is sometimes caused by a combination of different tissue/organ involvements.

Mitochondrial neuro-gastro-intestinal encephalomyopathy (MNGIE), caused by a deficiency of thymidine phosphorylase, due to mutations in *TYMP*, is characterised by severe

gastrointestinal dysmotility, and even a chronic intestinal pseudoobstruction (68). The syndrome frequently presents in adolescence or young adulthood and additional features are cachexia, peripheral neuropathy and/or ophthalmoplegia. Hearing impairment is common and most patients develop a leukoencephalopathy in adulthood (69). Similar phenotypes, with severe gastrointestinal dysmotility, have been reported with mutations in other genes involved in mtDNA maintenance, *POLG* being one example (70).

Diarrhoea, owing to exocrine pancreas insufficiency, is a cardinal feature of Pearson syndrome. Pearson syndrome is the most frequently seen phenotype in the early onset of a disease caused by a large-scale deletion in mtDNA. Additional symptoms in Pearson syndrome are transfusion-dependent anaemia and lactic acidosis (26). Liver failure, renal tubular acidosis and diabetes mellitus can further complicate the clinical picture.

Patients with Leigh syndrome, described in detail below, often have more diffuse gastrointestinal symptoms, such as failure to thrive, feeding difficulties and vomiting. The causative factors behind these symptoms are probably multiple in nature, including involvement of the CNS, gastrointestinal tract, muscles and peripheral nerves.

1.4.5 Endocrine dysfunction

Steroid hormones are synthesised within the mitochondria and a dysfunction of ATP production leads to impaired hormone production and endocrinological symptoms. Overall, endocrinological manifestations seem to be most common in the phenotypes caused by defects of mtDNA, particularly large-scale deletions and point mutations in tRNA genes. Patients with nuclear gene defects may also present with these symptoms, most frequently involving gene defects affecting mtDNA maintenance and translation (71).

Diabetes mellitus is the best described endocrine manifestation. The mechanism of diabetes in mitochondrial disease is not only a matter of decreased insulin secretion owing to a deficient ATP supply, but is also caused by the impairment of the mitochondrial role as a glucose sensor, connecting glucose metabolism to insulin release (72). Diabetes is reported in a substantial proportion of patients carrying the mutation m.3243A>G in *MT-TL1*, either as a dominant feature in the syndrome of maternally inherited diabetes and deafness (MIDD) or as a part of MELAS. The m.3243A>G mutation is estimated to cause 0.5-2.9% of diabetes mellitus in the population (73, 74). Diabetes mellitus is also frequently seen in Kearns-Sayre syndrome, caused by large scale deletions in mtDNA. In Pearson syndrome, exocrine pancreas dysfunction is a more prominent feature, but diabetes is seen as well (75).

Short stature is common in patients with mitochondrial disorders. In some of these patients a growth hormone deficiency can be established. In other patients, the underlying mechanisms are yet unknown.

Additional endocrinological manifestations that should be mentioned are hypothyroidism, hypoparathyroidism, adrenal insufficiency and hypogonadism.

1.4.6 ‘...any symptom from any organ or tissue’

Kidney

The kidney is highly dependent on aerobic metabolism and is therefore vulnerable to OXPHOS dysfunction. The cortical tubule is especially sensitive, the proximal tubule in particular, since it lacks the capacity to synthesise ATP anaerobically (76).

Renal manifestations of mitochondrial disease have been reported in association with mtDNA mutations, as well as numerous nuclear genes. Most usual is a tubular dysfunction, varying from a mild hyperaminoaciduria, which may only occur during illness or other catabolic situations, to a complete de Toni-Debré-Fanconi syndrome. The more pronounced tubulopathies are frequently associated with large-scale deletions in mtDNA and the clinical features of Pearson or Kearns-Sayre syndrome (77).

A subset of patients develops a glomerular disease. Focal segmental glomerulosclerosis, for one example, has been reported in patients with the mtDNA mutation m.3243G>A (78). Some defects in the CoQ biosynthesis pathway are also associated with glomerular disease and may respond to treatment with CoQ10 (79).

Heart

Cardiomyopathies are the most frequent cardiac manifestations of mitochondrial disease and are estimated to occur in 20-40% of the patients (40, 80). Hypertrophic cardiomyopathies are most common, but dilated, restrictive and other types are also seen. The severity ranges from asymptomatic, sometimes spontaneously reversible conditions, to a severe cardiomyopathy with an early, even prenatal, onset that causes death in early infancy. The presence of a cardiomyopathy in a mitochondrial disorder, regardless of its severity, is associated with a poorer prognosis (80).

Arrhythmias, conduction defects and pulmonary hypertension are examples of other more rare cardiac manifestations (81).

Hearing

Hearing impairment/deafness is a symptom of several mitochondrial phenotypes, caused by mutations in mtDNA, as well as in nDNA. The prevalence varies in different studies, but a minimal frequency is approximately 20% (40) (82). In contrast, hearing loss was found in 80% of patients in a study of 40 children with mitochondrial disease (83). Hearing impairment is not always part of a multi-systemic disorder. Nonsyndromic hereditary hearing loss sensitive to exposure to aminoglycosides, is an example. This clinical entity is caused by the mtDNA mutation m.1555G>A in *MT-RNR1* (84).

1.4.7 Leigh syndrome

Leigh syndrome (LS), or subacute necrotising encephalopathy, is a progressive neurodegenerative disorder of infancy and early childhood. It is the most common paediatric mitochondrial syndrome. In a study from Western Sweden the preschool incidence of LS was 1/ 34 000 (59).

The syndrome was first described in 1951 by the pathologist, Denis Leigh (85). He reported unique findings in the brain of an eight month-old boy that died of a rapidly progressive neurological disease. He had focal, bilaterally symmetrical necrotic lesions extending from the thalamus to the pons and the posterior columns of the spinal cord. Later reports have confirmed that LS is primarily a disease of the deep grey matter and sometimes involving the white matter. Lesions are characteristically seen in the basal ganglia, thalami, brainstem, cerebellum and spinal cord and consist of areas of demyelination, gliosis, necrosis, spongiosis and vascular proliferation (86).

In modern imaging techniques, the clinical diagnosis of the Leigh/Leigh-like syndrome is based on typical findings of bilateral, symmetric lesions in the basal ganglia and/or brainstem and other central structures seen on Magnetic Resonance Imaging (MRI) or Computed Tomography (CT) of the brain (*Figure 5*).

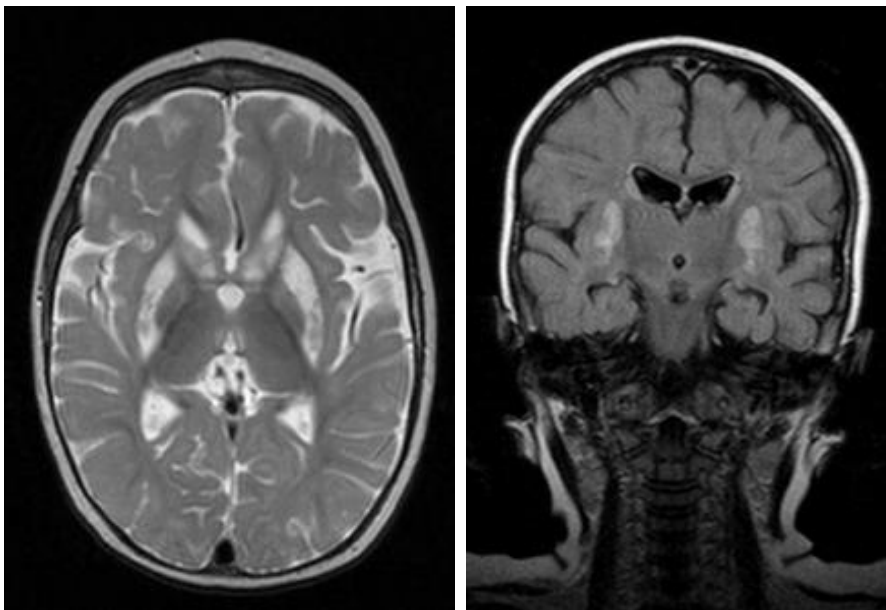


Figure 5. MRI of the brain in a boy with Leigh syndrome. The left picture shows axial T2 weighted images with bilateral symmetrical lesions in the putamen and caput nucleus caudatus. The right picture is a coronal FLAIR image showing bilateral symmetrical abnormalities in the putamen and corpus nucleus caudatus. Signs of atrophy.

Widely used additional clinical criteria for the diagnosis are: (i) progressive neurological disease with motor and/or cognitive delay and (ii) clinical signs or symptoms indicating brainstem and/or basal ganglia dysfunction. A third criterion initially included elevated serum or cerebrospinal fluid (CSF) lactate, indicating abnormal energy metabolism (87). Since the lactate levels are sometimes normal in patients with severe RC disease, the following revision of the criteria (iii) has been suggested: abnormal energy metabolism indicated by a severe defect in OXPHOS or PDH complex activity, a molecular diagnosis in a gene related to mitochondrial energy generation, or elevated serum or CSF lactate (88).

The onset of the disease is usually early, in the majority of patients before two years of age (89). Later forms do exist, although rarely (90).

In the typical clinical course, the initial development is normal. Symptoms often present during infections or other illnesses. The neurological symptoms include developmental delay/arrest, followed by loss of skills, axial hypotonia, increasing tonus in the arms and legs, ataxia and dystonia. Ophthalmological abnormalities, such as nystagmus and optic atrophy, are frequently seen (91), as well as sensorineural hearing impairment and epilepsy. Additionally, a diversity of non-neurological symptoms, such as cardiomyopathy, hepatopathy, renal tubular dysfunction or hormonal deficiencies, may constitute parts of the phenotype.

Leigh syndrome is most usually caused by a dysfunction of the mitochondrial respiratory chain, although the syndrome can be seen in other inborn errors of metabolism. It is a common phenotype in different conditions that causes severe failure of oxidative metabolism in the mitochondria of the developing brain.

The underlying genetic causes are heterogeneous. More than 75 different nuclear genes are reported to be causative (88). A number of mtDNA mutations are also known to cause LS. The phenotype is usually associated with high levels of heteroplasmy (>90 %). Most well-characterised are the mutations m.8993T>C/G in *MT-ATP6* (92) (93).

1.4.8 Alpers syndrome

Alpers syndrome (AS), also named Alpers-Huttenlocher syndrome, is another neurodegenerative mitochondrial encephalopathy of infancy and early childhood (94) (95). The phenotype is characterised by intractable epilepsy, developmental regression and hepatopathy with or without liver failure.

The disease primarily affects grey matter in the brain, particularly the cerebral cortex, cerebellum and thalami. Pathology in the brain includes spongiosis, astrocytosis and neuronal loss. In the liver, hepatitis with fatty degeneration, hepatocyte loss, bile duct proliferation and fibrous scarring, with or without cirrhosis, have been described (96).

The onset of the disease typically occurs in infancy, but later presentations also occur (97). Birth and initial development are usually normal, although some patients have a slight developmental delay. Failure to thrive and episodes of frequent vomiting are other unspecific, early signs of the disease. The onset may be acute/subacute, often with a preceding infection. Similar to LS, psychomotor developmental arrest and progressive loss of skills are common. In contrast to LS, patients with AS have a more pronounced loss of cognitive abilities owing to the cortical neurodegeneration. Seizures are the presenting features in 50% of the patients (98). Mixed types of seizures are seen. Focal motor seizures and myoclonia are the most common seizure types. A substantial proportion of the patients experience generalised or focal status epilepticus (99). Hepatopathy is usually not a presenting symptom, but occurs later in the disease. In approximately 50 % of the patients, the liver involvement is associated

with exposure to sodium valproate (100). Additional symptoms such as hypotonia, ataxia and cortical blindness are frequently present in the phenotype (101).

In 1999, AS was found to be associated with recessive mutations in the *POLG* gene (102). Although *POLG* mutations underlie the major portion of AS, mutations in other nuclear genes affecting replication, transcription or translation of mtDNA, have been reported to cause the phenotype (103). In several cases, the genetic aetiology remains unidentified.

1.5 DIAGNOSING MITOCHONDRIAL DISEASE

The diagnostic procedure, following a suspicion of mitochondrial disorder, is an extraordinary challenge owing to the extreme heterogeneity of the clinical and biochemical features and the fact that there is no single, specific test to confirm or exclude a diagnosis of mitochondrial disease. Relevant findings have to be merged to give a general picture.

1.5.1 Clinical phenotyping

A detailed medical and family history and a thorough examination are essential for the further diagnostics. The family history may help to discriminate between maternal (indicating an mtDNA defect) and Mendelian inheritance of the disease.

Clinical investigations include neurological, cardiac and ophthalmological evaluations and assessments of hearing, growth and psycho-motor development. The finding of multiple organ involvement, especially the brain and muscles, further strengthens the suspicion of a mitochondrial disorder. The mapping of clinical symptoms and signs also serves to establish the extent of disease in order to plan the management and follow-up of the specific individual.

Many of the more frequent symptoms, such as developmental retardation, hypotonia and failure to thrive, are non-specific and seldom raise the suspicion of a mitochondrial disorder. Other symptoms, or constellations of symptoms, are less frequent and more specific and point directly to the mitochondria. Ataxia, external ophthalmoplegia and renal tubulopathy are examples of 'red flags', signalling a potential mitochondrial disorder (104). Certain constellations of symptoms may even be clues to a specific mitochondrial syndrome, such as a combination of stroke-like episodes, diabetes and hearing impairment, strongly suggesting the MELAS syndrome.

'Paediatric mitochondrial disorders can be accompanied by normal muscle morphology, normal plasma lactate, normal mitochondrial enzymes on skeletal muscle, normal mtDNA mutation screening and a non-classical clinical presentation.'

F. Scaglia

1.5.2 Neuroimaging

Neuroimaging is important in all patients with CNS involvement. Structural MRI is the standard investigation. Modern functional brain imaging methods, such as magnetic

resonance spectroscopy, diffusion weighted imaging and perfusion MRI may provide valuable information regarding brain metabolism (105, 106).

Non-specific findings of cerebral atrophy or leukodystrophy are common. Certain imaging patterns are more distinct, and may be helpful in further biochemical and genetic investigations, such as identification of typical features of Leigh syndrome or Alpers syndrome. Stroke-like lesions, predominantly located in grey matter and not following vascular territories, suggest a MELAS syndrome (107, 108).

1.5.3 Clinical chemistry

Routine parameters, such as a full blood count, glucose, creatine kinase (CK) and laboratory parameters of liver, parathyroid, thyroid and renal function are evaluated to characterise the systemic involvement of the disease.

Lactic acid is an important, although non-specific biomarker of mitochondrial disease. A substantial proportion of patients may have consistently normal, or minimally elevated, lactate levels in the blood, as well as the CSF (39). Conversely, elevated blood or CSF lactate levels are seen in a range of conditions not linked to RC disorders. Inappropriate collection or handling of the samples may also result in a high concentration of lactate in the sample (109).

The more specific metabolic work-up serves to exclude other metabolic differential diagnoses and to find abnormalities, which further strengthen the suspicion of a mitochondrial disorder. Urinary organic acids are included in the diagnostics of virtually all types of metabolic disorders. Patients with mitochondrial disorders may have normal excretion, although abnormalities frequently occur. Most common is a non-specific increased excretion of lactate. Metabolites from the Krebs cycle, such as fumarate and malate, may indicate an RC dysfunction, but they are also found in normal urine, especially in catabolic situations (110). Excretion of 3-methyl glutaconic acid is normally hardly detectable in urine and is highly suggestive of an OXPHOS disorder (111). Dicarboxylic aciduria may occur owing to a secondary inhibition of mitochondrial fatty acid β -oxidation (112). The opposite scenario, a primary fatty acid oxidation disorder with secondary OXPHOS dysfunction, is also well-known. (113).

Quantitative analyses of amino acids can be performed in urine, plasma and CSF. Generalised hyperaminoaciduria is the sign of a tubulopathy, which is the most typical renal manifestation of mitochondrial disease (78). Alanine levels in plasma and/or CSF may be elevated, since alanine, like lactate, is derived from pyruvate in situations of metabolic de-compensation (12).

Carnitine levels in plasma may be low, occurring secondarily to a renal tubulopathy which causes carnitine loss via the urine. Another loss may result from increased consumption due to binding of acyl groups from acylCoA and excretion of acylcarnitine esters in the urine (114).

Acylcarnitine profiles may reveal primary organic acidemias, primary fatty acid oxidation disorders or a secondary fatty acid oxidation dysfunction due to the OXPHOS defect (115).

1.5.4 Muscle biopsy

Muscle biopsy is the golden standard procedure in investigations of mitochondrial function. Skeletal muscle is readily available for a percutaneous biopsy, being rich in mitochondria and among the most frequently affected tissues. We perform biopsies from *M. Tibialis anterior*, under local anaesthesia. Approximately 50 mg is the minimum for the complete investigation (Figure 6). A skin biopsy specimen is taken at the same time to obtain fibroblasts for future biochemical and molecular analyses.

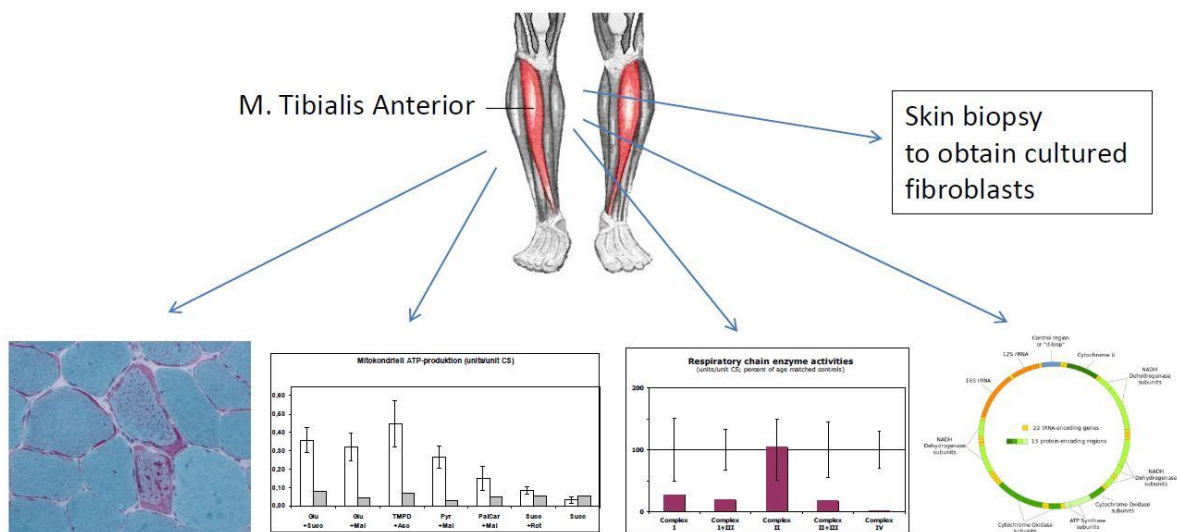


Figure 6. Percutaneous muscle biopsy. The tissue sample is used for (from the left): (i) Morphological investigations. A pattern of ragged red fibres is seen in the picture. (ii) Measurements of MAPR, results presented in filled bars, compared to healthy controls in open bars. (iii) Enzyme activity of the mitochondrial RC, results presented in red bars. Normal average $\pm 2SD$ indicated. (iv) MtDNA analyses.

Muscle morphology is studied by means of light and electron microscopy, using histochemical and immunohistochemical methods (116). The finding of ‘ragged red fibres’ (RRF) is strongly suggestive of a mitochondrial disorder. RRF is a pattern caused by subsarcolemmal accumulation of mitochondria. The presence of fibres deficient in COX activity is another hallmark of mitochondrial disease. Neither RRF nor COX-negative fibres are specific for a mitochondrial disorder, but they may appear secondarily to other, non-mitochondrial myopathies (117). Sequential staining for COX and succinate dehydrogenase (SDH) (complex II) is used to better see the sometimes mosaic pattern of COX-negative fibres (118). Electron microscopy may demonstrate a variety of abnormalities associated with mitochondrial disease. The mitochondria may appear enlarged, with abnormal shapes, absent cristae and paracrystalline inclusions (119).

The mean ATP production rate is determined in mitochondria from a fresh muscle sample. The analysis has to be performed within one hour from the biopsy, because the method requires intact, respiring mitochondria. A sensitive bioluminescence method is used (120).

Polarographic studies of oxygen consumption are an alternative for assessing the OXPHOS rate (121).

Spectrophotometric methods are used to analyse activities in isolated and combined enzyme complexes of the respiratory chain. At our centre, we measure activities of complex I (NADH coenzyme Q reductase), I+III (NADH cytochrome c reductase), II (succinate dehydrogenase), II+III (succinate cytochrome c reductase) and IV (cytochrome c oxidase). The results are reported in relation to citrate synthase, a mitochondrial matrix enzyme having good correlation with the mitochondrial mass (122).

1.5.5 Molecular genetics

Further genetic tests are performed based on the findings in the muscle biopsy. Isolated enzyme complex deficiencies indicate mutations in genes encoding subunits of the complex or assembly factors. Complexes I, III, IV and V contain subunits encoded by mtDNA as well as nDNA, whereas a complex II deficiency is expected to be caused by mutations in nuclear genes. Combined enzyme deficiencies including complexes I, III, IV and V indicate a deficiency of mtDNA expression or maintenance. Causative mutations may be found in mitochondrial tRNA genes, but more often in nuclear genes.

Occasionally, genetic analyses are performed without a preceding mitochondrial assay in muscle tissue. LHON is an example of that, with a specific clinical picture which, in more than 90% of the patients, is caused by one of three different mtDNA mutations.

Sequence analysis of the entire mitochondrial genome is usually the first step in the molecular part of the diagnostic procedure. It is relatively easily done and, if negative, rules out maternal (mitochondrial) inheritance of the disease. MtDNA mutation analyses are preferably performed in muscle tissue, since mtDNA molecules harbouring point mutations or large deletions tend to accumulate in non-dividing cells, such as muscle and nerve cells, but are eliminated in the rapidly dividing blood cells (123). Urinary epithelial cells and buccal mucosa cells are alternative cell types, with the advantage of a non-invasive sample collection (124).

A Southern blot analysis of mtDNA from muscle is often included. The analysis detects rearrangements (deletions and insertions) or mtDNA depletion in comparison with a normal control (125).

Once a causative mutation in mtDNA is excluded, hundreds of nuclear genes remain to be investigated. Sanger sequence analyses of selected genes are seldom cost-effective since identical signs and symptoms may be caused by mutations in many different genes.

The new techniques of massively parallel DNA sequencing have greatly increased our ability to establish a genetic diagnosis in patients with mitochondrial disorders, and have recently been implemented in the clinical setting (126, 127). The human genome contains three billion base pairs, approximately 1-2% of these being located in coding regions and are translated into proteins (128). The majority of disease-causing mutations are located in these coding regions (exons) and, therefore, whole exome sequencing (WES) was the analysis first introduced for clinical use. The method requires sequence capture (enrichment of specific regions of the genome) before sequencing (129). As prices have fallen and techniques have further developed, the use of whole genome sequencing (WGS) is preferred and has gradually been implemented at many centres for mitochondrial diagnostics. WGS enables variant detection also in non-coding regions, such as the introns. A WES analysis identifies approximately 20 000 single nucleotide variants (SNVs) per genome, whereas WGS identifies as many as four million. This large amount of data requires powerful bioinformatic tools. We use an in-house tool: Mutation Identification Pipeline (MIP) (<https://github.com/henrikstranneheim/MIP>), described in detail in Paper IV. In this pipeline, variants are scored according to allele frequency using dbSNP (<https://www.ncbi.nlm.nih.gov/SNP/>, (130)), Exome aggregation consortium (ExAC, <http://exac.broadinstitute.org>, (131)) and an in-house database. Variants with an allele frequency of >0.01 in the normal population are considered to be unlikely to cause these rare autosomal recessive disorders. The potentially damaging properties of a variant are determined *in silico*, using different software tools e.g., Combined annotation-dependent depletion (CADD, cadd.gs.washington.edu (132)), Sorting intolerant from tolerant (SIFT, <http://sift.jcvi.org/> (133)) and Polymorphism phenotyping v.2 (PolyPhen-2, <http://genetics.bwh.harvard.edu/pph2/> (134)). Filtered and scored genomic data are eventually evaluated in relation to the patient's clinical symptoms, biochemical findings and pattern of inheritance at weekly multidisciplinary meetings with clinicians, geneticists and bioinformaticians.

The clinical WES/WGS analysis is targeted on genes which have previously been described and validated as causative of mitochondrial or other metabolic disorders (135). We use an in-house, manually created, continuously updated database of currently >680 genes (dbCMMS, <http://www.karolinska.se/for-varldgivarer/kliniker-och-eheter-a-o/kliniker-och-eheter-a-o/karolinska-universitetslaboratoriet/cmms---centrum-for-medfodda-metabola-sjukdomar/genetisk-diagnostik/>). If no causative variants are detected, the analysis can be expanded to all known monogenic disease genes. With the written informed consent of the parents, the full genome can be analysed in the search for yet unknown genetic causes of disease, as part of a research project.

1.5.6 Prenatal diagnostics

Prenatal diagnostic analyses of mitochondrial disease are usually based on genetic findings, although prenatal biochemical analyses of OXPHOS function have been used (136).

In families with a disorder caused by identified mutations in a nuclear gene, molecular analyses in a chorionic villus sample (CVS) or cultured amniocytes can be performed in a customary manner. A preimplantatory genetic diagnosis (PGD) may also be an option for these families.

Prenatal diagnostics of mtDNA mutations are more complicated owing to the fact that most pathogenic mtDNA mutations are heteroplasmic. The fraction of mutated mtDNA in a CVS may not reflect the level of mutation in other fetal tissues. The mutation load may also change during development and throughout life (137). The more common mutations m.8993T>C/G are known to show an even tissue distribution and the mutation load of these variants does not appear to change significantly over time. The thresholds for a severe clinical expression in these mutations are reported to be 60-70% for the T>G and 80-90% for the T>C (138). Several prenatal diagnostic analyses have been performed successfully in families with these mutations (139).

Preimplantatory genetic diagnostics are currently used to a limited extent. Different percentage levels of mutated mtDNA are used as cut-offs in the decision to transfer an embryo to the uterus (140). Hellebrekers et al. studied mutation levels of different pathogenic mtDNA mutations in several families. They found that mutation levels of 18% or less were associated with a 95%, or higher, chance of being clinically unaffected (137). This percentage level may be used as a rather safe cut-off level in a PGD (141).

2 AIMS

The aims of the research presented here were:

- To increase understanding of the clinical phenotypes and pathophysiological mechanisms in patients with mitochondrial disease
- To identify correlations between genotypes and phenotypes in cohorts of patients with mitochondrial disease in order to generate better tools for predicting disease development and prognosis
- To identify novel disease-causing variants in mitochondrial, as well as nuclear, DNA in patients with mitochondrial disease in order to generate better tools for genetic counseling

3 PATIENTS AND METHODS

3.1 PATIENTS

Patients in all the studies were collected from a total of approximately 1200 children, admitted to the Centre for Inherited Metabolic Diseases (CMMS) with a suspected mitochondrial disorder.

Mitochondrial investigations have been performed at CMMS since 1990. A total of more than 2200 patients have been admitted for muscle biopsies, more than half of them being children under 18 years of age. Approximately 20-25% of the children were diagnosed with a verified or highly suspected mitochondrial disorder.

All results have been discussed at regular meetings with clinicians from paediatric, neuropaediatric and neurological units, pathologists, biochemists and molecular biologists. A plan for proceeding with genetic and other laboratory analyses, in order to establish the diagnosis, was made. Since the beginning, 25 years ago, there has been an amazing evolution regarding the possibilities of finding the genetic cause of the disease, especially with the introduction of Next Generation Sequencing (NGS) on a clinical platform.

In order to facilitate and organise the long-term, ongoing investigations of a considerable number of patients, we have built up an in-house clinical database for all patients admitted to the CMMS for a muscle biopsy. The database includes information on clinical signs and symptoms, neuroimaging findings, biochemical abnormalities, morphological and biochemical results from muscle biopsies and genetic findings. Data are collected from referral notes. We also contact the local doctor to obtain additional information. When a causative diagnosis is established, we include that in the database. In this database, we can search for patients with a particular phenotype, biochemical abnormality or genetic defect, in order to proceed with further analyses or to include patients in clinical studies.

Patients in Paper I were all under 18 years of age and had decreased activity of complex I (NADH dehydrogenase) of the mitochondrial respiratory chain.

In Paper II, we studied a group of 25 children (under 18 years of age at the time of investigation) with Leigh syndrome. We used the following inclusion criteria: (1) progressive neurological disease with motor and/or cognitive developmental delay, (2) signs or symptoms of brainstem and/or basal ganglia disease and (3) characteristic neuropathological findings at autopsy or characteristic features on neuroimaging. Typically seen abnormalities on imaging were either bilateral, symmetrical hypodensities in the basal ganglia/brainstem on CT or areas of hyperdensity in the basal ganglia/brainstem on T2-weighted MRI.

The patient in Paper III was a boy with Alpers syndrome, clinically defined by psychomotor developmental delay/arrest, epilepsy and hepatopathy.

In Paper IV, we studied a cohort of 55 children with combined enzyme deficiencies of the mitochondrial respiratory chain. We used the following inclusion criterion: activities below

the control range (± 2 SD of the average activity) of more than one of the enzyme complexes, measured in isolated mitochondria from muscle tissue. The patients in Papers V and VI belonged to the group described in Paper IV.

3.2 METHODS

3.2.1 Clinical history, neuroimaging and routine clinical chemistry

Patients in all studies were clinically characterised by reviewing their medical records. A substantial proportion of children came to our clinic for examination and a detailed history of the child and family was obtained. A careful neurological examination was performed in all patients and several were also subjected to ophthalmological and cardiac investigations. Depending on the clinical picture, selected cases were subjected to audiography, electromyography, nerve conduction studies and measurements of the visual evoked potential.

Magnetic resonance imaging of the brain was performed in most patients with symptoms from the CNS. A few patients in the first studies were only subjected to computed tomography.

The results of biochemical analyses performed at local laboratories, such as blood and liver function parameters, CK and lactate in blood and/or CSF, were obtained and documented.

3.2.2 Organic acids in urine

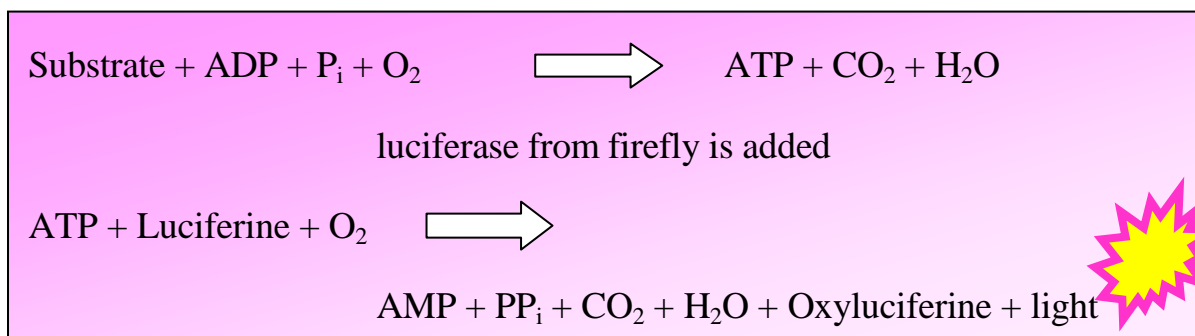
Organic acids in urine were analysed in a major portion of the patients in all studies. All analyses were performed and interpreted at the CMMS. We used gas chromatography combined with mass spectrometry, as described previously (142).

3.2.3 Mitochondrial investigations in muscle

All patients were subjected to a percutaneous muscle biopsy, except for two patients in Paper II. One of these patients was diagnosed with Leigh syndrome at autopsy. The other patient was a monozygotic twin brother of a patient included in the study.

ATP production rate and respiratory chain enzyme activities

A sensitive bioluminescence method was used to determine the mitochondrial ATP production rate (MAPR) in mitochondria isolated from muscle (120). Different substrates from the metabolism of carbohydrates and fat are used in the reaction below. Light is measured in a luminometer and the values correlate with the ATP production rate.



Respiratory chain complex activities were determined with standard spectrophotometric methods (122).

In patients investigated between 1991 and 2000, MAPR, respiratory chain enzyme activities, glutamate dehydrogenase and citrate synthase activities were determined according to the methods described by Wibom et al 1990 (120). Patients investigated later than 2000 were analysed with an improved set using the same method (122), which included the determination of complex I activity (NADH-coenzyme Q-reductase). Results diverging more than ± 2 standard deviations from the control group were considered pathological.

In Paper VI, mitochondrial oxygen consumption was determined in fibroblasts, instead of MAPR, as described in the Supplement of the paper.

Morphological analyses

Morphological examinations of skeletal muscle, including electron microscopy and enzyme histochemical stainings, were performed as described previously (116).

3.2.4 TK2 enzyme assay

In Paper V, an assay to measure TK2 activity was used, with the recombinant wild type enzyme and the enzyme translated from the mutated gene (mutation c.388C>T). The method is described in detail in Paper V.

3.2.5 Measurement of ubiquinone levels

Ubiquinone was quantified in mitochondria isolated from muscle biopsies, cultured fibroblasts and total cell extracts of fibroblasts (Paper VI). The analysis was performed using ultra-pressure liquid chromatography (UPLC)-tandem mass spectrometry. The method is described in detail in the Supplement of Paper VI.

3.2.6 Molecular genetics

The methods used for the genetic analyses are described in detail in the original publications.

DNA extraction

Total DNA (mtDNA and nDNA) was extracted from whole blood, cultured fibroblasts and skeletal muscle using standard commercial extraction kits.

Mitochondrial DNA analyses

Complete mtDNA sequence analyses were performed in patients in Papers I, II, IV and VI. We used muscle tissue preferentially, but occasionally fibroblasts as a DNA source. A previously described standard method was employed (143). Sequence data were compared with the revised Cambridge reference sequence for human mtDNA (https://www.ncbi.nlm.nih.gov/nucleotide/?term=NC_012920.1). Variants were searched for in the human mitochondrial genome databases: MITOMAP (A Human Mitochondrial Genome Database, www.mitomap.org 2016) and the mtDB Human mitochondrial genome database (www.mtodb.igp.uu.se, (144)). A blood sample from the mother was requested, when a variant suspected to be disease-causing was identified.

Six patients in Paper I were screened for mtDNA mutations by conformation-sensitive gel electrophoresis and subsequent sequence analysis (145).

Mitochondrial DNA in muscle tissue was analysed using Southern blot, to detect large-scale deletions and other rearrangements, after cleavage with the restriction enzyme PvuII (Paper IV). The method has been described previously by Larsson et al., 1990 (125).

Mutation levels were quantified with last-hot-cycle restriction fragment length polymorphism (RFLP) analyses. The method is described in detail in the Supplement of Paper II.

Sequence analyses of nuclear genes

Nuclear genes were sequenced by amplification of both strands of all coding exons with flanking intron regions, using M13-tailed primers. To validate findings from WES or WGS (Paper IV), only exons containing the variants were amplified and sequenced.

Multiplex Ligation-dependent Probe Amplification Analysis (MLPA)

An MLPA analysis was used in Paper III, to detect deletions or duplications in the *POLG* gene. We used the MLPA kit P010 (MRC Holland, Amsterdam, The Netherlands).

Whole exome and whole genome sequencing

Massively parallel WES or WGS was employed in the study on children with combined enzyme deficiencies of the respiratory chain (Paper IV). Sequencing was performed as previously described (146, 147). Data were analysed using the Mutation Identification Pipeline (MIP) (148). MIP performs quality control, alignment, coverage analysis, variant discovery, recalibration and annotation, sample/data integrity checks and ranking of the detected variants according to the disease potential. MIP also separates ‘clinical variants’ in genes known to cause inborn errors of metabolism from ‘research variants’ in genes not previously known to cause a metabolic or any other disease. The ‘clinical’ genes are included in an in-house database (dbCMMS), which is updated continuously. Genomic data from MIP are integrated with clinically relevant data in a visualisation tool (Scout: <https://github.com/Clinical-Genomics/scout>), with a user-friendly web browser-based interface for clinical evaluation.

4 RESULTS

Children with complex I deficiencies (Paper I)

This group of 11 patients, from seven families, was clinically heterogeneous. They had some features in common, such as early onset of disease, muscle weakness and exercise intolerance. All patients, except for patient 11 had, in addition, a progressive course of the disease, developmental delay and failure to thrive. Patients were classified into four different clinical subgroups. Three patients had Leigh or Leigh-like syndrome. Another three patients had neonatal lactic acidosis, encephalomyopathy and hypertrophic cardiomyopathy. Four siblings had varying degrees of encephalomyopathy, neuropathy, optic atrophy, hearing impairment and cardiac involvement. Patient 11 differed from the others in having a rather stable myopathic condition with hearing loss, cataract and hypertrichosis. There was no correlation between the clinical phenotype and residual complex I enzyme activity or MAPR.

Biochemically, all patients had a moderate decrease in MAPR, on average, 31% (range 0-63%), with substrates entering at the level of complex I (glutamate + malate). Other substrates yielded normal MAPR. The patients also had a reduced mean maximal MAPR (average 22%, range 0-56%). An increased succinate oxidation rate (in complex II) was seen in the absence of rotenone. The addition of rotenone did not result in any further increase in the rate. Elevated urinary excretion of malate and fumarate was observed in five of the patients.

Table I Clinical phenotypes and genetic findings.

Patients	Clinical phenotype	mtDNA mutation
1♀	Leigh-like syndrome	
2♀	Leigh syndrome	m.10191T>C <i>MT-ND3</i>
3♀	Leigh syndrome	m.14487T>C <i>MT-ND6</i>
4♂	Neonatal lactic acidosis, encephalomyopathy, hypertrophic cardiomyopathy	
5♂	Neonatal lactic acidosis, encephalomyopathy, hypertrophic cardiomyopathy	
6♂	Neonatal lactic acidosis, encephalomyopathy, hypertrophic cardiomyopathy	
7♂	Encephalomyopathy, hearing loss, optic nerve atrophy	m.11778G>A <i>MT-ND4</i>
8♂	Encephalomyopathy, hearing loss, optic nerve atrophy, cardiac involvement	m.11778G>A <i>MT-ND4</i>
9♂	Encephalomyopathy, hearing loss, optic nerve atrophy	m.11778G>A <i>MT-ND4</i>
10♀	Encephalomyopathy, cardiac involvement	m.11778G>A <i>MT-ND4</i>
11♀	Muscle weakness, hearing impairment, cataract, hypertrichosis	

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All probands were screened for mtDNA mutations. Pathogenic mutations were found in six patients from three families. The mutations m.10191T>C and m.14487T>C have been reported previously in patients with Leigh syndrome. The m.11778G>A mutation is one of the three most common ones in LHON. Patients with additional neurological symptoms have been described previously as LHON+, which was the clinical picture of the four siblings (patient 7-10).

Children with Leigh syndrome (Paper II)

During a period of eighteen years (1989-2006), a total of 25 children were clinically diagnosed with LS and referred to the Centre for Inherited Metabolic Diseases, Karolinska University Hospital, for further investigations.

Despite the use of the same diagnostic neurological criteria for LS, the cohort displayed a broad spectrum of clinical features (*Figure 7*). Developmental delay/intellectual disabilities, hypotonia, dyskinesia, failure to thrive and gastrointestinal symptoms were present in the majority of the children. Epileptic seizures were reported in 64% of the patients and progressed to drug resistance in a few of them. Different ophthalmological manifestations were present in 68 % and hearing impairment in 20%. A third of the patients had liver involvement. Renal tubulopathy was seen in 12 % of the patients.

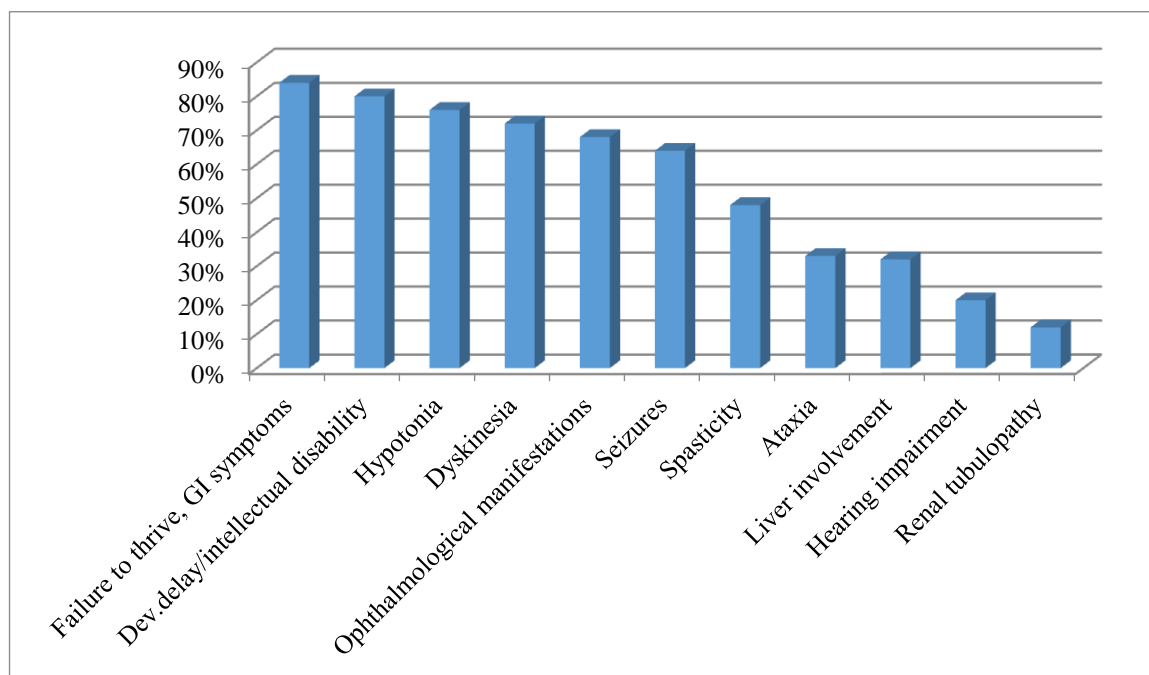


Figure 7. Clinical symptoms. Failure to thrive and/or gastrointestinal symptoms were frequent (84%). Non-neurological symptoms were seen in a substantial proportion of the patients. None of the patients had cardiac involvement.

The onset of disease was early, before six months of age in 80%. At two years of age, all but one patient had signs of disease. The patient presenting with symptoms at the oldest age

displayed mild motor problems at the age of seven. Disease progression varied considerably among the patients. Two brothers had only a couple of weeks between onset and death, whereas one patient had an onset at birth and is still living as a young adult with a rather stable condition. Seventeen patients are no longer alive. Ten of them died before five years of age and the remaining seven before the age of 15.

Lactate levels in blood and/or CSF were elevated in 21 of 25 patients (84%). Organic acids in the urine were analysed in all patients and abnormalities were detected in 15 out of 25 (60%). Metabolites from the Krebs cycle were observed in 11 patients. Three patients excreted 3-methylglutaconic acid and shared clinical features of developmental delay, hypotonia, dyskinesia, hearing loss and liver involvement. High excretion of methymalonic acid, not responding to treatment with vitamin B12, was detected in one patient.

Muscle biopsies were performed in 23 of the patients. Morphological investigations of these biopsies were mainly normal. None of the patients had COX-negative or RRF. Biochemical analyses showed decreased MAPR in seven patients, increased in two and normal rates in the remaining 14 patients. A total of ten patients had deficiencies of RC enzyme activities (*Figure 8*).

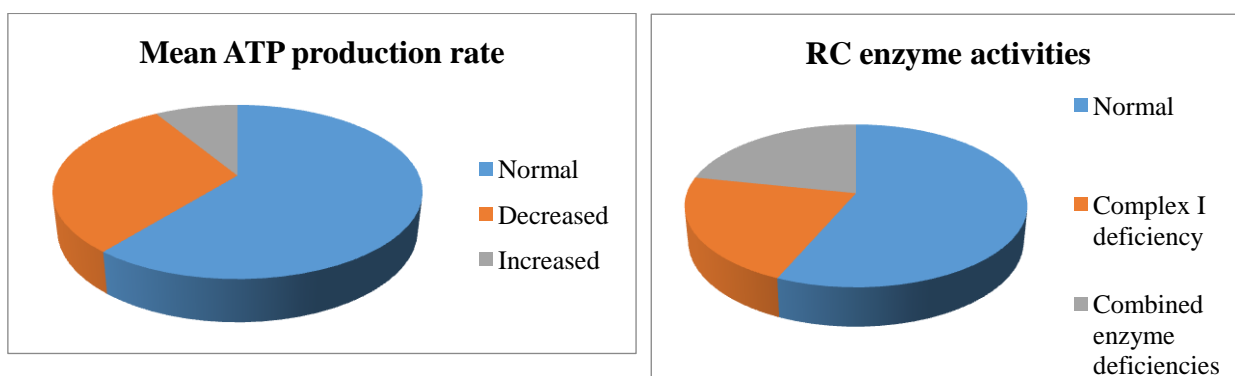


Figure 8. Biochemical measurements of MAPR and RC enzyme activities in muscle biopsies were normal in more than half of the cohort. Complex I deficiency was the most common defect. We did not find any patients with complex II, III or IV deficiency.

We performed complete mtDNA sequence analyses in all patients (only one of each pair of identical twins). Pathogenic mtDNA mutations were identified in eight (32%). All of these (six different mutations) had been previously reported to cause LS. One patient carried mutations in *POLG*, reflecting the phenotypic overlap between LS and AS.

We found no correlations between age of onset, rate of progression or survival on one hand, and genetic aetiology (mtDNA or nDNA) on the other

Genetic studies in a patient with Alpers syndrome (Paper III)

The patient presented at 18 months of age with epilepsy of the absence-type. He was treated with valproic acid and responded well. Three months later he developed a rapidly progressive, fatal liver failure. MRI of the brain showed the characteristic features of Alpers syndrome: cortical atrophy, cerebellar atrophy and bilateral, symmetrical high-signalling abnormalities in the thalami and the basal ganglia.

A muscle biopsy was performed. Morphological and biochemical investigations were normal.

In a sequence analysis of the entire *POLG* gene, the patient appeared to be homozygous for the previously described pathogenic mutation p.Trp748Ser. His father was a heterozygous carrier of the mutation, whilst his mother lacked the mutation. This caused us to proceed with an MLPA analysis in order to search for deletions or insertions in the gene. We detected a deletion comprising the entire maternal *POLG*-allele. The same deletion was detected in his mother.

Children with combined defects of the mitochondrial respiratory chain (Papers IV, V and VI)

The study included 55 children with deficiencies in more than one of the five enzyme complexes comprising the respiratory chain.

The cohort displayed a variety of clinical symptoms and presentations of disease. The onset of disease was generally early in life, at a median age of six weeks (range, birth-13 years). Details are shown in *Figure 1*, Paper IV.

The clinical presentations varied with the age of onset. Lactic acidosis was seen in five patients, all presenting in the first month of life. The patients with LS and AS presented with symptoms within the first year of life, whereas the patients with MELAS, MELAS/MERRF-overlapping syndrome and CPEO+ had a later onset (later than four years) (*Figure 2*, Paper IV). The majority of children could not be categorised into distinct mitochondrial syndromes. Most of them had a non-specific encephalopathy or encephalomyopathy, with additional symptoms from other organs.

The most frequently reported symptoms in the group were muscle weakness (80%), hypotonia (76%) and developmental delay/intellectual disability (71%). A variety of ophthalmological presentations were seen in 60% and 45% of the patients had epilepsy. Symptoms from organs outside the nervous system were common. Cardiac symptoms were seen in 13 patients, liver involvement in 12 and renal manifestations in 11 (*Figure 3*, Paper IV).

Metabolic clinical chemistry determinations included lactate. Blood lactate levels were elevated in 72% of the patients. Lactate levels in the CSF were analysed in 21 patients and were increased in five.

Urinary organic acids were analysed in 45 patients and abnormalities were found in 18. Elevated excretion of lactate was the most common finding. Elevated excretion of Krebs cycle intermediates were seen in seven patients and in another seven we detected elevated levels of 3-methylglutaconic acid. One patient excreted increased amounts of intermediates from the fatty acid oxidation, especially 3OH-compounds. Another patient excreted thymine, dihydrothymine, uracil and dihydrouracil, which are normally hardly detectable in the urine.

Muscle morphology, including histochemical stainings, was normal in 31 of the patients. COX-negative fibres were found in 13 samples and RRF in five of them. Ultrastructural abnormalities were observed in five patients.

The MAPR was decreased in 33 of the 55 patients. Four patients had a slightly abnormal ATP production rate from certain substrates, but, in total, a normal rate. Decreased activity in enzyme complexes I, III and IV or I and IV were seen in 26 of the patients. The remaining patients displayed deficiencies including complex II.

Genetic findings are summarised in *Figure 9*. In total, a genetic diagnosis was established in 34 of the 55 patients (62%). In six of these, we detected pathogenic point mutations in mtDNA. One patient harboured a large-scale deletion in mtDNA.

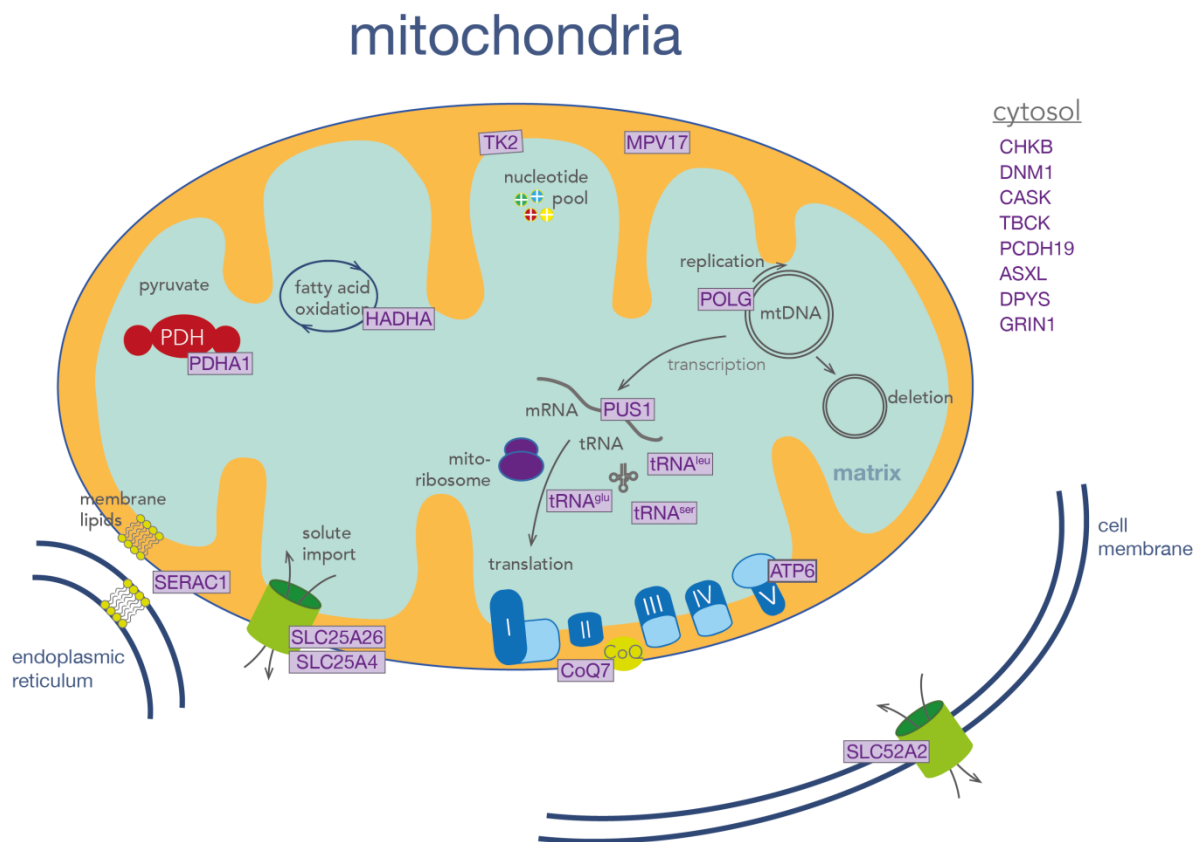


Figure 9. Mutations were identified in 19 different nuclear genes and five mitochondrial ones. One patient had a large-scale deletion of mtDNA.

Illustration: Christoph Freyer.

In a subset of patients, single genes were sequenced, based on clinical and/or biochemical findings. Two patients in the group suffered from a thymidine kinase-2 (TK-2) deficiency, an early onset fatal skeletal myopathy, caused by mutations in the *TK2* gene. One of these patients is reported on in Paper V. The girl presented, at less than three weeks of age, with failure to thrive, fatigue and muscle weakness. Seizures were observed from six weeks and she rapidly deteriorated into refractory epilepsy. Levels of CK were markedly increased in the blood and biochemical analyses in muscle showed pronounced deficiencies in complexes I, III, IV and V. Severe depletion of mtDNA was seen, with less than 5% of the levels present in an age-matched control. A suspicion of TK-2 deficiency was raised and sequence analysis of the gene revealed two novel mutations. The parents were heterozygous carriers. The first mutation, a CG insertion in exon 3 (c.219insCG), resulted in a frameshift and a subsequent downstream stop codon, leading to an inactive protein. The other was a missense mutation in exon 6 (c.388C>T). This second mutation resulted in a protein with virtually no residual activity *in vitro*.

Thirty-one patients in the cohort were subjected to WES/WGS. In 16 of these (32%), causative variants were found. Among the 19 different genes, in which we found disease-causing variants, eight had not been previously linked to primary or secondary dysfunction of the mitochondrial respiratory chain.

We diagnosed two patients with novel mitochondrial gene defects, *SLC25A26* (149) and *COQ7* (Paper VI). The patient in Paper VI was a boy who presented already *in utero* with fetal lung hypoplasia and growth retardation. He was born full-term but small for gestational age. He had a muscular hypotonia and respiratory distress with persistent pulmonary hypertension. Lung hypoplasia was confirmed, and ultrasound revealed small dysplastic kidneys. Secondary to that, there was a systemic hypertension and left ventricular cardiac hypertrophy. Within the first year of life, blood pressure and renal and lung function normalised and the cardiac hypertrophy regressed. The boy had moderate developmental retardation and, at the current age of ten years, he has a mild intellectual disability. Additionally, he has hearing and visual impairments and progressive weakness due to a sensori-motor polyneuropathy of the axonal and demyelinating type. Whole exome sequencing revealed a homozygous mutation, c.422T>C (p.Val141Glu), in the *COQ7* gene. The gene encodes a di-iron oxidase, which is part of the biosynthesis pathway of coenzyme Q. CoQ10 levels were severely reduced in isolated mitochondria from skeletal muscle and fibroblasts, as well as in total cell extracts from fibroblasts. Transfection of patient cells with wild type *COQ7* resulted in improved function of the mitochondrial respiratory chain.

Benzoic acid derivatives have previously been shown to bypass certain deficient steps in the CoQ biosynthesis (150). We used resorcylic acid, 2,4-dihydroxybenzoic acid (2,4-dHB), which is known to bypass the enzymatic step performed by CoQ7, as a supplement to the culture medium of the patients fibroblasts. After seven days of incubation, we could demonstrate increased cellular CoQ10 levels and improved mitochondrial respiration.

5 DISCUSSION

In this thesis we have studied children with mitochondrial disorders, with a focus on symptoms, clinical courses, biochemical abnormalities and genetic causes of the disease. Patients and patient groups with certain clinical phenotypes, or biochemical features, have been selected from our in-house clinical database.

The diagnostic procedure has undergone considerable development during the more than 25 years we have investigated patients with suspected mitochondrial disorders at the CMMS. The implementation of WES and WGS in clinical use has been the most revolutionary advance.

Clinical phenotyping and family history are still the foundation of the diagnostics. Whole genome analyses generate a large number of potentially disease causing variants, which need to be evaluated in relation to the clinical picture. Other inherited disorders may mimic mitochondrial RC defects clinically and may be diagnosed via WES/WGS analyses.

The clinical chemistry may add important clues to the diagnosis, as exemplified in the discussion of urinary organic acids below. Differential diagnoses, such as peroxisomal disorders, CDG syndromes, biotinidase deficiency or defects in the fatty acid β -oxidation can be excluded before more invasive investigations are performed. Findings of elevated levels of lactate in the blood or the CSF strengthen the suspicion of a mitochondrial disease, but lactate is not a very sensitive or specific biomarker for RC dysfunction. New, more reliable biomarkers have been introduced recently, although further studies are needed before they can be used more widely in the clinic. Fibroblast Growth Factor 21 (FGF21) in serum has proved to be a useful biomarker for mitochondrial disorders involving muscles (151). Growth Differentiation Factor 15 (GDF15) in serum is another promising candidate (152).

The muscle biopsy continues to be the golden standard investigation in the diagnostic procedure. The method used at the CMMS does not require general anaesthesia and is, by all accounts, a rather uncomplicated procedure. The fact that the biopsy specimen has to be taken immediately before analysis brings the opportunity to meet and examine the patient and complete the clinical history. Abnormal muscle biopsy findings steer further analyses. The mitochondrial assay in muscle may, however, be completely normal, despite a severe mitochondrial disease. We then rely on clinical symptoms, neuroimaging features or biochemical abnormalities in the continued search for the genetic diagnosis.

Complex I deficiency

Isolated complex I deficiency is the most common biochemical defect in our total cohort of children with mitochondrial disease. This is consistent with reports from other centres that have found defects in complex I to account for approximately one third of the biochemical findings in mitochondrial patients (153). Complex I is the largest enzyme of the RC, built up from approximately 37 nDNA- and seven mtDNA-encoded subunits. A number of nuclear encoded assembly factors are also needed. Mutations in genes encoding the subunits and

assembly factors result in an isolated complex I deficiency. Also, complex I contains the highest number of mtDNA encoded subunits of all RC complexes. Therefore, dysfunction in mtDNA replication, transcription and translation may initially show up as a complex I deficiency. This is seen in defects of mitochondrial tRNA genes, as well as nuclear gene defects, such as *POLG* and *MTFMT* (154). Later in these disorders, a deficiency of multiple enzyme complexes may evolve. Complex I deficiency has also been reported as a secondary phenomenon in various neurodegenerative and neuromuscular disorders, such as Parkinsons disease (155, 156).

Patients with a complex I deficiency display a variety of clinical pictures. The spectrum ranges from early-onset fatal disorders with multi-organ involvement, to single-organ presentations, as in the classical LHON syndrome. More frequently recognised phenotypes are fatal infantile lactic acidosis, Leigh syndrome, cardiomyopathy, non-specific leukoencephalopathy and MELAS syndrome (157). This is consistent with the clinical phenotypes we observed in our study of complex I patients (Paper I). One patient in the cohort (patient 11) had a phenotype clearly differing from the other ones. Her condition has been stable, including a moderate muscle weakness, hearing loss, cataract and hypertrichosis. She was investigated before we analysed isolated complex I activity. She had a defect in NADH-cytochrome c reductase (complex I+III). A whole exome analysis has later revealed two causative mutations in *BCSIL*, a nuclear gene encoding an assembly factor of complex III. Her clinical picture had similarities with the Bjornstad syndrome, which is a clinical presentation at the milder end of the disease spectrum of *BCSIL* defects (158).

Urinary organic acids in patients with mitochondrial disorders

The analyses of organic acids in the urine have provided valuable information in many of the patient investigations reported in this thesis. The analysis is considered to be an important part of the diagnostic procedure in patients with a suspected mitochondrial disorder (110).

Elevated levels of metabolites from the Krebs cycle, such as malate and fumarate, were observed in several of the patients with complex I deficiency, Leigh syndrome or combined enzyme deficiencies. In Paper I, we suggest that the increased NADH/NAD⁺ ratio in complex I deficiency affects the Krebs cycle negatively. NAD⁺ is required for the conversion of malate to oxaloacetate. Since oxaloacetate acts as a complex II feedback inhibitor, the reduced levels probably explain the increased succinate oxidation rate seen in the patients (Paper I, *Figure 2*). This altered regulation of the Krebs cycle may contribute to the disease mechanism in patients with complex I defects.

The finding of 3-methylglutaconic aciduria is clearly suggestive of a mitochondrial disease and may sometimes pin-point a specific gene defect (111). Four patients included and described in Paper II (patients 1, 23 and 24) and Paper IV (patients 7, 8, 9 and 10) excreted high levels of 3-methylglutaconic acid in repeated urine samples. They had similar clinical phenotypes, including developmental retardation, hypotonia, dyskinesia, hearing loss, hepatic disorder and features of Leigh syndrome in MRIs of the brain. This phenotype was

previously described as a MEGDEL association (159). In collaboration with Wortmann et al., we were able to establish a *SERAC1* deficiency in these patients (67).

High excretion of methylmalonic acid (MMA) was found in the urine of patient 12 in Paper II. The levels were not lowered by treatment with high doses of vitamin B12. Disorders of cobalamin metabolism were excluded and the patient has later been diagnosed as having a *SUCLA2* defect. The gene encodes a β -subunit of the enzyme, succinate CoA-ligase. The constellation of elevated levels of MMA in urine and Leigh/Leighlike syndrome is also seen in *SUCLG1* defects, the gene encoding the α -subunit of the same enzyme (160).

Intermediates of the nucleotide metabolism are hardly detectable in normal urine. Among the OXPHOS disorders caused by an imbalance in the nucleotide pools, is the thymidine phosphorylase (TP) deficiency, causing the MNGIE syndrome. Patients with this condition excrete thymidine and deoxyuridine in the urine, which is a key to the diagnosis. Patient 19 in Paper IV excreted thymine, dihydrothymine, uracil and dihydrouracil, which indicated a defect in the pyrimidine metabolism. Further genetic analyses detected causative variants in *DPYS*, the gene encoding the dihydropyrimidinase (DPYS) enzyme. Her dominant clinical symptom was gastrointestinal dysmotility, which is typically also seen in MNGIE patients. We hypothesise that DPYS defects, similar to TP defects, could induce an imbalance in the nucleotide pool, resulting in impaired replication of mtDNA. It could be the same for other defects in purine or pyrimidine metabolism, and OXPHOS dysfunction might then be a part of the disease mechanism in these disorders. In accord with the findings of Frangini et al., we did not find increased excretion of thymidine in the urine of our patient with TK2 deficiency. Frangini et al. demonstrated unaltered cytosolic and mitochondrial dTTP pools, as well as a normal composition of total dNTP pools in fibroblasts from patients with TK2 deficiencies (161).

There are pitfalls in the interpretation of urinary organic acid abnormalities. Patient 20 in Paper IV excreted high amounts of intermediates from the fatty acid oxidation, particularly long-chain 3OH-compounds. He had a multi-systemic disorder including muscle weakness, cardiomyopathy, hypothyroidism, nephrotic syndrome, liver involvement and failure to thrive. His clinical picture was highly suggestive of a primary RC disorder. He also had COX-negative fibres in muscle, further strengthening the suspicion. The abnormal findings in urine were initially interpreted as being secondary to his RC disorder, which has been described previously (112). Further genetic analyses identified variants in the *HADHA* gene, resulting in a long chain 3-hydroxyacyl-CoA dehydrogenase (LCHAD) deficiency. This secondary OXPHOS dysfunction in a primary fatty acid β -oxidation disorder has also been reported previously (113). It is hypothesised that the accumulated fatty acid metabolites and their carnitine esters act as detergents and dissolve membrane structures, thereby compromising the respiratory chain.

POLG disease

The *POLG* gene is the most frequently affected gene in mitochondrial disorders. *POLG* is a nuclear gene encoding the polymerase replicating mtDNA (162). The first report of a *POLG*

mutation associated with disease described a family with CPEO (38). Since then, a wide spectrum of phenotypes has been associated with *POLG* mutations, Alpers syndrome being at the severe end (163). Patient 17 in Paper II was compound heterozygous for three mutations in *POLG*, previously described as causing AS. He was included in the Leigh study, since he was considered to fulfil the criteria for Leigh syndrome, although he had a more Alpers-like clinical picture. The phenotypic overlapping of these two different mitochondrial encephalopathies of childhood is not surprising. Both are conditions resulting from a severe failure of oxidative metabolism within the mitochondria of the developing brain.

To date, more than 150 pathogenic mutations have been identified in the *POLG* gene (<http://tools.niehs.nih.gov/polg>). Most cases of Alpers syndrome are caused by either of the two mutations, p.Trp748Ser or p.Ala467Thr, in compound heterozygosity with another *POLG* mutation. Patients who are homozygous for the mutation p.Trp748Ser tend to have a milder disease, with a later onset (164). The patient described in Paper III had one deleted *POLG* allele and the p.Trp748Ser mutation on the other allele. In comparison with patients homozygous for the p.Trp748Ser mutation, he had an earlier onset of disease at 18 months. On the other hand, he did not display the typical severe phenotype seen in patients who are compound heterozygous for the mutation p.Trp748Ser and another *POLG* mutation. This is in agreement with previous reports on the occurrence of Alpers syndrome in patients with monoallelic expression of p.Ala467Thr, owing to nonsense-mediated decay of the transcripts from the corresponding allele. These patients had a much earlier onset than patients homozygous for the p.Ala467Thr mutation (165, 166). The authors suggest that gene dosage plays an important role in the later onset of symptoms in homozygous p.Ala467Thr patients, while the clinical course in patients who are compound heterozygous with p.Ala467Thr is steered by the mutation on the corresponding allele. Our findings in Paper III support the hypothesis that the *POLG* gene dosage is an important determinant of the phenotype in *POLG* disease.

The patient described in Paper III died from liver failure, induced by medication with valproic acid (VPA). Bicknese et al described VPA toxicity in children with Alpers syndrome as early as in 1992 (63). Since then, numerous cases have been reported (167). The mechanisms of VPA-induced liver failure are not fully understood. VPA is known to affect mitochondrial RC function by inhibition of the β -oxidation of fatty acids (168). In a recent study, Li et al used induced pluripotent stemcell-derived hepatocyte-like cells from patients with Alpers syndrome to study mechanisms of VPA toxicity. They demonstrated that the Alpers-derived cells were more sensitive, than control cells, to VPA-induced apoptosis. This was mediated by opening of the mitochondrial transition pore.

The case reported in Paper III highlights the importance of considering *POLG* disease in paediatric seizure disorders of unknown aetiology. Typical, but not obligatory, signs of *POLG* disease are refractory focal or generalised *status epilepticus*, an initial EEG with an occipital lobe predilection, developmental arrest or regression, stroke-like episodes or ataxia. Mutation analyses of *POLG* should be performed liberally. Hopefully, in this era of

massively parallel sequencing, a substantial proportion of epilepsy patients will be investigated genetically. Patients with *POLG* variants will be diagnosed and VPA treatment can be avoided.

MtDNA mutations

In Paper I, we studied seven probands (11 patients) with complex I deficiency. Three of the probands (43%) carried pathogenic mutations in mtDNA. In the group of patients with Leigh syndrome, studied in Paper II, causative mtDNA mutations were identified in 32% (8 out of 25). Among the children with combined deficiencies of the respiratory chain, seven patients (13%) harboured a pathogenic mutation in mtDNA. In several larger cohorts, the prevalence of causative mtDNA mutations has amounted to 20-30% (169, 170). Altogether, these prevalence figures emphasise the importance of including mtDNA sequence analyses in the diagnostic procedure, regardless of the clinical phenotype. This enables confirmation or exclusion of maternal (mitochondrial) inheritance in the genetic counselling of the family.

Nuclear gene defects

The broad spectrum of clinical presentations, the diversity of underlying genetic defects and the large number of proteins involved in the OXPHOS make the mitochondrial disorders suitable for massively parallel sequencing. A number of studies show a considerably higher yield of specific genetic diagnoses after implementation of WGS/WES in the diagnostic procedure (171-173).

Sanger sequencing of single genes may, occasionally, still be rational. In a few mitochondrial entities, the clinical and/or biochemical phenotype is strongly suggestive of a specific genetic defect. Two patients in Paper IV (patients 22 and 45) suffered from a TK2-deficiency. They both had hypotonia and severe muscle weakness, although refractory epileptic seizures were the dominant feature of patient 45 (case reported in Paper V). Mitochondrial assays in muscle showed pronounced deficiencies of complexes I, III, IV and V in both patients. CK levels were 5-20 times normal ones, which is unusually high, compared to other patients with mitochondrial myopathies. These biochemical findings led us to sequence the *TK2* gene, in which causative mutations were found. Patient 22 had a typical clinical phenotype, with onset of disease at six months of life, pronounced muscle weakness, but no obvious signs of CNS disease. He died from a respiratory insufficiency at the age of 11 months. In a mutation analysis of *TK2*, the previously described mutation, c.368G>C (p.Arg123Pro), was detected in homozygosity. The other patient (45), reported on in Paper V, had two novel mutations in the *TK2* gene. The mutation c.219insCG generated an early stop codon, which prevented the synthesis of a functional protein. The second mutation, c.388C>T (p.Arg130Trp), resulted in an enzyme with severely reduced activity (<4%, *Figure 4A* in Paper V). We suggest that the severe and atypical clinical presentation of the disease, including overwhelming CNS symptoms, was due to a virtual lack of mitochondrial TK2 activity.

In the study on children with combined enzyme deficiencies of the mitochondrial RC (Paper IV), we used massively parallel sequencing in a subset of patients. Thirty-one of them have,

so far, been subjected to WES and/or WGS. In 16 of these patients (52%), we have thereby established a genetic diagnosis. We expect more cases to be solved.

We identified disease-causing variants in two novel genes, *COQ7* and *SLC25A26*.

Patient 29 in Paper IV has been reported also in Paper VI. He was investigated with WES and was diagnosed with a homozygous variant in *COQ7*, a gene encoding one of the enzymes of CoQ biosynthesis. The defect resulted in severely reduced levels of CoQ. The patient had an intrauterine presentation of disease, with growth retardation, foetal lung hypoplasia and oligohydramnios. Postnatal investigations revealed renal dysplasia and dysfunction. The boy successively developed a multi-systemic disorder. Remarkably, his renal function normalised within the first week and follow-up ultrasounds of the kidneys showed normal growth and appearance within one year. There are no signs of glomerular or tubular dysfunction so far. Renal manifestations, particularly glomerular dysfunction, have been reported in other patients with primary CoQ deficiencies (174, 175). Most of the previously reported patients suffered from a glomerular disease with nephrotic syndrome and progression to chronic renal failure. There are no reports of spontaneous recovery from the renal disease. The response to supplementary CoQ10 was not as successful for the renal manifestations, as it was for other symptoms. One exception was a girl with a *COQ2* defect who received treatment very early on in the course the disease (176). An early start of treatment, with doses of 30-50 mg/kg/day, has been recommended (79). It is not known why renal involvement is prevalent in primary CoQ deficiencies. The findings in our patient support the hypothesis that CoQ is particularly important for renal function and development.

The other novel gene defect, identified in WES, was the pathogenic variants in *SLC25A26*. The gene encodes the transport protein for S-adenosylmethionine (SAM) into the mitochondria (149). SAM is an essential donor of methyl groups and we were able to demonstrate that a lack of SAM influences several important processes, among them, RC function, the activity of the PDH complex and CoQ biosynthesis.

In summary, we were able to establish a definite genetic diagnosis in 34 of the 55 patients (62%) in our cohort with multiple enzyme deficiencies (Paper IV). The most frequently affected genes were *SERAC1* (four patients) and *PDHA1* (four patients). The global incidence of *SERAC1* disease is not known, but it is believed to be very low. Ongoing multicentre studies will shed more light on this question. PDH complex deficiency is a more common metabolic disease, in most cases caused by mutations in the gene *PDHA1*. This gene is located on the X-chromosome, but a high percentage of female carriers develop a neurological disease. The disorder is not strictly a mitochondrial RC disorder, but it is usually included in this group. Clinically, there are many similarities and, in Paper IV, we could also demonstrate decreased activities of several OXPHOS enzyme complexes in the patients.

The patients described in Paper IV had disease-causing variants in 19 different nuclear genes. Defects in *SERAC1*, *MPV17*, *SLC25A4*, *TK2*, *POLG* and *PUS1* have previously been reported to cause a primary defect in the mitochondrial RC. Defects in the genes, *HADHA*, *CHKB* and *SLC52A2*, result in the disorders LCHAD-deficiency, congenital muscle

dystrophy of the megaconial type and Brown-Vialetto-van Laere syndrome type 2 (a riboflavin transporter deficiency), respectively, and have been previously reported to cause a secondary RC dysfunction (113, 177, 178).

A substantial proportion of the children had causative mutations in genes encoding proteins active outside the mitochondria and not previously linked to OXPHOS dysfunction. One boy (Patient 44) had a *de novo* mutation in the *DNM1* gene. He had the clinical features of early infantile epileptic encephalopathy, which is the phenotype reported in patients with this gene defect (179). Mitochondrial analyses in muscle showed decreased activity in complexes II, III and IV. Blue Native Gel electrophoresis showed clearly diminished amounts of complexes II and IV. *DNM1* encodes dynamin-1, a GTP:ase, mainly expressed in the CNS and known to have a critical role in endosomal trafficking and synaptic vesicle recycling in the nerve terminals (180). DNMI belongs to the dynamin family of GTP-ases, which mediate membrane remodelling in a variety of cellular processes. Otsuga et al. demonstrated (in 1998), that the DNMI protein in *Saccharomyces cerevisiae* is essential for the maintenance of mitochondrial morphology and that disruption of the *DNM1* gene caused severe abnormalities in the mitochondrial membrane network of yeast cells (181). We can, so far, only speculate that this might be the mechanism of the OXPHOS dysfunction seen in our patient. Further studies on mitochondrial function and morphology have to be performed in patients with DNMI defects to clarify this matter.

Two patients in our cohort (patients 39 and 31) were homozygous for a mutation in the *TBCK* gene. The families were of Syrian ancestry, but they were not known to be related with each other. Patient 39 presented at one month with respiratory difficulties. He is now 16 years old and has a severe intellectual disability, profound muscle weakness and hypotonia, prominent strabismus and visual impairment. His respiratory insufficiency, owing to hypoventilation, has continued. The mitochondrial assay in muscle demonstrated decreased activity of complexes I, II and III. The ATP-production rate was reduced. Patient 31 presented in a similar manner, at two months of age, with respiratory symptoms. She had the same features of muscle weakness, hypotonia, intellectual disability, strabismus and visual impairment. At the current age of eight years, she requires nocturnal ventilatory support. A normal ATP-production rate was observed in analyses of mitochondrial function in muscle, but the activities of complexes I and IV were decreased. MRI of the brain demonstrated signs of a white matter disease in both patients. Previous reports of patients with pathogenic variants in *TBCK* describe clinical phenotypes strikingly similar to what is seen in the two patients in our cohort. Moderate mitochondrial dysfunction, without further details, has been described in a few patients by Chong et al. (182, 183). The *TBCK* gene encodes a GTPase-activating protein, TBCK, which plays a role in cell proliferation, cell growth and actin-cytoskeleton dynamics, by modulation and regulation of components of the mammalian target of rapamycin (mTOR) complex (184). Activation of the mTOR complex stimulates cell growth, cell proliferation and mitochondrial biogenesis (185), whereas autophagy and mitophagy are down-regulated (186). A loss of function in TBCK could potentially lead to the opposite scenario. Chong et al hypothesise that OXPHOS dysfunction, caused by decreased

mitochondrial biogenesis and increased mitophagy, might contribute to the disease progress in TBCK deficiency. Our findings support this hypothesis.

Disease-causing variants in other genes, not previously linked to mitochondrial disease, will be identified in whole genome analyses in this group of patients. New mechanisms of disease remain to be elucidated.

6 CONCLUSIONS

Mitochondrial disorders are clinically heterogeneous. Identical biochemical deficiencies or molecular defects result in a broad variety of clinical presentations. Conversely, a specific clinical phenotype may be caused by variants in a diversity of genes.

The most common symptoms are often non-specific. More specific signs or symptoms are often present and are discovered in the clinical characterisation.

Lactate levels in blood and CSF are widely used biomarkers of mitochondrial disease. Several patients display normal lactate levels. We therefore conclude that lactate cannot be used as a criterion for the diagnosis of Leigh syndrome or any other mitochondrial disease.

An analysis of organic acids in urine is helpful in the diagnostic procedure.

Deficiencies of complex I cause secondary alterations of Krebs cycle metabolism and impair the feed-back inhibition of complex II. This may influence the disease process in patients.

Alpers syndrome is, in a certain proportion of the patients, caused by mutations in the *POLG* gene. The mutation analyses of *POLG* ought to include methods capable of detecting copy number changes and intragenic deletions. Gene dosage plays a role in the severity of the phenotype.

Liver failure, induced by valproic acid, is a severe complication of Alpers syndrome. The possibility of a *POLG* disease in paediatric seizure disorders of unknown aetiology has to be considered before starting treatment with valproic acid.

MtDNA mutations are a common cause of severe disease in Leigh syndrome and in children with complex I deficiency. It is also fairly common in other groups of mitochondrial disorders, such as the combined enzyme deficiencies. Sequencing of the entire mtDNA is therefore recommended, irrespective of the severity of the disease, biochemical defects or morphological findings.

Age of onset, clinical symptoms and prognosis did not differ significantly between patients with mitochondrial and nuclear mutations among the patients with Leigh syndrome.

Massively parallel sequencing of the whole human exome/genome is an excellent tool for the diagnosis of mitochondrial disorders. The clinical phenotyping and biochemical investigations continue to be essential elements, since they are prerequisites for the interpretation of the results of WES/WGS.

CoQ deficiencies are treatable disorders and are therefore important to diagnose as early as possible.

7 FUTURE PERSPECTIVES

Complex I deficiency is the most common biochemical defect in our cohort of patients with confirmed or suspected mitochondrial disease. Some of these patients have a genetic diagnosis, while the diagnosis remains unknown in others. We will employ the same strategy as for the patients with combined enzyme deficiencies. The patients will be subjected to WGS and the genetic data will be, in the first step, evaluated in the dbCMMS. In selected cases we can continue on a research basis.

The participation in the Mitochondrial Clinical Research Network (MCRN) will enable collaborative studies on clinical phenotypes, biochemical features and genetic disease mechanisms also in more rare conditions of OXPHOS dysfunction.

The future use of WES/WGS in clinical diagnostics will continue to reveal novel genes causing OXPHOS disease. New mechanisms of disease will also be elucidated.

Following the ‘diagnostic revolution’, we look forward to a ‘therapeutic revolution’. New therapies are under development, targeting different disease mechanisms. Various small molecules are currently being evaluated in clinical studies (187).

Future therapies will probably be individually tailored, depending on the specific gene defect and disease mechanism in each patient. An example from our own studies is the CoQ7 defect, in which we demonstrated a bypass of the particular enzymatic step by the use of 2,4-dHB. The results encourage continuation with a clinical trial in humans suffering from this defect.

The role of autophagy and mitophagy in mitochondrial health and disease is an area of interest. Two patients with combined enzyme deficiencies had causative mutations in *TBCK*. Their disease process is suggested to be influenced by inhibition of the mTOR complex and the regulation of autophagy, mitophagy and mitochondrial biogenesis. This opens up possibilities for new treatment strategies.

Clinical trials are, in general, difficult to perform, owing to the broad spectrum of clinical pictures, clinical courses, biochemical features and genetic aetiologies. There is also a lack of biomarkers and other objective, validated outcome measures, suitable for this group of patients. Collaboration in the MCRN will create larger cohorts of patients with similar phenotypes or genetic causes of disease, for inclusion in future clinical trials.

Supplementation with CoQ10 is widely used in the treatment of mitochondrial disorders. Clinical trials show diverging results of the effect. It would be interesting to study the effect in defined groups of patients with different mitochondrial disorders. A hypothesis could be that certain individuals suffer from secondary CoQ deficiency and would therefore benefit more than others from supplementary CoQ10.

8 SVENSK SAMMANFATTNING

Mitokondrier är små enheter i våra celler. De brukar liknas vid kraftverk. Här omvandlas beståndsdelar från nedbrytningen av kolhydrater, protein och fett i födan, med hjälp av syre, till adenosintrifosfat (ATP), koldioxid och vatten. ATP är cellens energivaluta.

Energibildningen sker via ett stort antal kemiska reaktioner. Processen avslutas i den mitokondriella andningskedjan (även kallad elektrontransportkedjan).

Den energialstrande processen är beroende av ett stort antal (ca 1500) proteiner/enzymer. Bristfällig funktion i något av dessa ger en energibrist i kroppens celler, en mitokondriell sjukdom. Generna för dessa proteiner finns i kärnans DNA (nDNA) eller i mitokondriens eget DNA (mtDNA). Det mitokondriella genomet innehåller 37 gener och finns i ett stort antal kopior i varje cell. I kärnans DNA finns över 20 000 gener. Vi har två kopior av dessa, undantaget de som är belägna på X- eller Y-kromosomen. Cirka 75 % av de mitokondriella sjukdomarna orsakas av mutationer i nDNA.

Avhandlingen handlar om mitokondriella sjukdomar hos barn och målsättningen var att öka kunskapen om olika sjukdomsbilder och bakomliggande mekanismer. Vi ville studera mitokondriella sjukdomar orsakade av olika genetiska defekter, för att bättre kunna bedöma patienters sjukdomsförlopp och prognos. Vi använde nya genetiska metoder för att finna nya gener kopplade till mitokondriell sjukdom

Mitokondriella sjukdomar kan ge mycket varierande sjukdomsbilder, med vilket symptom som helst, från vilket organ som helst. Framför allt drabbas de organ som förbrukar mest energi, till exempel hjärnan och muskulaturen. Andra organ/funktioner som ofta drabbas är hjärta, njurar, syn och hörsel. När sjukdomen debuterar tidigt i livet är den i regel svårare, med engagemang av många olika organ och med en sämre prognos.

I det första delarbetet studerade vi 11 barn med nedsatt aktivitet i det första enzymkomplexet i den mitokondriella andningskedjan. De hade varierande sjukdomsbilder, alla med tidig sjukdomsdebut och symptom från musklerna i form av svaghet och ansträngningsintolerans. Tio av elva patienter hade en psykomotorisk utvecklingsförsening och svårigheter med födointaget. Sex patienter visade sig ha en mutation i mtDNA, vilket är en relativt hög andel. Vid tiden för studien undersöktes inte patienterna avseende mutationer i kärnans gener. Biokemiska analyser av mitokondrier isolerade från muskel, visade hos samtliga patienter en nedsatt aktivitet i komplex I och nedsatt ATP-produktion från de substrat som är beroende av komplex I. ATP-produktionen från substrat via komplex II var däremot ökad och vi spekulerar i mekanismerna bakom detta.

Leighs syndrom är den vanligaste, enhetligt beskrivna, sjukdomsbilden vid mitokondriell sjukdom hos barn. Det är en svår, fortskridande neurologisk sjukdom med karaktäristiska skador i hjärnans djupare strukturer. I delarbete II studerade vi 25 barn med Leighs syndrom. Vi kunde bekräfta att sjukdomen drabbar tidigt i livet, då 80 % av barnen debuterade före sex månaders ålder. Sjukdomen kan i vissa fall vara snabbt fortskridande och leda till tidig död. I vår grupp avled 40 % före fem års ålder. En andel av patienterna uppvisade en mer stationär

sjukdomsbild och har överlevt in i vuxen ålder. Vi kunde fastställa mutationer i mtDNA hos åtta patienter (32 %). Dessa patienter hade en lika svår sjukdomsbild som de övriga i gruppen. Hos 16 patienter förblev den genetiska orsaken okänd.

Alpers syndrom drabbar också tidigt i livet. Jämfört med Leighs syndrom ger den ofta mer påverkan på den mentala utvecklingen, samt en svår epilepsi. Alpers syndrom beror, i många fall, på mutationer i *POLG*-genen. *POLG* styr bildningen av det polymeras som ser till att mtDNA kopieras och repareras. I delarbete III beskriver vi en patient med Alpers syndrom, vars sjukdom orsakats av en mutation i en genkopia av *POLG* och ett bortfall av hela den andra genkopian. Ett bortfall av hela *POLG*-genen har inte beskrivits hos någon patient tidigare. Patientens sjukdomsbild var något mildare än hos patienter som har olika mutationer i respektive genkopia, men svårare än hos patienter med samma mutation i båda genkopiorna. Patientens sjukdom försämrades akut vid behandling med epilepsiläkemedlet valproat. Han avled vid två års ålder i en svår leversvikt. Vi vill betona risken för leversvikt av detta läkemedel hos patienter med sjukdom orsakad av mutationer i *POLG*-genen.

Delarbete IV är en studie av 55 barn med bristande funktion i flera av de enzymkomplex som bygger upp den mitokondriella andningskedjan. Man vet att mutationer i ett stort antal olika gener kan orsaka denna typ av sjukdom. Vi använde en ny genetisk teknik, där man med en parallell sekvensanalys undersöker alla gener i kärnans DNA. Vi fastställde de sjukdomsorsakande mutationerna hos totalt 34 av patienterna i gruppen. Två patienter hade mutationer i gener som inte tidigare beskrivits ge sjukdom. Hos sjutton patienter fann vi mutationer i gener som tidigare är kända att orsaka en primär eller sekundär påverkan på den mitokondriella andningskedjan. Nio patienter visade sig ha sjukdomar utan tidigare känt samband med mitokondriell sjukdom, där vi nu kunde visa en sekundär påverkan på mitokondriens energiproduktion.

Tymidinkinasbrist ger en obalans i tillgången på byggstenar till mtDNA (nukleotider). Detta ger en svår mitokondriell sjukdom med bl.a. uttalad muskelsvaghet. Vi beskriver i delarbete V en flicka med tymidinkinasbrist orsakad av två, tidigare icke beskrivna, mutationer i genen *TK2*. Hennes sjukdomsbild var ovanlig, med en tidigt debuterande mycket behandlingsresistent epilepsi.

Ny genetisk teknik gör att mutationer i nya gener successivt visar sig orsaka sjukdom och att nya sjukdomsmekanismer upptäcks. I delarbete VI rapporterar vi en ny sjukdom; Coenzym Q7-brist. Bristen orsakar en dysfunktion i bildningen av coenzym Q10 som är en viktig cofaktor i den mitokondriella andningskedjan. Sjukdomen kan förbättras med tillskott av coenzym Q10, vilket var fallet för vår patient. Vi kunde i odlade hudceller från patienten också visa hur substansen 2,4-dihydroxybensoat kan överbrygga defekten. Detta kan bli en ny form av behandling vid denna sjukdom.

Avhandlingen illustrerar, och bidrar till, den stora kunskapsutveckling som sker inom området mitokondriella sjukdomar. Nya genetiska tekniker förbättrar diagnostiken så att fler

patienter kan få en specifik sjukdomsdiagnos. Nya gener upptäcks och därmed nya mekanismer för mitokondriell sjukdom.

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