Genotypic and phenotypic spectrum of mitochondrial diseases with focus on early onset mitochondrial encephalopathies

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Cover illustration: Mitochondrion (greek: Μιτοχόνδριο). By Kalliopi Sofou.

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ABSTRACT

Early-onset mitochondrial encephalopathies comprise a challenging group of neurodegenerative disorders. This is due to their progressive nature, often leading to major disability and premature death, as well as their diagnostic complexity and lack of customized treatments.

The overall aim of the research presented in this thesis was to explore the phenotypic and genotypic spectrum of childhood-onset mitochondrial diseases with central nervous system involvement. The present thesis focuses on early-onset mitochondrial encephalopathies with particular emphasis on Alpers and Leigh syndromes.

We studied 19 patients with Alpers syndrome and showed specific genotype-phenotype correlations depending on the presence or not of *POLG1* mutations. We have further identified, with the help of whole exome sequencing, mutations in *NARS2* and *PARS2* in two of our patients with Alpers syndrome not associated to *POLG1*, being the first to link mutations in these genes to human disease and to Alpers syndrome.

We also present the natural history data on a unique cohort of 130 patients with Leigh syndrome, along with predictors of long-term outcomes. Disease onset before six months of age, failure to thrive, brainstem lesions on neuroimaging and intensive care treatment were associated with poorer survival. Based on the findings from this study, we suggest revised diagnostic criteria for Leigh syndrome.

We also studied the brain MRIs of 66 patients with mitochondrial disorders with central nervous system involvement. We describe the optimal use of brain neuroimaging in the diagnostic work-up of suspected mitochondrial disorders, as well as its role in the differential diagnosis among mitochondrial encephalopathies and from other diseases with similar features.

This thesis advances our knowledge of the phenotypic and genotypic spectrum of early-onset mitochondrial encephalopathies and discusses the applicable diagnostic methods, from the diagnostic criteria used to define clinical syndromes, to the role of the traditional and modern methodologies in the diagnostic work-up of these complex disorders. The study of patients with Leigh syndrome is the first joint research work between eight centers from six European countries specializing in mitochondrial diseases, creating a strong platform for ongoing collaboration on mitochondrial research projects.

Keywords: mitochondrial encephalopathy, Alpers syndrome, Leigh syndrome, neuroimaging, whole exome sequencing

Sammanfattning på svenska

Tidigt debuterande mitokondriella encefalopatier är en grupp av neurodegenerativa sjukdomar som kännetecknas av ett progressivt förlopp som oftast leder till grav funktionsnedsättning och för tidig död. Diagnostiken av dessa sjukdomar är komplex och behandlingsmöjligheterna är begränsade.

Forskningen som presenteras i denna avhandling har som övergripande mål att studera det kliniskt uttrycksättet (fenotypen) och de genetiska orsakerna (genotypen), för mitokondriella sjukdomar som engagerar det centrala nervsystemet hos barn. Avhandlingen fokuserar på tidigt debuterande mitokondriella encefalopatier och i synnerhet på Alpers och Leigh syndrom.

Vi studerade 19 patienter med Alpers syndrom och visar specifika genotyp-fenotyp korrelationer beroende på närvaron eller frånvaron av *POLG1* mutationer. Vi har dessutom, med hjälp av helexomsekvensering av två patienter med Alpers syndrom, hittat mutationer i *NARS2* respektive *PARS2*. Mutationer i dessa gener har därigenom för första gången associerats till sjukdom hos människa och till Alpers syndrom.

I avhandlingen presenteras naturalförlopp och riskfaktorer hos 130 patienter med Leigh syndrom. Sjukdomsdebut före sex månaders ålder, tillväxthämning, neuroradiologiska tecken till hjärnstampåverkan och behandling inom intensivvård var kopplat till sämre överlevnad. Baserat på studiens resultat, föreslår vi reviderade diagnostiska kriterier för Leigh syndrom.

Vidare eftergranskade vi MR-undersökningar av hjärnan från 66 patienter med mitokondriella encefalopatier. Vi beskriver hur neuroradiologiska metoder kan användas vid diagnostik av misstänkt mitokondriell sjukdom och vid differentialdiagnostik av olika former av mitokondriell encefalopati och för att skilja dessa från andra sjukdomar med liknande bild.

Avhandlingen tillför ny kunskap om de fenotypiska formerna av och de genetiska orsakerna till tidigt debuterande mitokondriella encefalopatier. Diagnostiken av dessa sjukdomar diskuteras utifrån de kriterier som finns för olika kliniska mitokondriella syndrom och utifrån traditionell och nyare diagnostisk metodik. Studien om Leigh syndrom är den första som genomförs inom ett kollaborativt nätverk av åtta centra från sex europeiska länder som driver forksning om mitokondriella sjukdomar.

Nyckelord: mitokondriell encefalopati, Alpers syndrom, Leigh syndrom, neuroradiologi, helexomsekvensering

LIST OF PAPERS

This thesis is based on the following papers, referred to in the text by their Roman numerals.

I. **Sofou K**, Moslemi AR, Kollberg G, Bjarnadóttir I, Oldfors A, Nennesmo I, Holme E, Tulinius M, Darin N.

Phenotypic and genotypic variability in Alpers syndrome. Eur J Paediatr Neurol, 2012. 16(4): p. 379-89.

II. **Sofou K**, Kollberg G, Dávila M, Darin N, Gustafsson C, Holme E, Oldfors A, Tulinius M, Asin-Cayuela J.

Whole exome sequencing reveals mutations in NARS2 and PARS2, encoding the mitochondrial asparaginyl-tRNA synthetase and prolyl-tRNA synthetase, in patients with Alpers Syndrome. Submitted.

III. **Sofou K**, De Coo IF, Isohanni P, Ostergaard E, Naess K, De Meirleir L, Tzoulis C, Uusimaa J, De Angst IB, Lönnqvist T, Pihko H, Mankinen K, Bindoff LA, Tulinius M, Darin N.

A multicenter study on Leigh syndrome: disease course and predictors of survival. Orphanet J Rare Dis, 2014. 9(1): p. 52.

IV. **Sofou K**, Steneryd K, Wiklund LM, Tulinius M, Darin N.

MRI of the brain in childhood-onset mitochondrial disorders with central nervous system involvement. Mitochondrion, 2013. 13(4): p. 364-71.

CONTENTS

ABBREVIATIONS

AARS2	Alanyl-tRNA synthetase 2
ADC	Apparent diffusion coefficient
ATP	Adenosine triphosphate
BBGD	Biotin-responsive basal ganglia disease
CNS	Central nervous system
COX	Cytochrome C oxidase
CRF	Case report form
CSF	Cerebrospinal fluid
CT	Computed tomography
DARS2	Aspartyl-tRNA synthetase 2
DWI	Diffusion-weighted imaging
EDC	Electronic data capture
FADH	Flavin adenine dinucleotide
FARS2	Phenylalanine-tRNA synthetase 2
FLAIR	Fluid attenuated inversion recovery
GAMT	Guanidinoacetate methyltransferase
GRACILE	Growth retardation, aminoaciduria, cholestasis, iron overload, lactic acidosis, early death
IBSN	Infantile bilateral striatal necrosis
ILAE	International league against epilepsy
IOSCA	Infantile onset spinocerebellar ataxia
IUGR	Intrauterine growth restriction
KSS	Kearns-Sayre syndrome
LDH	Lactate dehydrogenase
LHON	Leber hereditary optic neuropathy
LSBL	Leukoencephalopathy with brainstem and spinal cord involvement and lactate elevation
MCRN	Mitochondrial Clinical and Research Network
MDS	mtDNA depletion syndrome
MEGDEL	3-methylglutaconic aciduria with deafness, encephalopathy and Leigh-like syndrome
MELAS	Mitochondrial encephalomyopathy, lactic acidosis and stroke-like episodes
MERRF	Myoclonus epilepsy with ragged red fibers
MILS	Maternally inherited Leigh syndrome
MIRAS	Mitochondrial recessive ataxia syndrome
MRI	Magnetic resonance imaging
MRS	Magnetic resonance spectroscopy
MSCAE	Mitochondrial spinocerebellar ataxia and epilepsy
mt-AARS	Mitochondrial aminoacyl-tRNA synthetase
mtDNA	Mitochondrial DNA

NADH	Nicotinamide adenine dinucleotide
NARP	Neuropathy, ataxia and retinitis pigmentosa
NARS2	Asparaginyl-tRNA synthetase 2
nDNA	Nuclear DNA
OXPHOS	Oxidative phosphorylation
PARS2	Prolyl-tRNA synthetase 2
PDHc	Pyruvate dehydrogenase complex
PEO	Progressive external ophthalmoplegia
POLG1	Polymerase gamma 1
RRFs	Ragged red fibers
rRNA	Ribosomal RNA
tRNA	Transfer RNA
WES	Whole-exome sequencing

1 SCIENTIFIC BACKGROUND

1.1 Introduction

Mitochondria were first recognized as unique intracellular structures by Altmann in 1890, described under the name 'bioblasts', as elementary organisms living inside cells and carrying out vital functions. The name mitochondrion was introduced in 1898 and originates from the Greek 'mitos' (thread) and 'chondros' (granule), a descriptive term of their morphology during spermatogenesis [1]. Their role in the evolution of complex species has been essential, as cells without mitochondria would have been dependent exclusively upon anaerobic glycolysis for energy production, which is unlikely to support complex multicellular organisms [2]. As a result of their fundamental role in the evolution to the present-day species, mitochondria have been the focus of intense morphological, biochemical and molecular research.

Mitochondria were first linked to human disease in 1962 by the Swedish endocrinologist Rolf Luft, who described a condition of childhood-onset hypermetabolism with biochemical and histological findings of mitochondrial dysfunction [3]. One year later, it was shown that mitochondria carry their own genome, known currently as the mitochondrial DNA (mtDNA) [4, 5]. However, it wasn't until 1981 that the human mitochondrial genome was fully sequenced [6]. In the following years, it was shown that the mitochondrial structure and functions are under dual genomic control, mitochondrial (mtDNA) and nuclear (nDNA). To date, mutations in 228 protein-encoding nDNA genes and 13 mtDNA genes have been linked to human disorders, while novel genes are continuously being identified [7].

Mitochondrial disorders comprise a clinically and genetically heterogeneous group of disorders caused by defects in mtDNA or nDNA, which impair the cellular energy production [8]. Multiple organs and tissues can be affected, but those with the highest aerobic demand, such as the brain and the skeletal muscles are the most vulnerable [9]. Childhood-onset mitochondrial disorders typically present with central nervous system (CNS) involvement, often manifesting as diffuse encephalopathy, with a devastating and rapidly progressive disease course [9, 10].

The present thesis reviews the current knowledge and provides novel data on the phenotypic and genotypic spectrum of childhood-onset mitochondrial disorders with a special focus on early-onset mitochondrial encephalopathies.

We discuss further the diagnostic approach to mitochondrial encephalopathies, from the diagnostic criteria used to define clinical syndromes, to the role of the traditional and modern methodologies in the diagnostic work-up.

1.2 Mitochondria: Structure and functions

Mitochondria are intracellular organelles that regulate critical cellular processes, from energy production to apoptosis. They are remarkably mobile and plastic, constantly changing shape through fusion and fission, and forming networks, in response to the highly intricate relationship between mitochondrial dynamics, structure and function [11]. Their number per cell varies from a few hundred to several thousand depending upon the energy requirements of the cell. Their structure is bounded by two phospholipid bilayer, highly specialized membranes; an outer membrane with protein channels permeable to molecules smaller than 5 kDa and a highly-convoluted inner membrane that separates the intermembrane space from the matrix. The mitochondrial respiratory chain is composed of multi-heteromeric protein complexes in the inner membrane of the mitochondrion, known as complexes I to V. The mitochondrial genome (mtDNA) resides in multiple copies in the mitochondrial matrix. It is a 16.569 base pair, double-stranded, closed-circular molecule that encodes 13 structural subunits and 24 RNAs – of which 22 are transfer RNAs (tRNAs) and two are ribosomal RNAs (rRNAs)-, that are essential for intramitochondrial protein synthesis. These 13 structural subunits interact with approximately 79 nuclear-encoded subunits to form the respiratory chain complexes I to V [12]. The mitochondrial matrix also contains mitochondrial ribosomes, tRNAs and a large variety of enzymes, including those required for the expression of mitochondrial genes, as well as those that mediate the oxidation of pyruvate and fatty acids for the citric acid cycle [2]. Approximately two thirds of the mitochondrial proteins are located in the matrix [2].

Mitochondria are the major source of energy production in the form of adenosine triphosphate (ATP). ATP is synthesized through the process of oxidative phosphorylation (OXPHOS) carried out by the mitochondrial respiratory chain. The entire process is driven by an electrochemical proton gradient across the inner membrane, with electron transport, proton pumping, and ATP formation occurring simultaneously. The electron donors are the products of the Krebs cycle, nicotinamide adenine dinucleotide (NADH) and flavin adenine dinucleotide ($FADH_2$). The Krebs cycle takes place inside the mitochondrial matrix to oxidize the acetyl-CoA which is produced from the metabolism of pyruvate and fatty acids entering from the cytosol. For every

molecule of acetyl-CoA, the Krebs cycle produces energy in the form of 3 NADH, 1 $FADH_2$ and 1 ATP. The electrons from NADH are transferred through complex I (NADH: ubiquinone oxidoreductase) to ubiquinone (coenzyme Q10). The electrons from $FADH_2$ are also transferred to ubiquinone through complex II (succinate-ubiquinone oxidoreductase). Subsequently, electrons are transferred to complex III (ubiquinol:cytochrome c oxidoreductase) and then, through cytochrome C, to complex IV (cytochrome C oxidase, COX), where these are eventually accepted by oxygen atoms to form oxygen ions which in their turn form water. In parallel to electron transport, protons are also pumped through the complexes I, III and IV, from the matrix to the intermembrane space, creating an electrochemical proton gradient. When these protons flow down the concentration gradient through channels of the inner membrane, the ATP synthase (complex V) uses this energy to further generate ATP. For each molecule of glucose entering the cell, the aerobic cellular respiration produces a total of 36 ATP, as summarized in the following three stages:

Glycolysis (cytosol): 1 glucose + 2 NAD^+ + 2 ATP → 2 pyruvates + 2 NADH + 4 ATP

Krebs cycle (matrix): 2 pyruvates + 8 NAD^+ + 2 FAD + 2 ADP → 6 CO2 + 8 NADH + 2 $FADH_2$ + 2 ATP

OXPHOS (inner membrane): 6 O2 + 8 NADH + 4 $FADH_2$ + 32 ADP → 8 NAD^+ + 4 FAD + 12 H_20 + 32 ATP

Under anaerobic conditions, pyruvate is converted by lactate dehydrogenase (LDH) to lactate according to the following reaction: Pyruvate + NADH ↔ Lactate + NAD^+

The lactate-to-pyruvate ratio is therefore correlated with the cytoplasmic NADH: NAD^+ ratio and is used as a surrogate measure of oxidative phosphorylation [13, 14]. In order to keep the NADH levels low, so that pyruvate keeps on metabolizing to acetyl-CoA that continues into the Krebs cycle, shuttles are used to assist the oxidative phosphorylation and the oxidation of NADH back to NAD^+ [15]. The malate–aspartate shuttle is the principle mechanism. Impairment of the oxidative phosphorylation or increased rate of glycolysis or both, result in an increased NADH: NAD^+ ratio and a shift of the LDH equilibrium toward increased production of lactate and increased lactate-to-pyruvate ratio [14, 15].

1.3 The genetics of mitochondrial disease

As mitochondrial function is under dual genomic control, mitochondrial and nuclear, genetic defects in either the mitochondrial or the nuclear genome may give rise to a mitochondrial disease. The mitochondrial genome is maternally inherited. The human cells contain between 100 and 10.000 copies of mtDNA. In the majority of cases, mtDNA copies share identical sequence known as homoplasmy. As mtDNA is often subject to mutation, it is common that the mutated mtDNA co-exists with the wild-type counterpart, known as heteroplasmy. The relative proportion of mutant to wild-type genome that causes a mitochondrial disease, known as threshold, varies depending upon the type of mutation and the tissue. Pathogenic mtDNA mutations may occur either as (i) point mutations or (ii) mtDNA rearrangements (deletions and insertions).

The vast majority of pathogenic mtDNA point mutations occur in the tRNA genes and they are typically heteroplasmic. Examples of mitochondrial diseases due to mtDNA point mutations in tRNA genes are (i) mitochondrial encephalomyopathy, lactic acidosis and stroke-like episodes (MELAS) commonly caused by an A>G transition at m.3243 in tRNA-leucine, and (ii) myoclonus epilepsy with ragged red fibers (MERRF), mainly caused by an A>G transition at m.8344 in tRNA-lysine. Pathogenic mtDNA point mutations may also occur in protein coding genes, affecting the subunits of the respiratory chain complexes. An example is the mutation at m.8993 in the gene encoding the subunit 6 of the ATP synthetase (complex V), which depending on the level of heteroplasmy, may give rise to the maternally inherited Leigh syndrome (MILS) or to a milder phenotype of neuropathy, ataxia and retinitis pigmentosa (NARP) syndrome. Pathogenic mtDNA rearrangements are typically large-scale deletions, which are mainly sporadic. The major clinical phenotypes associated with mtDNA large-scale deletions are (i) Kearns-Sayre syndrome (KSS), (ii) progressive external ophthalmoplegia (PEO) and (iii) Pearson syndrome.

Mutations in the nuclear genome account for the majority of mitochondrial disorders, as the nuclear genome encodes the majority of mitochondrial proteins. The pattern of inheritance in this case is usually autosomal recessive; however, autosomal dominant and occasionally X-linked patterns of inheritance, are also found in nDNA-associated mitochondrial disorders. Mutations in the nuclear genome may cause mitochondrial disease via five distinct pathways, i.e. mutations affecting (i) the nuclear-encoded subunits of the respiratory chain complexes; (ii) the biogenesis and regulation of OXPHOS; (iii) the mtDNA replication, transcription and translation; (iv) the

mtDNA stability and maintenance; and (v) the mitochondrial network dynamics.

An overview of the biological pathways involved in mtDNA- and nDNA-associated mitochondrial diseases with CNS involvement, as well as the genes involved and their major phenotypes, are summarized in Figure 1 and Table 1.

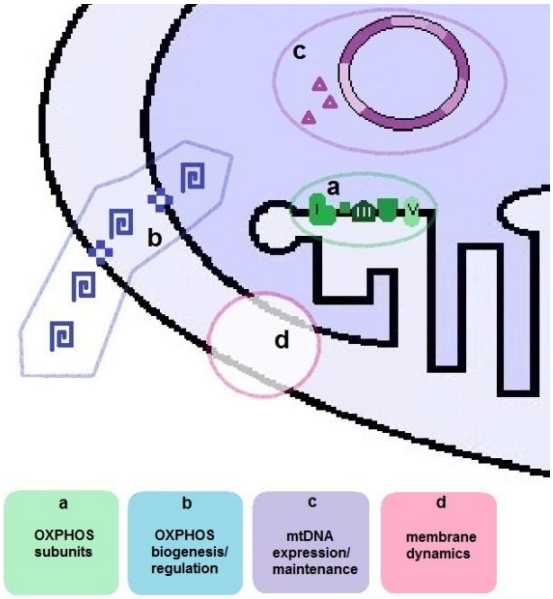


Figure 1. Biological pathways of mitochondrial disease.

Table 1. Biological pathways of mtDNA- and nDNA-associated mitochondrial diseases with CNS involvement, the genes involved and their major phenotypes. Adapted from Chinnery et al. [12], Rouault et al. [105], Pearce et al. [106] and Calvo et al. [107].

	Pathways	Components	Genes	Major phenotypes
a	OXPHOS subunits encoded by nDNA	Complex I	*NDUFS1, 2, 3, 4, 6, 7, 8; NDUFV1, 2; NDUFA2, 10, 12*	LS, leukoencephalopathy
		Complex II	*SDHA*	LS
		Complex II	*SDHB*	Leukoencephalopathy, paraganglioma
		Complex II	*SDHC, SDHD*	Paraganglioma
		Complex III	*UQCRB, UQCRQ*	Encephalopathy, metabolic decompensation
		Complex IV	*COX6B1*	Leukoencephalopathy, hydrocephalus, cardiomyopathy
	OXPHOS subunits encoded by mtDNA	**Complex I**	***ND1, 2, 3, 4, 4L, 5, 6***	**LS, MELAS, LHON**
		Complex III	***CYTB***	**Encephalopathy, (cardio)myopathy, exercise intolerance, septooptic dysplasia**

		Complex IV	***COX1, 2, 3***	**Encephalopathy**
		Complex V	***ATP6***	**LS, MILS, NARP**
b	OXPHOS biogenesis/regulation	Complex I	*NDUFAF2; C20ORF7; C8ORF38*	LS
		Complex II	*SDHAF1*	Leukoencephalopathy
		Complex II	*SDHAF2*	Paraganglioma
		Complex III	*BCS1L*	GRACILE
		Complex III	*UQCC2*	IUGR, neonatal LA, renal tubular dysfunction
		Complex III	*LYRM7/MZM1L*	Early-onset encephalopathy with LA
		Complex III	*UQCRC2*	Neonatal-onset recurrent metabolic decompensation
		Complex III	*TTC19*	Encephalopathy
		Complex IV	*SURF1*	LS
		Complex IV	*SCO1, 2; COX 10, 15*	Neonatal-onset encephalopathy, cardiomyopathy, LA, LS
		Complex IV	*COX20*	Recessive dystonia-ataxia syndrome
		Complex IV	*LRPPRC*	LS French-Canadian
		Complex V	*ATP5A1*	Neonatal encephalopathy
		Complex V	*TMEM70*	3-MGA, LA, IUGR, encephalopathy, (cardio)myopathy, cataracts
		Complex V	*ATPAF2*	3-MGA, LA, neonatal encephalopathy, dysmorphy
		Fe/S cluster biogenesis	*NUBPL*	Encephalopathy with complex I deficiency
		Fe/S cluster biogenesis	*FRDA*	Friedreich's ataxia
		Fe/S cluster biogenesis	*NFU1*	Early-onset encephalopathy, LA, vasculopathy
		Fe/S cluster biogenesis	*BOLA3*	Early-onset encephalopathy, LA, cardiomyopathy
		OXPHOS regulation	*SPG7*	Spastic paraplegia 7
c	mtDNA replication, transcription, translation	mtDNA replication	*POLG1*	Alpers-Huttenlocher syndrome, MIRAS, MSCAE, ad/arPEO
			POLG2	adPEO
			Twinkle (C10orf2)	IOSCA, hepatocerebral MDS, adPEO
		mtDNA-related transcription and translation	***MTTL1 (tRNA-leucine)***	**MELAS**
			MTTK (tRNA-lysine)	**MERRF**
			MTTS (tRNA-serine)	**myoclonus, epilepsy, ataxia, sensorineural hearing loss**
			Single large-scale mtDNA deletions	**KSS, Pearson syndrome, PEO**
		mtDNA translation: aminoacyl-tRNA synthetases	*AARS2*	Neonatal LA, cardiomyopathy
			DARS2	LBSL
			EARS2	LTBL
			FARS2	Alpers syndrome
			HARS2	Perrault syndrome
			MARS2	Leukoencephalopathy with spastic ataxia
			RARS2	Pontocerebellar hypoplasia type 6
			YARS2	MLASA2, metabolic decompensation
		mtDNA translation: tRNA-modifying	*MTFMT*	LS

		enzymes		
			MTO1	Neonatal LA, cardiomyopathy
		mtDNA translation: ribosomal proteins	*MRPS16*	Neonatal LA, hypotonia, agenesis of corpus callosum
			MRPS22	Leukoencephalopathy, cardiomyopathy, tubulopathy, dysmorphy
		mtDNA translation: elongation factors	*GFM1*	Encephalopathy with or without liver involvement
			TUFM	Leukoencephalopathy, LA, polymicrogyria
			TSFM	Encephalopathy, hypertrophic cardiomyopathy
		mtDNA translation: termination factors	*C12orf65*	Leukoencephalopathy
		mRNA stability and activation	*TACO1*	LS
			MTPAP	Progressive spastic ataxia with optic atrophy
	mtDNA maintenance	mtDNA depletion: defects affecting nucloeside pool regulation	*DGUOK*	Hepatocerebral MDS
			RRM2B	Encephalomyopathic MDS, tubulopathy
			TYMP	MNGIE
			SLC25A4	adPEO, cardiomyopathic MDS with exercise intolerance
		mtDNA depletion	*MPV17*	Hepatocerebral MDS
			FBXL4	Early-onset encephalopathy, LA, IUGR, dysmorphy, cataracts
		mtDNA depletion: enzymes in production of succinate-CoA ligase	*SUCLA2, SUCLG1*	Encephalomyopathic MDS, MMA, LS
d	Membrane dynamics and other	Mitochondrial fusion	*OPA1*	adOA, deafness, PEO, ataxia
			MFN2	Charcot-Marie-Tooth disease type 2A1 and 2A2
		CoQ10 biosynthesis	*COQ2, PDSS1*	Encephalopathy, cardiomyopathy, renal failure
			COQ9, PDSS2	Neonatal-onset encephalopathy, LS
			CABC1	Spinocerebellar ataxia-9 (SCAR9)
			ADCK3	Childhood-onset cerebellar ataxia and seizures
		Sulfide metabolism in mitochondrial matrix	*ETHE1*	Ethylmalonic encephalopathy
		Pyruvate metabolism in mitochondrial matrix	*PDHA1*	LS, neonatal LA and encephalopathy
			PDHX	LS
		Mitochondrial import	*SLC19A3*	LS
			SLC25A3	Hypertrophic cardiomyopathy, LA
			SLC25A12	Encephalopathy with global cerebral hypomyelination
			DDP1 (TIMM8A)	Mohr-Tranebjaerg syndrome
		Mitochondrial membrane repair	*SERAC1*	MEGDEL

Genes encoded by mtDNA and the associated phenotypes appear in bold text

adOA: Autosomal dominant optic atrophy; adPEO: Autosomal dominant progressive external ophthalmoplegia; arPEO: Autosomal recessive progressive external ophthalmoplegia; IOSCA: Infantile onset spinocerebellar ataxia; IUGR: Intrauterine growth restriction; LA: Lactic acidosis; LBSL: Leukoencephalopathy with braistem and spinal cord involvement and lactate elevation; LHON: Leber hereditary optic neuropathy; LS: Leigh syndrome; LTBL: Leukoencephalopathy with thalamus and

brainstem involvement and high lactate; MDS: mtDNA depletion syndrome; MILS: Maternally inherited Leigh syndrome; MIRAS: Mitochondrial recessive ataxia syndrome; MLASA2: Myopathy, lactic acidosis and sideroblastic anemia-2; MNGIE: Mitochondrial neurogastrointestinal encephalopathy; MMA: Methylmalonic aciduria; MSCAE: Mitochondrial spinocerebellar ataxia and epilepsy; NARP: Neuropathy, ataxia and retinitis pigmentosa; PEO: Progressive external ophthalmoplegia; 3-MGA: 3-methylglutaconic aciduria

1.4 Early-onset mitochondrial encephalopathies

Early-onset mitochondrial encephalopathies comprise a group of mitochondrial disorders that present in infancy or early childhood and primarily involve the CNS. An overview of the major phenotypes of early-onset mitochondrial encephalopathies along with the associated genetic defects, are displayed in Table 2.

1.4.1 Alpers syndrome

Alpers syndrome is a progressive neurodegenerative disorder of infancy and early childhood that has over the years been designated various names, such as diffuse progressive degeneration of the cerebral gray matter, progressive (infantile) cerebral poliodystrophy, spongy glio-neuronal dystrophy, and progressive neuronal degeneration of childhood. The syndrome was neuropathologically described by Bernard Alpers in 1931 as diffuse, progressive degeneration of the gray matter of the cerebrum [16]. This cerebral degeneration has been shown to predominantly affect the cerebral cortex and to a lesser degree the cerebellar gray matter, thalamus and basal ganglia, while atrophy of the white matter is typically less striking. Alpers syndrome initially presents with hypotonia, failure to thrive, epileptic seizures and psychomotor regression, often deteriorating during infections. The disease course is severe and often rapidly progressive, characterized by intractable epilepsy, sever psychomotor regression, spasticity and cortical blindness. Liver dysfunction has been associated with Alpers syndrome in variable degree.

Historically, the neurodegenerative process underlying Alpers syndrome has occasionally been attributed to causes other than genetic, such as extensive perinatal cortical damage due to anoxia in relation to a complicated delivery, postepileptic atrophy or inflammatory processes [17-19]. It was not until 2004, that mutations in *POLG1*, the gene encoding the gamma subunit of the mtDNA polymerase, have been found to cause Alpers syndrome with hepatic involvement, a syndrome that is better known as Alpers-Huttenlocher syndrome [20-22]. Deficient polymerase gamma results in impaired mtDNA replication, which may lead to reduction in mtDNA copy number, a condition known as mtDNA depletion. Indeed, Alpers-Huttenlocher syndrome is one of

the most common phenotypes of hepatocerebral mtDNA depletion syndromes (MDS) [23]. The depleted mtDNA is apparent in the liver and may be apparent in fibroblasts, whereas muscle mtDNA content is usually normal [23]. Antiepileptic treatment with valproate has been associated with fulminant liver failure in the presence of *POLG1* mutations [24, 25].

The genetic etiology underlying Alpers syndrome is basically unknown. Recently, mutations in *FARS2*, a gene encoding the mitochondrial phenylalanine-tRNA synthetase, have been identified in two Finnish patients with Alpers syndrome [26]. This enzyme belongs to the class II aminoacyl-tRNA synthetase (mt-aaRS) family and is responsible for charging the mitochondrial tRNA-phenylalanine. This function, like that of all the other mitochondrial aminoacyl-tRNA synthetases, is essential for efficient mitochondrial protein synthesis [27].

1.4.2 Leigh syndrome

Leigh syndrome or subacute necrotizing encephalomyelopathy, is a progressive neurodegenerative disorder that is usually associated with defects involving mitochondrial OXPHOS. Leigh syndrome primarily affects infants and young children and is considered to be the most common distinct phenotype among OXPHOS disorders in children [9]. It was first described by Denis Leigh in 1951, as a distinct neuropathological entity with focal, bilaterally symmetrical, subacute necrotic lesions extending from the thalamus to the brainstem and the posterior columns of the spinal cord [28]. As opposed to the poliodystrophy in Alpers syndrome that mainly affects the cerebral cortex, the lesions in Leigh syndrome mainly affect the central gray matter, i.e. the basal ganglia, diencephalon, brainstem, cerebellum and/or spinal cord [29, 30]. Onset of disease occurs typically between three and 12 months of age, with disease progression and death within two years. Later onset and slower progression have also been reported [29-33]. Clinical manifestations include psychomotor delay, hypotonia, dyskinesia, akinesia, ataxia, dystonia and brainstem dysfunction, including respiratory abnormalities, swallowing dysfunction, ophthalmological manifestations and abnormal thermoregulation [29, 34].

Leigh syndrome is genetically heterogeneous and can be inherited as a mitochondrial trait, as an autosomal recessive trait due to mutations in nuclear genes encoding mitochondrial respiratory chain complex subunits or complex assembly proteins [35] and X-linked related to defects in pyruvate dehydrogenase complex (PDHc) due to mutations in the *PDHA1* gene [36].

Table 2. Early-onset mitochondrial encephalopathies: Major phenotypes, typical age of onset, cardinal features and associated genotypes.

Phenotypes	**Age of onset**	**Cardinal features**	**Genotypes**
Alpers syndrome	Neonatal, infantile	Hypotonia, seizures, psychomotor regression	*FARS2*
Alpers-Huttenlocher syndrome	Infantile, occasionally later	Hypotonia, refractory seizures, epilepsia partialis continua, psychomotor regression, hepatic failure	*POLG1*
Leigh syndrome	Infantile, occasionally later	Hypotonia, dystonia, dyskinesia, ataxia, brainstem dysfunction. Phenotypic overlap with GRACILE syndrome (see below), biotin-responsive basal ganglia disease and MEGDEL	*mtDNA* *ND1, ND2, ND3, ND4, ND5, ND6, ATPase 6* *nDNA* *NDUFS1, NDUFS2, NDUFS3, NDUFS4, NDUFS7, NDUFS8, NDUFV1, NDUFA2, NDUFA10, NDUFA12, NDUFAF2, C20orf7, C8orf38, SDHA, BCS1L, SURF1, COX10, COX15, TACO1, LRPPRC, PDSS2, PDHA1, PDHX, SLC19A3, SUCLA2, SUCLG1, MTFMT, SERAC1*
GRACILE	Neonatal, infantile	Growth retardation, aminoaciduria, cholestasis, iron overload, lactic acidosis, early death. Phenotypic overlap with Leigh syndrome.	*BCS1L*
Hepatocerebral MDS	Infantile	Hypotonia, failure to thrive, hepatopathy, psychomotor delay/regression	*DGUOK* *MPV-17*
	Infantile, occasionally later	Ataxia, hypotonia, athetosis, ophthalmoplegia. Also known as infantile onset spinocerebellar ataxia (IOSCA)	*C10orf2*
	Infantile, occasionally later	*POLG1*-associated, also known as Alpers-Huttenlocher syndrome (see above)	*POLG1*

Encephalo-myopathic MDS	Infantile	Hypotonia, psychomotor delay/regression, dystonia, hearing impairment, respiratory distress. Phenotypic overlap with Leigh syndrome (see above)	*SUCLA2, SUCLG1*
	Neonatal, infantile	Hypotonia, lactic acidosis, progressive muscle weakness, seizures, tubulopathy, respiratory distress	*RRM2B*
Other MDS	Neonatal, infantile	IUGR, hypotonia, microcephaly, craniofacial abnormalities, cataracts	*FBXL4*
Neonatal lactic acidosis with cardiomyopathy	Neonatal	Lactic acidosis, hypotonia, failure to thrive, heart disease, psychomotor delay, myopathy, respiratory distress, oculomotor findings	*AARS2, MTO1, TMEM70, SCO2*
Infantile-onset leukoencephalopathy	Neonatal, infantile	Psychomotor delay, ataxia, spasticity	*SDHAF1, COX6B1, DARS2, NDUFS1, NDUFV1*

The main genetic defects affecting the respiratory chain complexes that have been associated with Leigh syndrome are summarized below:

- Complex I: (i) mutations affecting mtDNA-encoded subunits, i.e. *MT-ND1, MT-ND2*, *MT-ND3, MT-ND4, MT-ND5, MT-ND6* [10, 37, 38]; (ii) mutations affecting nDNA-encoded subunits, i.e. *NDUFS1, NDUFS2, NDUFS3, NDUFS4, NDUFS7, NDUFS8, NDUFV1, NDUFA2, NDUFA10, NDUFA12* [10, 39-41]; (iii) mutations affecting nDNA-encoded assembly factors, i.e. *NDUFAF2, C20orf7, C8orf38* [10]

- Complex II: mutations in the *SDHA* encoding flavoprotein subunit A [10]

- Complex III: mutations in *BCS1L* gene, encoding an assembly factor, see detailed description in paragraph 1.4.3

- Complex IV: mutations in nDNA-encoded COX assembly factors, i.e. *SURF1, COX10, COX15* and mRNA translation activator for COX subunit I, i.e. *TACO1* [10, 42-45]

- Complex V: mutations in mtDNA affecting the ATP synthetase 6, the most frequent being T8993G [10]

- The French Canadian (or Saguenay-Lac-Saint-Jean) type of Leigh syndrome with tissue-specific COX deficiency is caused by mutations in the *LRPPRC* gene, which encodes for a mitochondrial protein involved in mtDNA expression and in mRNA stability and processing [46]

- Mutations in genes affecting the biosynthesis of coenzyme Q10, i.e. *PDSS2* [47]

Besides defects in PDHc, other genetic mitochondrial pathways indirectly affecting OXPHOS have been associated with Leigh syndrome, such as: (i) mutations in the thiamine transporter *SLC19A3* impair the import of thiamine in mitochondria, leading to thiamine metabolism dysfunction syndrome-2 or biotin-responsive basal ganglia disease (BBGD) [48]; (ii) mutations in nDNA-encoded enzymes for the production of succinate-CoA ligase, *SUCLA2* and *SUCLG1*, may cause Leigh syndrome with methylmalonic aciduria, often in the context of an encephalomyopathic MDS, see also paragraph 1.4.5; (iii) mutations in *MTFMT* affect the initiation and elongation of mitochondrial translation [49]; (iv) mutations in *SERAC1* affect the phosphatidylglycerol remodeling in mitochondria, causing 3-methylglutaconic aciduria with deafness, encephalopathy and Leigh-like syndrome (MEGDEL) [50].

1.4.3 GRACILE syndrome

GRACILE syndrome is a fatal, neurodegenerative disease characterized by fetal growth retardation, aminoaciduria, cholestasis with iron overload in the liver, lactic acidosis and early death [51, 52]. This syndrome is caused by mutations in the *BCS1L* gene, which is the chaperone needed to incorporate the catalytic subunit, Rieske iron-sulfur protein, into complex III at the final stage of its assembly. The resulting phenotype of infantile onset encephalopathy with lesions in the basal ganglia and brainstem overlaps with Leigh syndrome [52].

1.4.4 Hepatocerebral mtDNA depletion syndromes (MDS) not associated to *POLG*

The *POLG1*-related hepatocerebral MDS is better known as Alpers-Huttenlocher syndrome and is presented separately in paragraph 1.4.1. Other hepatocerebral MDS presenting early in childhood include those related to pathogenic mutations in (i) *DGUOK*, (ii) *MPV-17* and (iii) *C10orf2* genes. The *DGUOK* gene encodes for the deoxyguanosine kinase, a kinase responsible for the salvage of deoxyribonucleotides for mtDNA replication inside mitochondria. *DGUOK*- and *MPV-17*-related hepatocerebral MDS

have overlapping phenotypes, both characterized by infantile onset hepatopathy, often rapidly progressive to liver failure, and neonatal or infantile onset of hypotonia, failure to thrive and psychomotor delay [53-55]. Hypoglycemia, lactic acidosis and cholestasis are typically present at onset [53, 54]. Abnormal ophthalmological findings –mainly nystagmus- and seizures are less common [53]. Lesions in the reticular formation and globus pallidi have been found in both *DGUOK*- and *MPV-17*-related MDS [53], while white matter lesions and initial normal brain MRI have also been reported [55].

C10orf2-related hepatocerebral MDS is a rare cause of early-onset encephalopathy. The *C10orf2* gene encodes the mtDNA helicase Twinkle, which works in close connection with *POLG* in mtDNA replication. Recessive *C10orf2* mutations give rise to a hepatocerebral MDS, known as infantile onset spinocerebellar ataxia (IOSCA) [56, 57]. This syndrome presents with ataxia, hypotonia, athetoid movements and loss of deep tendon reflexes in infancy [56, 57]. Age of onset is typically between six and 14 months [57]. Ophthalmoplegia and hearing deficit develop soon after the onset of the disease. Sensory axonal neuropathy, psychomotor delay, migraine-like headaches, psychotic episodes and catastrophic epilepsy develop later in the disease course [58]. Liver involvement occurs early, but is typically less striking than in the other hepatocerebral MDS and not associated with valproate treatment [58]. Neuropathologically, the syndrome is characterized by spinocerebellar neurodegeneration, with moderate atrophy of the brain stem and the cerebellum and severe atrophy of the dorsal roots, the posterior columns and the posterior spinocerebellar tracts [56, 57]. The clinical and neuropathological findings in IOSCA are typically milder than those seen in Alpers-Huttenlocher syndrome [58].

1.4.5 Encephalomyopathic and other mtDNA depletion syndromes (MDS)

The encephalomyopathic MDS present two different phenotypes, one related to mutations in *SUCLA2* and *SUCLG1* genes and another related to mutations in *RRM2B*. *SUCLA2* and *SUCLG1* are the two subunits of succinyl-coenzyme A ligase, the enzyme that catalyzes the reversible conversion of succinyl-coenzyme A to succinate in the Krebs cycle. The phenotype in *SUCLA2*- and *SUCLG1*-related MDS is characterized by infantile onset hypotonia and psychomotor delay, dystonia, choreoathetosis, feeding and sucking difficulties, sensorineural hearing impairment and respiratory insufficiency [59, 60]. The patients may also develop abnormal ophthalmological findings and seizures, while neonatal lactic acidosis and

elevated methylmalonic acid in the urine are characteristic findings [59-61]. Bilateral lesions in the basal ganglia are a common neuroimaging finding, resulting in a phenotypic overlap with Leigh syndrome [62].

The *RRM2B* gene encodes the R2 subunit of the p53-controlled ribonucleotide reductase (p53R2) that catalyzes the biosynthesis of deoxyribonucleotides for mtDNA replication. The *RRM2B*-related MDS is characterized by neonatal or infantile onset hypotonia, lactic acidosis and progressive muscle weakness [63, 64]. The majority of patients develop renal proximal tubulopathy, seizures and respiratory distress during infancy [63, 64].

Another phenotype of MDS was recently reported, owing to mutations in *FBXL4*, encoding an F-box protein important for the maintenance of mtDNA [65]. Affected patients develop fatal mitochondrial encephalopathy and lactic acidosis with typical onset within the first year of life. Intrauterine growth restriction, microcephaly, hypotonia, craniofacial abnormalities and cataracts are common clinical features. Severe mtDNA depletion has been found in the muscle resulting in combined respiratory chain enzyme deficiencies [65].

The phenotype and natural course of MDS with encephalopathy are believed to depend upon the severity of the causative mutations, which also reflects to the severity of the mtDNA depletion and the subsequent impairment of OXPHOS [64, 65].

1.4.6 Neonatal lactic acidosis with cardiomyopathy

Neonatal lactic acidosis in combination with cardiomyopathy or heart failure is seen in neonates with severe, usually fatal, mitochondrial disorders, while encephalopathy develops in those patients who survive a fatal outcome early in infancy. The phenotypic spectrum of these disorders is overlapping and includes a combination of features, such as severe myopathy, central hypotonia, failure to thrive, respiratory distress, psychomotor delay, ataxia and oculomotor findings. An overview of the most common genotypes implicated in these disorders is summarized below.

Mutations in *AARS2*, encoding the mitochondrial alanyl-tRNA synthetase, have been associated with neonatal lactic acidosis and hypertrophic cardiomyopathy resulting in infantile cardiac failure [66].

MTO1 encodes one of the two subunits of the enzyme that catalyzes the 5-carboxymethylaminomethylation (mnm5s2U34) of the uridine base in the

mitochondrial tRNAs specific to glutamine, glutamic acid, lysine, leucine, and possibly tryptophan. Mutations in *MTO1* have been associated with neonatal lactic acidosis, hypertrophic cardiomyopathy, hypotonia, muscle weakness and failure to thrive, while features of encephalopathy, dystonia and optic atrophy have been found in patients surviving into early childhood [67, 68].

Mutations in *TMEM70*, a gene that encodes the structural subunits of ATP synthase (complex V) have been associated with neonatal lactic acidosis, respiratory distress, hypotonia, cardiomyopathy and psychomotor delay [69, 70].

SCO2 is a gene which together with *SCO1* code for metallochaperones that deliver copper to a subunit in the catalytic core of cytochrome c oxidase. Mutations in *SCO2* have been associated with neonatal lactic acidosis and cardiomyopathy with fatal outcome in infancy [71, 72].

Mutations in two genes involved in the biogenesis of the Fe-S clusters in mitochondria, *BOLA3* and *NFU1* have been linked to neonatal encephalopathy with lactic acidosis, hyperglycinemia and fatal outcome in infancy [73-75]. Cardiomyopathy has been seen in *BOLA3* mutations, while patients with *NFU1* mutations suffer from pulmonary hypertension owing to obstructive vasculopathy [75].

1.4.7 Infantile onset leukoencephalopathy

The appearance of clinical and neuroimaging findings that predominantly involve the cerebral white matter occurs in a heterogeneous group of mitochondrial disorders known as leukoencephalopathies. Infantile onset leukoencephalopathy is often associated with defects of complex I or complex II. The most common genotypes underlying this disorder are: (i) defects in *SDHAF1,* the gene encoding for the SDH assembly factor 1, causes an infantile leukoencephalopathy with accumulation of lactate and succinate in the white matter [76]; (ii) mutations in *COX6B1* have been linked to infantile onset leukodystrophic encephalopathy, myopathy and growth retardation [77]; (iii) leukoencephalopathy with brainstem and spinal cord involvement and lactate elevation (LBSL) due to mutations in the mitochondrial aspartyl-tRNA synthetase *DARS2* present a broad phenotypic spectrum, including a more severe phenotype of infantile onset [78]; (iv) mutations in the nDNA-encoded subunits *NDUFS1* and *NDUFV1* have been shown to cause infantile onset cavitating leukoencephalopathy associated with complex I deficiency [79, 80].

1.5 The diagnostics of mitochondrial disease

The genotypes of childhood-onset mitochondrial disorders is extremely heterogeneous and the resulting phenotypes extremely broad and overlapping. As a consequence, the diagnosis of mitochondrial diseases is a multi-step process with molecular diagnostics being the verification step of the entire process. The diagnosis of a mitochondrial disease is clear cut when a pathogenic mutation is found along with compatible clinical findings. In the absence of identified pathogenic mutations, the diagnosis rests upon the combination of characteristic phenotypic, morphological and biochemical findings. The major steps in the diagnostic approach of mitochondrial disorders are described below.

1.5.1 Identifying the clinical phenotype

The diagnostic process starts upon clinical suspicion. As the phenotypic spectrum of mitochondrial disorders is broad and complex, the physical examination should include a thorough neurological examination of both the central and peripheral nervous system, as well as evaluation of the involvement of other systems or organs, suggestive of a mitochondrial disorder. In order to facilitate the clinical evaluation, a number of 'red-flags' have been proposed, as the clinical features that should prompt a diagnostic work-up for suspected mitochondrial disease [81]. The clinical suspicion is strengthened by a positive family history, which assists in recognizing the inheritance pattern; a negative family history, however, does not exclude the presence of mitochondrial disease.

1.5.2 Metabolic laboratory work-up

The laboratory work-up aims to identify biochemical markers suggestive of mitochondrial disease, such as the lactate levels, to evaluate involvement of other organs, such as measurement of liver and renal function and to help differentiate from other metabolic disorders with similar phenotypes, i.e. urea cycle defects, aminoacidopathies, organic acidopathies and fatty acid oxidation disorders. The measurement of lactate and occasionally pyruvate in the blood and the cerebrospinal fluid (CSF) is included in the initial work-up, along with the estimation of the lactate-to-pyruvate ratio. Despite their lack of specificity, an elevated plasma lactate or pyruvate level can be suggestive of mitochondrial disease. CSF lactate levels are considered more reliable as they are less influenced by external factors, such as the collection technique. In some patients, the levels of lactate and/or pyruvate rise only during episodes of metabolic decompensation and are otherwise normal [81].

1.5.3 Neuroimaging

Both structural magnetic resonance imaging (MRI) and functional brain imaging methods, e.g. magnetic resonance spectroscopy (MRS), diffusion-weighted imaging (DWI) and perfusion MRI, have helped to increase our knowledge of mitochondrial disorders by allowing a good assessment of the anatomic lesions, metabolism and hemodynamics of the brain [82]. MRI signal abnormalities, as acquired with T1- and T2-weighted sequences, can reveal specific or 'signature' disease features, non-specific features or leukodystrophy-like features suggesting a mitochondrial disorder [83-86]. However, a brain MRI may occasionally be normal [83-86]. MRS provides a unique in vivo evaluation of brain metabolism and may also be used to monitor disease progression. Lactate accumulation and N-acetyl-aspartate (NAA) reduction are the most prominent MRS signal abnormalities in mitochondrial disorders. DWI, on the other hand, detects the random Brownian motion of water protons in the brain. The diffusion of water protons can be quantitated by a parameter known as the apparent diffusion coefficient (ADC). The combination of DWI and ADC helps differentiate between acute and chronic ischemia and between cytotoxic and vasogenic edema [84]. Combined with clinical indices, neuroimaging of the brain can assist in identifying and differentiating between mitochondrial disorders [87]. This is of great importance in non-syndromic mitochondrial disorders, where brain imaging features are characteristically diverse and non-specific [88].

1.5.4 Specific tissue biopsies

In general, a biopsy has higher chance to provide a representative sample for diagnostic testing when performed in the tissues that are most profoundly affected by the disease. In mitochondrial encephalopathies, the most affected organ is usually the brain and the second most affected organ is the muscle. The skeletal muscle biopsy is an essential diagnostic tool, as it provides optimal sampling for morphological, biochemical and molecular testing. Portions of the biopsy are utilized for histochemical, immunohistochemical and electron microscopy studies to evaluate for morphological evidence of primary mitochondrial disease and assist in the differential diagnosis from other neuromuscular diseases [82]. Morphological findings highly suggestive of a mitochondrial disease are the identification of ragged red fibers (RRFs), decreased SDH reaction suggesting complex II deficiency, and decreased COX reaction suggesting complex IV deficiency. Muscle tissue is also used for biochemical analysis of the respiratory chain enzyme activities and for the extraction of DNA for genetic testing. Biochemical analysis includes polarographic studies of oxygen consumption and spectrophotometric analysis of the mitochondrial respiratory chain enzymes in cultured cells,

tissue homogenates or whole cells. To help distinguish primary electron transport chain defects from secondary deficiencies, the enzyme activity measurements are reported relative to a marker enzyme, such as citrate synthase, a mitochondrial matrix enzyme considered to be a good indicator of the mitochondrial mass [82].

In mitochondrial encephalopathies, the absence of pathognomonic morphological or biochemical findings from the skeletal muscle does not exclude a mitochondrial etiology. A skin biopsy is usually performed at the time of muscle biopsy and the cultured skin fibroblasts can also be used for biochemical and molecular analysis. When affected, the liver is a biopsy site that allows not only for morphological and biochemical analysis, but also for detection of mtDNA depletion in the case of hepatocerebral MDS. A heart biopsy can be informative when signs of mitochondrial cardiomyopathy are present.

1.5.5 Molecular diagnostics

The molecular diagnosis of suspected mitochondrial disease has evolved rapidly over the past two decades. Genetic testing of mtDNA, that was once limited to a panel of common mtDNA point mutations underlying well-recognized syndromic mitochondrial disorders, such as MELAS, MERRF and NARP, has now evolved to whole mtDNA genome sequencing; the latter permits identification of all known and potentially novel disease-causing mutations in a single platform [89]. When the phenotype, family history and biochemical findings raise the suspicion of maternally inherited mitochondrial disease, the testing of mtDNA is usually the first step towards a molecular diagnosis. The increasing number of nuclear genes causing primary mitochondrial disease, now counting 1500 nuclear genes, has made targeted sequencing of nuclear genes on an individual step-wise basis expensive, time-consuming and of low diagnostic yield [89].

The development of Next Generation Sequencing (NGS) has revolutionized the diagnostic approach of mitochondrial diseases. The highly advanced NGS technology is capable of sequencing a group of target genes in parallel and is therefore an ideal approach for the diagnosis of complex dual genome mitochondrial disorders [90]. Whole-exome sequencing (WES) is the application of the next-generation technology to determine the variations of all coding regions, or exons, of known genes. WES provides coverage of more than 95% of the exons, which contains 85% of disease-causing mutations in Mendelian disorders [91]. WES can identify not just known mitochondrial disease genes, but also mutations in a wide range of genetic

disorders with overlapping clinical manifestations that may directly or indirectly cause secondary mitochondrial dysfunction, thus offering an excellent diagnostic tool in the demanding field of mitochondrial disorders [89, 90].

1.5.6 Post-mortem investigation

Post-mortem investigation is pivotal both for diagnostic and research purposes, as it discloses the underlying neuropathology in mitochondrial encephalopathies. Both Alpers syndrome and Leigh syndrome were first described as neuropathological entities and later shown to be genetic disorders. The poliodystrophy seen in Alpers syndrome is a characteristic finding seen on brain biopsy. The role of post-mortem investigation is also essential, as it may reveal tissue-specific findings in the absence of muscle pathology, such as in hepatocerebral MDS, where the mtDNA depletion may only be seen in the brain or the liver.

2 AIMS

The overall aim of this thesis was to explore the genotypic and phenotypic spectrum of childhood-onset mitochondrial diseases with CNS involvement, with focus on early-onset mitochondrial encephalopathies. The present work describes this spectrum with emphasis on Alpers and Leigh syndrome.

The basic aims of this thesis were:

i) To present the genotypic and phenotypic spectrum of Alpers syndrome and to identify genotype-phenotype correlations – Paper I

ii) To present novel genetic defects underlying Alpers syndrome along with the respective phenotypes – Paper II

iii) To present the genotypic and phenotypic spectrum of Leigh syndrome, to characterize the clinical course and identify predictors of survival – Paper III

iv) To present the brain MRI findings in childhood-onset mitochondrial disorders with CNS involvement – Paper IV

3 PATIENTS AND METHODS

3.1 Patients

3.1.1 Patients in Papers I, II and IV

The patients who participated in our studies were collected from a total of approximately 170 patients who were investigated for suspected mitochondrial disease at the Queen Silvia's Children Hospital between January 1st 1984 and December 31st 2012 and were found to meet the diagnostic criteria of mitochondrial encephalopathy, as follows [9]:

Presence of known pathogenic mutations of mtDNA or nDNA, together with compatible clinical findings;

Or, in the absence of other known disorders with secondary effects on the mitochondrial respiratory chain, at least two out of four criteria should be fulfilled:

1. Oximetry: Decreased respiratory rates indicating isolated or combined complex deficiencies, as follows: Respiratory rates below the control range in the presence of the nicotinamide adenine dinucleotide (NAD)-linked substrates pyruvate and glutamate but with normal rates in the presence of succinate and ascorbate plus N,N,N',N'-tetra methyl-p-phenylene-diamine (TMPD), indicating a deficiency of reduced NAD (NADH)-Co Q reductase (complex I). Respiratory rates below the control range in the presence of pyruvate, glutamate, and succinate but with normal rates in the presence of ascorbate plus TMPD, indicating a deficiency of Co Q-cytochrome c reductase (complex III). Decreased respiratory rates in the presence of all substrates tested, indicating a deficiency of cytochrome c oxidase (COX, complex IV).

2. Spectrophotometry: enzyme activities below the control range of NADH ferricyanide reductase (complex I), succinate-cytochrome c reductase (complex II and/or III) or COX (complex IV).

3. Histochemical evidence of COX deficiency.

4. Abundant, ultra-stucturally abnormal mitochondria.

Patients with Alpers syndrome were considered those who in addition to the above mentioned diagnostic criteria, exhibited early-onset diffuse progressive

cerebral atrophy with cortical predominance, based on their clinical features in combination with neuroimaging and/or neuropathological findings. Siblings to study participants that had presented similar clinical features and disease course were also included.

3.1.2 Patients in Paper III

The patients who participated in the study presented in Paper III arose from a collaboration between eight centers specializing in mitochondrial diseases in Europe; Gothenburg, Rotterdam, Helsinki, Copenhagen, Stockholm, Brussels, Bergen and Oulu. We included patients with Leigh syndrome that were diagnosed and followed at the participating centers and that fulfilled both of the following inclusion criteria: (i) clinical features compatible with Leigh syndrome, such as psychomotor regression, dystonia, ataxia and/or brainstem dysfunction and (ii) MRI or CT or neuropathological findings of Leigh syndrome, as follows: bilateral symmetrical lesions in the basal ganglia, and/or thalamus, and/or brainstem. Patients were excluded from this study if they had known syndromic mitochondrial phenotypes other than Leigh syndrome.

3.2 Methods

This research work was performed as a retrospective analysis of longitudinal data, including clinical, laboratory, morphological, histochemical and (neuro)pathological data. The author performed neurological examination on only a few patients. All patients had been clinically assessed by at least one of the neurologists involved in the studies. A set of tools and procedures were applied to ensure that all study-related data were collected in a standardized and valid manner, as described below.

3.2.1 Methods in Papers I, II and IV

3.2.1.1 Clinical evaluation, laboratory investigations, investigations of the muscle and post-mortem investigations

The clinical evaluation and investigations performed in our clinic for suspected mitochondrial disease follow a specific protocol, which has been implemented in collaboration with a research team of experts in mitochondrial diseases at the Sahlgrenska University Hospital. The clinical and laboratory findings for our studies were collected from the patients' medical records with the help of a standardized case report form (CRF). The biochemical, morphological and histochemical investigations of the muscle samples were performed in collaboration with the research team at

Sahlgrenska University Hospital according to standard protocols, essentially as described previously [92]. The pathologists involved in the studies reported the pathological findings from specimens obtained post-mortem.

3.2.1.2 Genetic investigations

The methodology applied for the genetic investigations is described in detail in the original publications.

DNA extraction

Total genomic DNA was extracted from skeletal muscle tissues or peripheral blood using standard commercial extraction kits. Total DNA was extracted from frozen skeletal muscle tissues and post-mortem tissues using commercially available kits.

***POLG1* analysis**

All patients with Alpers syndrome were investigated for *POLG1* mutations. The only exception was the siblings to patients who were found negative for mutations in *POLG1* and thus they were considered to be negative as well.

Mitochondrial DNA analysis

Muscle mtDNA was analyzed by Southern blotting to detect large-scale deletions and duplications after cleavage with the restriction enzymes PvuII or BamHI, and for the point mutations A3243G, T8993C/G and A8344G. mtDNA was also analyzed for the presence of large-scale deletions by long expand PCR and depletion using real-time quantitative PCR in selected patients. The ratio of mtDNA copy number to nDNA copy number in each patient was compared to the mean relative ratio in a control group of children (n=8) younger than two years of age and without evidence of neuromuscular disease. Values below 0.2 were considered as mtDNA depletion. Complete mtDNA sequence analysis was performed in all patients with Alpers syndrome without *POLG1* mutations (Paper I). Identified mutations considered to be potentially pathogenic were further investigated in the mothers' mtDNA. The primer sequences and PCR conditions are available upon request.

Whole exome sequencing

Whole exome sequencing (WES) was performed in selected patients with Alpers syndrome without *POLG1* mutations. The results in two of these

patients are presented in Paper II. WES in these two patients was carried out using high-throughput sequencing technology, as described in detail in Paper II. Quality assessment of the sequence reads was performed by generating QC statistics with FastQC (http://www.bioinformatics.bbsrc.ac.uk/projects/fastqc). For the identification of potentially pathogenic variants, the Ingenuity® Variant AnalysisTM software was used (www.ingenuity.com/variants). All mutations identified by WES were verified by Sanger sequencing using primers amplifying the exons harbouring the mutations in *NARS2* and *PARS2*. Sequencing analysis was performed using an ABI PRISM® 3100 Genetic analyzer and the BigDye Terminator v.1.1 Cycle Sequencing Kit (Applied Biosystems). A description of the sequencing data is provided in Paper II. Primer sequences and PCR conditions are available upon request.

3.2.1.3 Neuroimaging investigations

All brain MRI scans, performed either at our hospital or elsewhere, were re-evaluated by the author together with a pediatric neuroradiologist, who were both blinded to the patients' diagnosis, as well as a pediatric neurologist. Since this was a retrospective study, patients' MRI scans were not performed under a standardized protocol. Axial and sagittal T1-weighted images were assessed in particular for the presence of cortical atrophy, agenesis/hypoplasia of the corpus callosum, enlargement/dilatation of the ventricles and/or subarachnoid spaces. Axial and coronal T2-weighted images, including fluid attenuated inversion recovery (FLAIR) images, were studied to identify signal intensity changes. Myelination was assessed with the aid of conventional MRI images. Myelination was compared to age-matched healthy controls and classified as described in detail in Paper IV. Additional imaging sequences, such as DWI, ADC and MRS, were evaluated where available.

For the diagnostic approach used in Paper IV, i.e. from the neuroanatomical and/or neurofunctional lesion to disease identification, the abnormal MRI findings were categorized on the basis of their predominant location in the brain as follows: lesion predominance in the cerebral cortex/limbic system; white matter; basal ganglia/diencephalon; posterior fossa; diffuse lesions (more than one anatomic region involved with no apparent predominance).

3.2.2 Methods in Paper III

Paper III is the first collaborative research work within the Mitochondrial Clinical and Research Network (MCRN). This network was established in 2011 and constitutes a European network of research centers specializing in

mitochondrial diseases. The centers that participated in this study were from Gothenburg, Rotterdam, Helsinki, Copenhagen, Stockholm, Brussels, Bergen and Oulu. Our center in Gothenburg had the leadership in the design and coordination of this research project.

For the collection of patient data, an electronic-Case Report Form (e-CRF) was designed, which is shown in paper form in the Appendix. The design of the CRF was a multi-step procedure involving quarterly meetings between all investigators from the participating sites. The development of the electronic database for the electronic data capture (EDC) was undertaken by the Contract Research Organisation (CRO) Qualitis Ltd (http://www.qualitis.gr/). Qualitis' assigned personnel in collaboration with the author, set the validation rules and interim monitoring procedures to ensure the validity, completeness and integrity of the collected data and provided comprehensive training to all designated investigators prior to study initiation. Data were entered electronically via the e-CRF using web-based secure server by the designated investigators. The e-CRF was completed by one designated investigator at each center. Prior to study initiation, a detailed data management plan was issued describing the process to be followed for validating, processing and cleaning all study data. When all data were properly validated and the quality control procedure had been completed, the database was locked. The data management plan, the data validation plan and the standard operating procedures (SOPs) that were applied for quality control are available upon request. A detailed list with the definitions of the terms used in this study was provided as an attachment in the e-CRF (see Appendix).

3.2.3 Ethical considerations

The present research work is part of ongoing studies on childhood-onset mitochondrial diseases undertaken by the department of Child Neurology at the Queen Silvia Children's Hospital in collaboration with the Sahlgrenska University Hospital with the approval of the ethics committee at the University of Gothenburg. Additionally, for the multicenter study on Leigh syndrome (Paper III), each participating center received approval by the local ethical committee, as per the standing regulatory requirements, prior to the initiation of the study. The web-based EDC application (e-CRF) that was specifically designed for the needs of the multicenter study shown in Paper III adhered to all applicable data protection regulations and requirements with regard to electronic records.

4 RESULTS

4.1 Alpers syndrome – Paper I and II

4.1.1 Genotypic spectrum

Nineteen patients with Alpers syndrome were studied, among them four non-twin sibling pairs. Six patients were found to have pathogenic mutations in *POLG1* in compound heterozygosity as shown in Table 3. In two of the 13 patients without *POLG1* mutations, whole exome sequencing revealed mutations in two mitochondrial aminoacyl-tRNA synthetases (mt-aaRS), as follows: patient AS-13 was found to have a homozygous mutation (c.641C>T, p.P214L) in *NARS2*, encoding the mitochondrial asparaginyl-tRNA synthetase, while patient AS-15 was compound heterozygous for two mutations (c.1130dupC, p.K378fs*1 / c.836C>T, p.S279L) in *PARS2*, a gene encoding the mitochondrial prolyl-tRNA synthetase. The results were verified by Sanger sequencing and the parents of patients AS-13 and AS-15 were shown to be heterozygous for the respective mutations. The underlying cause for the remaining 11 patients with Alpers syndrome remains elusive.

4.1.2 Phenotypic spectrum and genotype-phenotype correlations

4.1.2.1 Onset

Patients with *POLG1* mutations had a median disease onset at 6.5 months of age, while patients without *POLG1* mutations exhibited a median disease onset in the perinatal period. The most common initial symptoms among all patients were seizures (13/19) and failure to thrive (5/19). Patient AS-13 with mutations in *NARS2* presented in early infancy with inconsolable crying, tendency to opisthotonus posturing and delayed head control. The patient's psychomotor development reached its maximum level at the age of six months, which corresponded to a developmental age of three months. Then, the patient showed the first signs of psychomotor regression, with loss of previously acquired motor skills and feeding difficulties. Patient AS-15 with mutations in *PARS2* was admitted to the hospital at 2.5 months of age because of feeding difficulties following an upper respiratory tract infection. During hospitalization, the patient developed seizures and signs of psychomotor regression.

Table 3. Clinical phenotypes and genetic findings in our 19 patients with Alpers syndrome

Patient id	Age at on set	Psycho-motor regres sion	Micro-cephaly	Hypo-tonia	Spasti-city	Ataxia	Stroke-like episodes	Seizures[1]	Hepatic failure	Genetic findings
AS-1§	12m	+	-	+	-	+	+	R/EPC/SE/M	+	*POLG1* A467T/R574W
AS-2§	7m	+	-	NA	NA	NA	+	R/EPC/SE/M	+	*POLG1* A467T/R574W
AS-3§	4m	+	-	+	-	+	+	R/EPC/SE/M	+	*POLG1* A467T/G848S
AS-4	11m	+	-	+	+	-	+	R/EPC/SE/M	+	*POLG1* A467T/G303R
AS-5§	5m	+	-	+	-	+	-	EPC/M	-	*POLG1* W748S, E1143G[2] /R232H
AS-6§	6m	-	NA	+	-	-	+	R/EPC/M	+	*POLG1* W748S, E1143G[2] /M1163R
AS-7	IU[3]	-	+	+	+	-	-	NS/IS/G/M	-	Y831C[2]
AS-8	2m	+	-	+	+	-	-	G	-	-
AS-9	IU	+	+	+	+	-	-	EPC/G/M	-	Q1236H[2]
AS-10	5m	+	-	+	+	-	-	G/M	-	NA
AS-11	Nb[4]	-	-	+	+	-	-	IS/M	-	-
AS-12	1m	+	+	+	+	-	-	IS/G/M	-	-
AS-13	IU	+	+	+	+	-	-	G	-	*NARS2* p.P214L homozygous
AS-14	IU	+	-	+	+	-	-	NS/IS	-	Y831C[2]
AS-15	2m	+	+	+	-	-	-	IS/G	-	*PARS2* p.K378fs*1/p.S279L
AS-16	Nb	+	+	+	+	-	-	IS/G	-	-
AS-17	Nb	+	+	+	+	-	-	R/G/SE/M	-	Q1236H[2]
AS-18	4m	+	+	+	-	-	-	IS/G/M	-	-
AS-19	Nb	-	+	+	+	-	-	NS/IS/G	-	NA

[1]Type of seizures: R= Refractory; EPC= Epilepsia Partialis Continua; SE= Status Epilepticus; M= Myoclonus; G= Generalized Seizures; NS= Neonatal Seizures; IS= Infantile Spasms
[2] The E1143G, Y831H and Q1236H are single-nucleotide polymorphisms (SNPs) according to the NCBI SNP database
[3]IU= Intrauterine, [4]Nb= Newborn, [5]NA= Not Applicable
§ Patient #1, 2, 3, 5 and 6 are included in the publication by Kollberg et al [21]

4.1.2.2 Clinical course and outcome

All patients developed seizures. Patients with *POLG1* mutations developed epilepsia partialis continua (6/6), myoclonus (6/6), refractory seizures (5/6) and status epilepticus (4/6). Patients without *POLG1* mutations developed in decreasing order of frequency; generalized tonic-clonic seizures (11/13), infantile spasms (8/13), myoclonus (7/13) and neonatal seizures (3/13).

All patients with *POLG1* mutations developed hepatic dysfunction. In the majority of them, hepatic dysfunction led to liver failure (5/6) and was related to preceding valproate treatment (4/6). Most of the patients without *POLG1* mutations developed hepatic dysfunction (9/13), regardless of valproate treatment, which mainly presented as abnormal liver function tests and did not lead to liver failure.

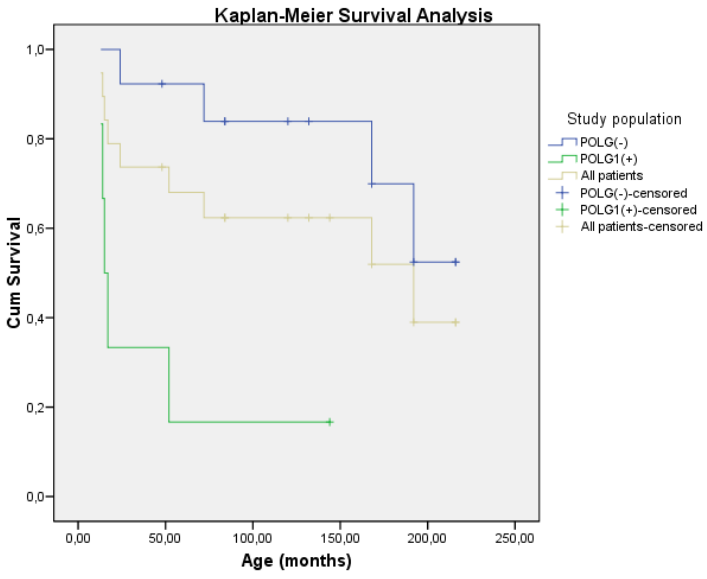
The majority of patients with Alpers syndrome developed muscular hypotonia (18/19), psychomotor regression (15/19) and failure to thrive (15/19). Ataxia (3/19) and stroke-like episodes (5/19) were only seen in patients with *POLG1* mutations. Progressive microcephaly and spasticity were more common in patients without *POLG1* mutations.

Cardiomyopathy was found in one patient with *POLG1* mutations and in four patients without *POLG1* mutations. One of these patients was patient AS-15 with *PARS2* mutations, who at the age of two years developed dilated cardiomyopathy and left ventricular hypertrophy and died of heart failure shortly afterwards.

Renal impairment was found in two patients with *POLG1* mutations and in one patient without *POLG1* mutations, patient AS-13 with *NARS2* mutations. This patient developed hypochloremic metabolic alkalosis and increased urinary glucose and salt excretion due to renal tubulopathy.

Our patients with Alpers syndrome were followed up for a period of seven years (range: 4m-18y). At the time of data analysis, nine patients had died (five with *POLG1* mutations and four without) at a median age of two years (range: 1y-16y). Patients with *POLG1* mutations exhibited a shorter life span, as five of them had died at a median age of 15 months (range: 1y-4.3y). The median age of death for the four patients without *POLG1* mutations was 10 years (range: 2y-16y). Patients AS-13 and AS-15 are among those four patients who had died. The survival analysis for all 19 patients and separately for those with versus without *POLG1* mutations is depicted in Figure 2.

Figure 2: Kaplan-Meier survival curve for the 19 patients with Alpers syndrome and separately for those with and without POLG1 mutations



4.1.2.3 Morphological, histochemical and biochemical findings

The morphological, histochemical and biochemical findings from the muscle investigations are detailed in Papers I and II. Abnormal respiratory chain enzyme activity was found in 15 of 17 examined patients with the most prevalent being complex I deficiency. Abnormal morphology and/or enzyme histochemistry of the muscle biopsy specimens were found in 10 out of 17 examined patients; four patients with *POLG1* mutations and six patients without *POLG1* mutations. Patients AS-1 and AS-2 who were siblings had normal muscle investigations without signs of OXPHOS deficiency; they were found to have *POLG1* mutations in compound heterozygosity (A467T/R574W).

Liver biopsy was performed in 12 patients at different stages during the course of the disease. Inflammation, steatosis, fibrosis, bile ductal proliferation and increased matrix density were the main findings in patients with *POLG1* mutations. Inflammation, steatosis and fibrosis were also found in patients without *POLG1* mutations, but to a lesser degree.

4.1.2.4 Neuroimaging findings

CT and/or MRI-investigations of the brain showed progressive atrophy of the cerebral cortex (17/19); in addition, these patients had lesions in the white matter (9/19), cerebellum (4/19), basal ganglia (3/19), thalamus (2/19) and agenesis/hypoplasia of the corpus callosum (2/19). Two patients with *POLG1* mutations had no atrophy on brain neuroimaging. One of them had a normal CT of the brain but experienced a detrimental disease progress that led to death shortly afterwards. The other patient presented with a unilateral cerebral lobar infarction and died shortly afterwards.

4.1.2.5 Post-mortem findings

The major neuropathological finding was a profound degeneration of the cerebral cortex, characterized by neuronal loss, spongiosis and gliosis frequently accompanied by signs of capillary proliferation, as described in detail in Paper I. Basal ganglia, thalamus and cerebellum were involved to variable extent in several patients. Microscopic vacuolation in the thalamus and basal ganglia was prominent in most patients with *POLG1* mutations. Several patients without *POLG1* mutations showed severe brain atrophy, which might be related to disease duration before death.

Post-mortem examination of the kidneys in patient AS-13 with *NARS2* mutations revealed an increased number of sclerotic glomeruli and occasional focal segmental sclerosis, along with a few atrophic tubules, without signs of increased interstitial fibrosis.

Post-mortem examination of the heart in patient AS-15 with *PARS2* mutations showed marked enlargement with dilation of the ventricles and increased thickness of the ventricular walls. There was both macroscopic and microscopic interstitial fibrosis of variable degree.

4.2 Leigh syndrome – Paper III

4.2.1 Perinatal history

A total of 130 patients with Leigh syndrome were enrolled into the study, among whom 11 sibling pairs. 19 patients (14.6%) were born preterm. Most pregnancies were uneventful (77.7%); the most common causes of complicated pregnancy were preeclampsia and oligohydramnios. 13 patients (10.0%) were born small for gestational age, while two patients presented intrauterine growth restriction. Microcephaly was evident at birth in six

patients (4.6%). The median Apgar score at 1, 5 and 10 minutes was 9-9-10 (Q25: 7-9-9; Q75: 9-10-10). The most common pathological signs at birth in decreasing order of frequency were respiratory difficulties (n=9), hypotonia/floppiness (n=7), cardiac complications (n=5), lactic acidosis (n=4) and feeding/sucking difficulties (n=3).

4.2.2 Onset

The median age of disease onset was 7 months (range: intrauterine-19y), with 80.8% presenting by the age of 2 years. Perinatal onset of disease was reported in 17 patients (13.1%), while three patients were reported to have intrauterine onset. Leigh syndrome presented initially with abnormal motor findings in the vast majority of patients (82.8%), with the most common being hypotonia (59.2%), abnormal tendon reflexes (14.6%) and ataxia (12.3%). Other presenting features were abnormal ocular findings (25.0%), feeding/sucking difficulties (14.1%), epileptic seizures (13.3%) and failure to thrive (10.2%). The clinical features at onset in relation to age of onset are shown in detail in Paper III.

4.2.3 Clinical course and outcome

4.2.3.1 Clinical features throughout the disease course

The vast majority of patients exhibited abnormal motor findings (99.2%), with the most common being hypotonia (74.6%), abnormal tendon reflexes (47.7%) and dystonia (44.6%). Dystonia, spasticity, hypertonia and choreoathetosis were less frequent at disease onset but developed later in the disease course.

Abnormal ocular findings were present in 79 patients (60.8%), the most prevalent being nystagmus (23.8%), followed by strabismus (19.2%), visual impairment (16.2%), optic atrophy (14.6%), ptosis (13.1%) and ophthalmoplegia (12.3%).

Epileptic seizures were reported in 51 patients (39.2%) and were classified according to ILAE as follows: generalized seizures (22.3%), focal seizures (14.6%) and epileptic spasms (6.1%). Resistance to antiepileptic treatment was reported in 16 patients.

Respiratory dysfunction was present in 37.7% with hyperventilation and/or abnormal breathing pattern being the most prevalent type (20.0%), followed by apnoea (16.1%), obstructive or restrictive respiratory disease (13.8%) and central hypoventilation (10.0%).

Cardiac dysfunction was present in 23 patients (17.7%), with more than half having hypertrophic cardiomyopathy (9.2%). Arrhythmia/conduction defects were reported in five patients and dilated cardiomyopathy was reported in two patients.

59 patients manifested feeding difficulties (45.4%) sufficient to necessitate tube feeding and/or gastrostomy. Mental retardation was found in 48 patients (36.9%). The severity of mental retardation was classified as mild in 11 patients, moderate in 17, severe in 15, profound in three and unspecified in two patients. Hearing impairment was identified in 25 patients and was sensorineural in 22, conductive in two and mixed in one patient. Hepatic dysfunction was present in 16 patients (12.3%), with elevated liver transaminases in 12; structural abnormalities defined by ultrasound or biopsy, including liver steatosis and/or fibrosis in four; severe liver failure in two and hepatomegaly in two patients. Microcephaly was present in 15 patients (11.5%).

4.2.3.2 Acute exacerbations and survival outcome

The study population was followed up for a median time of 9.6 years from disease onset. In total, 56.9% of patients experienced at least one acute exacerbation requiring hospitalisation during their disease course, 43.8% during the previous year. Of these, one fourth had at least three exacerbations during the previous year. Intensive care was required in 39.2% of hospitalized patients. The main cause of acute exacerbation was infection (60.8%); other causes included respiratory complications (13.5%), stroke-like episodes (4.0%) and poor nutrition or dehydration (4.0%).

53 patients were alive at the time of data analysis (40.8%), 51 were dead (39.2%) and 26 (20.0%) were lost to follow-up. Median age at death was 2.4 years (range: 1 month – 21 years). The elapsed median time from disease onset to death was 1.8 years. Main causes of death were respiratory complications (51.0%), progression of Leigh syndrome (17.6%) or infection (17.6%). The survival analysis for the entire population is seen in Figure 3.

4.2.4 Morphological, biochemical and histochemical findings

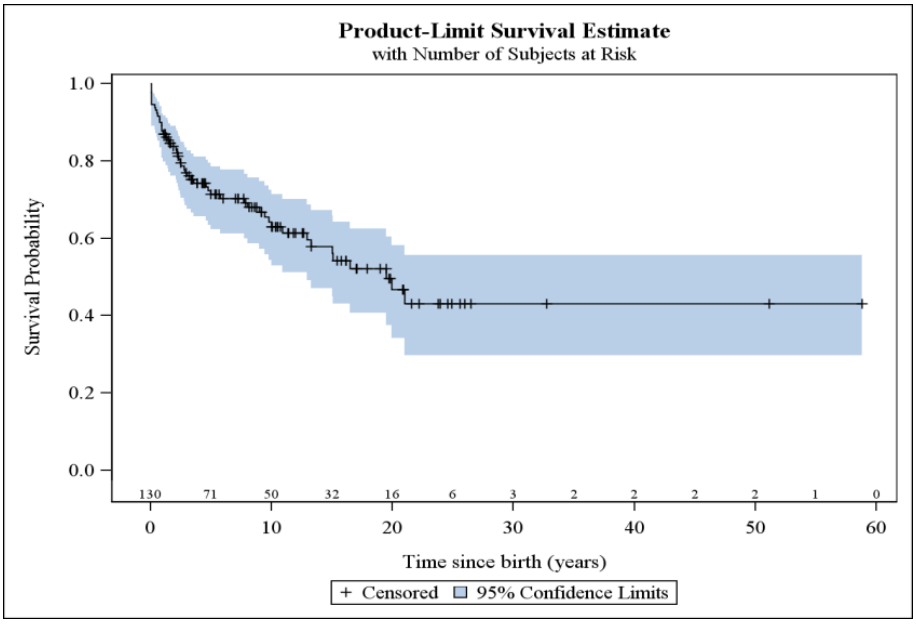
Abnormal respiratory chain enzyme activity was found in 70% of examined patients, with the most prevalent being complex I deficiency. In 24 of 57 patients with abnormal histopathology of the muscle, at least one of the following was found: cytochrome c oxidase deficiency, succinate

dehydrogenase deficiency, ragged red fibers and signs of abnormal mitochondrial proliferation. 25% of the patients with available lactate values had a maximum lactate in blood and/or CSF below or equal to 2.4 mmol/l, which was considered to be normal.

4.2.5 Genetic findings

Genetic etiology was confirmed in 77 patients (59.2%) of which nDNA mutations were much more common than mtDNA mutations (37.7% and 21.5% respectively). A detailed description of the genetic findings is provided in Paper III.

Figure 3: Kaplan-Meier survival curve for the 130 patients with Leigh syndrome



4.2.6 Predictors of disease severity and long-term prognosis

The presence of pathological signs at birth and a history of epileptic seizures are the two factors significantly associated with higher occurrence of acute exacerbations and/or relapses (p=0.0081 and p=0.0005, respectively).

Predictors of poorer long-term prognosis were: onset before six months of age, failure to thrive, brainstem lesions on neuroimaging and requirement of

intensive care treatment. The respective figures and p-values are included in detail in Paper III.

25% of patients had normal lactate levels throughout the disease course; 10 of them had genetically verified disease. Elevated lactate in CSF (> 2.4 mmol/l) was associated with early onset of Leigh syndrome before six months of age (p=0.013), presence of hypotonia (p=0.002), acute exacerbations and/or relapses (p=0.014), brainstem lesions on neuroimaging (p=0.002) and absence of dystonia (p=0.011).

4.3 Neuroimaging in childhood-onset mitochondrial disorders with CNS involvement – Paper IV

4.3.1 Overview of the study population

In this study, we re-evaluated the brain MRIs from 66 patients who were diagnosed with the following mitochondrial disorders: 19 patients with Leigh syndrome; 10 patients with Alpers syndrome; nine patients with leukoencephalopathy; six patients with MELAS; three patients with MERRF; three patients with KSS; two patients with Leber hereditary optic neuropathy (LHON); one patient with GRACILE; seven patients with congenital lactic acidosis; and six patients with mitochondrial encephalomyopathy with isolated or multiple respiratory chain enzyme disorders.

4.3.2 Predominant lesions in the cerebral cortex/limbic system

Sixteen patients had abnormal MRI findings predominantly affecting the cerebral cortex, including all patients with MELAS (6/6), six patients with Alpers syndrome (6/10), one patient with MERRF (1/3), one with congenital lactic acidosis (1/7) and two with non-syndromic mitochondrial encephalomyopathy (2/6). The majority of MELAS patients had bilateral findings on MRI, representing acute and chronic stroke-like lesions with variable involvement of the subcortical white matter. One of the patients with MELAS showed abnormal MRI findings mainly on DWI, with bilateral occipital increased diffusion and low attenuation on ADC, while only a discrete hyperintense signal was present on conventional T2-weighted sequence.

The main MRI findings in Alpers syndrome were moderate to severe cerebrocortical atrophy with variable degree of accompanying atrophy of the

white matter, basal ganglia and cerebellum (Figure 4). Two patients with Alpers syndrome had a first normal MRI performed at the age of two days and one week respectively, with an abnormal follow-up MRI, eight and 13 months later.

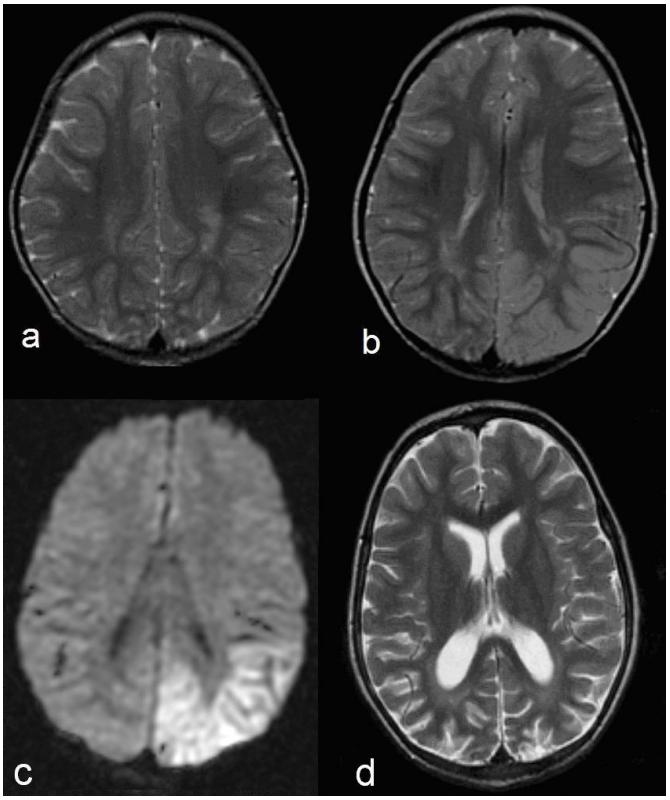


Figure 4. Patient AS-1 with Alpers syndrome due to POLG1 mutations: This patient had a normal axial T2-weighted image at 1.5 years of age (a). Follow-up MRI at 3 years of age showed a swollen left occipital cortex on T2-weighted image (b) with decreased diffusion (c). Follow-up MRI at 3.5 years of age showed atrophy of the occipital cortex at T2-weighted image (d).

4.3.3 Predominant lesions in the basal ganglia/diencephalon

Fifteen patients had predominant lesions in the basal ganglia and/or diencephalon, including 12 patients with Leigh syndrome (12/19), one patient with GRACILE (1/1), one patient with congenital lactic acidosis (1/7) and one patient with non-syndromic mitochondrial encephalomyopathy (1/6). All patients with Leigh syndrome showed bilateral T2 signal hyperintensity in the lentiform nuclei, while the caudate nuclei were affected in seven of them (Figure 5).

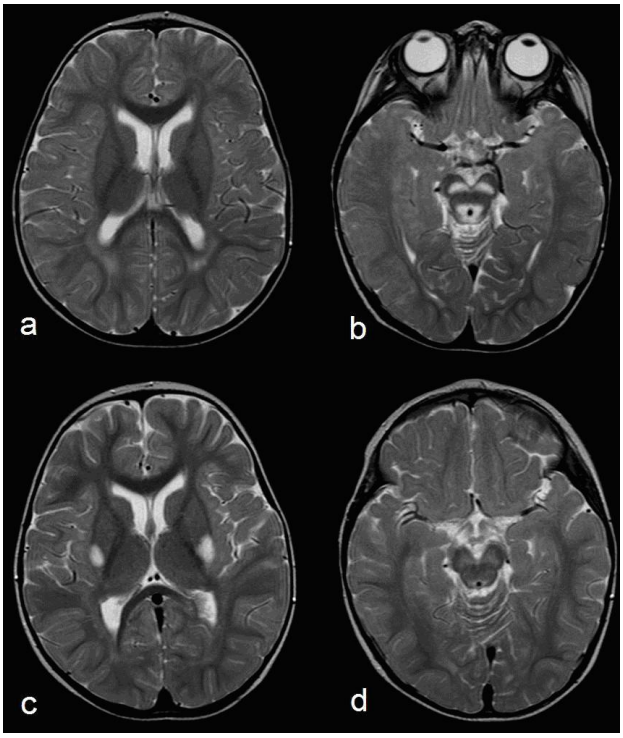


Figure 5. Patient with Leigh syndrome caused by the G13513A mutation in ND5 of mtDNA. An MRI with axial T2-weighted images at 16 months of age showed unaffected basal ganglia (a), bilateral symmetrical hyperintense lesions in the substantia nigra and periaqueductal gray matter (b). Follow-up MRI eight months later showed new bilateral symmetrical lesions in the posterior putamen (c), with partial regression of the previous lesions (d).

4.3.4 Predominant lesions in the white matter

Fifteen patients had pathological MRI findings predominantly affecting the white matter, including all patients with leukoencephalopathy (9/9), one patient with KSS (1/3) and five patients with congenital lactic acidosis (5/7). Patients with leukoencephalopathy showed signal abnormalities of the white matter with abnormal myelination, cystic lesions (4/9) and abnormal MRS in all examined patients (6/9). The patients with congenital lactic acidosis had in their majority, moderate-to-marked reduction in the periventricular white matter with enlargement of the subarachnoid spaces and ventricles, hypoplasia or complete agenesis of the corpus callosum and delayed myelination. The patient with KSS had signal hyperintensities in T2-weighted sequences in the subcortical white matter both supra- and infratentorially, as well as in the deep gray matter.

4.3.5 Predominant lesions in the posterior fossa

Three patients with Leigh syndrome presented with MRI lesions predominantly affecting the posterior fossa; marked signal abnormalities in the tectum (3/19), pons and medullar involvement (2/19), as well as involvement of the diencephalon and basal ganglia (2/19).

4.3.6 Diffuse lesions

Diffuse lesions were seen in four patients with Leigh syndrome (4/19), three patients with Alpers syndrome (3/10), one patient with KSS (1/3) and one patient with non-syndromic mitochondrial encephalomyopathy (1/6).

4.3.7 Normal MRI

Eight patients presented with a normal MRI, as follows: two patients with MERRF with a mutation in A8344G tRNALys (2/3); two patients with LHON (2/2); one patient with KSS due to an mtDNA-deletion (1/3); one patient with Alpers syndrome with mutations in *POLG1* (1/10); two patients with non-syndromic mitochondrial encephalomyopathy, with COX deficiency and complex I and V deficiencies respectively (2/6).

5 DISCUSSION

Early-onset mitochondrial encephalopathies comprise a challenging group of neurodegenerative disorders. This is due to their progressive nature, often leading to major disability and premature death, as well as their diagnostic complexity and lack of customized treatments.

In this work, we present our findings from studying 19 patients with Alpers syndrome. Our studies describe the clinical spectrum of Alpers syndrome, along with the neuroimaging and pathological features, and present specific genotype-phenotype correlations depending on the presence or not of *POLG1* mutations. We have further identified, with the help of WES, mutations in *NARS2* and *PARS2* in two of our patients with Alpers syndrome, being the first to link mutations in these genes to human disease and to Alpers syndrome.

We also present natural history data on a unique cohort of 130 patients with Leigh syndrome. This study is the first joint research work between eight centers from six European countries specializing in mitochondrial diseases, creating a strong platform for ongoing collaboration on mitochondrial research projects. This study helps define the natural history of Leigh syndrome and identify novel factors that predict outcome and long-term prognosis.

Finally, we studied the brain MRIs of 66 patients with mitochondrial disorders with CNS involvement. The diagnostic approach used in this study — from the neuroanatomical/ neurofunctional lesion to disease identification — assists the physician in the use of brain neuroimaging early in the diagnostic work-up of suspected mitochondrial encephalopathies.

5.1 Genotype-phenotype correlations in Alpers syndrome

In our study, six patients had pathogenic mutations in *POLG1*, a syndrome better known as Alpers-Huttenlocher [20-22]. These patients exhibited infantile disease onset at around six months of age, followed by a severe and rapidly progressive disease course, characterized by psychomotor regression, ataxia, refractory seizures, epilepsia partialis continua, convulsive status epilepticus and stroke-like episodes [93]. All patients developed moderate to severe hepatic dysfunction, which was mainly related to valproate treatment [93]. All but one of the patients with Alpers-Huttenlocher syndrome died at a median age of 15 months [93].

We also showed that mutations in *POLG1* only explain the mitochondrial respiratory chain deficiency in some patients with Alpers syndrome, while the genetic cause of the disorder in the majority still remains obscure. Patients with Alpers syndrome with no mutations in *POLG1* present symptoms earlier in life, often perinatally, and exhibit a more protracted disease course, characterized by neonatal seizures, infantile spasms or generalized seizures, while they usually develop spasticity and progressive microcephaly with accompanying severe mental retardation [93]. Liver dysfunction may also be present in Alpers syndrome not associated to *POLG1* mutations, but is usually mild, not associated to valproate treatment, and sometimes spontaneously resolving [93]. The absence of *POLG1* mutations was associated with better survival outcomes in these patients [93].

Alpers syndrome not associated to *POLG1* mutations has been recently linked to mutations in *FARS2*, a gene encoding the mitochondrial phenylalanyl-tRNA synthetase [26]. With the help of WES, we identified mutations in mt-aaRS in two of our patients with Alpers syndrome.

Patient AS-13 presented a homozygous mutation in *NARS2*. This gene encodes 2 isoforms, named isoform 1 and isoform 2. The mutation is located in the 3'-UTR region of isoform 2 (c.641C>T), whereas in isoform 1 it causes a change of amino acid affecting a conserved residue (c.641C>T, p.P214L). Based on the crystal structure of the protein homolog in Pyrococcus horikoshii [94], we can predict that the affected proline is located in the stem of a loop from which two conserved aromatic residues (tyrosine and/or phenylalanine) protrude and physically participate in monomer-monomer interaction. The structural change caused by the mutation in this loop can therefore be expected to affect the activity of the protein by destabilizing the interaction between the two monomers, still leaving some residual activity.

Patient AS-13 also developed renal disease in the form of focal segmental glomerulosclerosis and tubulopathy with accompanying hypochloremic metabolic alkalosis. Renal disease has been associated with another mt-aaRS, *SARS2*, the gene encoding the mitochondrial seryl-tRNA synthetase. Patients with mutations in *SARS2* show infantile onset of a multiorgan syndrome known as HUPRA syndrome. The renal disease in HUPRA syndrome is characterized by distal tubular dysfunction and renal failure [95]. Renal manifestations in mitochondrial disorders are uncommon and have been reported mainly in patients with mtDNA-associated mitochondrial disorders, such as MELAS, MERRF, KSS and Pearson syndrome [96]. To our

knowledge, *POLG1* mutations have not been associated with kidney disease so far.

Patient AS-15 presented compound heterozygosity for two mutations in *PARS2*. The first mutation (c.1130dupC, p.K378fs*1) is an one base insertion at codon 377 which creates a premature stop codon at position 378. This mutation is predicted to generate a truncated protein 98 residues shorter than the wild type form. The predicted missing part contains motif 3, which is part of the active site and part of the dimer interface. Even assuming that the truncated protein is stable, it can be predicted that the lack of such motifs totally abolishes the enzymatic activity of the protein. The second mutation (c.836C>T, p.S279L) is a missense mutation, predicted by SIFT to be damaging. It is located in a highly conserved motif which shares homology with a fragment of the bacterial-type prolyl-tRNA synthetase editing domain. This fragment is flanked by CXXC motifs, which is characteristic of functional domains inserted in larger proteins [97]. From this evidence it has been postulated that mitochondrial prolyl-tRNA synthetases first acquired and then lost their cis-editing domain at some point during evolution [98]. It is therefore tempting to speculate that the function of the cluster in which the mutation is located is somehow related to editing, perhaps by interacting with a free standing editing domain in trans-.

Patient AS-15 developed mitochondrial cardiomyopathy at two years of age. This patient had increased lactate levels at disease onset, but did not develop lactic acidosis. The disease course was milder than the one described in the mitochondrial disorders with neonatal lactic acidosis and cardiomyopathy in infancy.

Even though there is in silico evidence to propose that the mutations identified in our patients are responsible for their respective clinical phenotypes, further experimental work needs to be carried out to confirm these observations. It is important to further investigate whether these phenotypes are specific for the identified candidate genes *NARS2* and *PARS2*.

5.2 Neuropathological and neuroimaging findings in Alpers syndrome and correlation to the genotype

The neuropathology is pathognomonic in Alpers syndrome and pertains to major degenerative changes in the cerebral cortex, characterized by neuronal loss, spongiosis and gliosis. The deep gray matter, the white matter and the cerebellum may be involved to a variable degree. However, the extent of the

brain lesions and accompanying atrophy are considered to be related to disease duration. These findings are uniform in Alpers syndrome regardless of the underlying genotype. In our study, patients with *POLG1* mutations also had vacuolation in the thalamus and basal ganglia. This finding was absent in those patients with Alpers syndrome with no mutations in *POLG1*.

Brain neuroimaging plays a pivotal role in capturing the neurodegenerative changes, with the sensitivity increasing with disease duration. The progressive degeneration of the cerebral cortex in Alpers syndrome is mainly seen as cortical atrophy on brain MRI. However, depending on the duration of the disease, the MRI findings vary from normal, early in the disease course, to generalized cortical or diffuse atrophy at later stages [99]. As a result, patients develop progressive microcephaly. Furthermore, the brain MRI of patients with *POLG1* mutations may show features compatible with stroke-like episodes, similar to those seen in MELAS [99].

5.3 The differential diagnosis of Alpers syndrome

The differential diagnosis of Alpers syndrome includes other progressive neurodegenerative disorders characterized by early-onset encephalopathy and similar neuroimaging and/or neuropathological findings. The early-onset mitochondrial encephalopathies that pose the greatest challenge in the differential diagnosis from Alpers syndrome are IOSCA and Leigh syndrome. IOSCA and Alpers-Huttenlocher syndrome share similar age of onset and clinical features, making the differential diagnosis on clinical grounds difficult (see also paragraph 1.4.4). The neuropathology of IOSCA typically affects the posterior fossa, while the poliodystrophy in Alpers-Huttenlocher syndrome predominates in the cerebral cortex. However, both syndromes may present with stroke-like cortical lesions. Both *POLG1* mutations in Alpers-Huttenlocher syndrome and Twinkle mutations in IOSCA cause tissue specific mtDNA depletions. The differential diagnosis from Leigh syndrome is discussed separately in paragraph 5.6.

Late-onset Alpers syndrome is uncommon. Other *POLG1*-related encephalopathies with typical onset in adolescence or adulthood are mitochondrial spinocerebellar ataxia and epilepsy (MSCAE) and mitochondrial recessive ataxia syndrome (MIRAS), which both present with ataxia, epilepsy –often epilepsia partialis continua-, ptosis and/or external ophthalmoplegia, sensory peripheral neuropathy and exacerbation episodes of rapidly progressive encephalopathy. The MRI findings suggestive of these syndromes are cerebellar cortical atrophy with or without white matter

involvement, and thalamic lesions, while the basal ganglia are typically spared. The stroke-like lesions seen in *POLG1*-related encephalopathies are similar to those seen in MELAS. The latter is caused by mtDNA point mutations, mostly in the tRNA gene for leucine (MT-TL), but also in other tRNAs or MT-ND5. These stroke-like episodes, which are typical for MELAS, are seen as stroke-like cortical lesions on brain MRI, often affecting the occipital and temporal lobes. There is often a co-existence of acute and chronic lesions in MELAS patients [99]. Calcifications of the basal ganglia and dentate nuclei may also be seen in MELAS, but are not typically found in *POLG1*-associated encephalopathies [100].

5.4 The diagnostic criteria of Leigh syndrome

Almost two decades have passed since Rahman and colleagues proposed diagnostic criteria for Leigh syndrome [30]. Based on our findings and the findings from other studies performed since then, we believe that the following set of criteria is more appropriate to capture the wide phenotypic and genotypic spectrum of Leigh syndrome [101, 102]:

a) clinical features of a progressive neurodegenerative disorder resulting from affected deep gray matter structures and/or the brainstem, i.e. psychomotor delay, disturbed muscle tone (hypotonia, hypertonia, dystonia), disturbed movement (dyskinesia, akinesia, chorea, athetosis), ataxia and brainstem dysfunction (dysfunction of swallowing/sucking, ophthalmological manifestations, abnormal respiration and thermoregulation);

b) neuroimaging or neuropathological findings of symmetrically affected deep gray matter structures and/or the brainstem;

c) defects in mitochondrial function shown on laboratory or molecular testing, in the absence of inflammatory, autoimmune, vascular, toxic or other metabolic etiology.

As Leigh syndrome is an umbrella term of an increasing number of genetically identifiable mitochondrial diseases, we believe that the term ‘Leigh-like syndrome’, which is widely used in the literature, is not a suitable term to an already wide spectrum of diseases. We therefore suggest the use of the term ‘suspected Leigh syndrome’ in those patients that do not fulfill all three of the above mentioned criteria.

5.5 The natural history of Leigh syndrome and factors of disease severity and survival

In our study, four of five patients with Leigh syndrome presented with symptoms by the age of two years. This is in accordance with other studies and it is the age that we propose as a cut-off age between early- and late-onset Leigh syndrome [101-104]. The survival rate in our patients was better than in previous studies, with 41% of patients being alive at a median follow-up of 11.4 years from birth. However, in the group of patients that had died, an early mortality was found at a median age of 2.4 years.

Predictors of survival in Leigh syndrome have not previously been assessed, probably due to the small number of patients per study. Factors associated with poorer survival were found to be: disease onset before six months of age, failure to thrive, brainstem lesions on neuroimaging and intensive care treatment. The negative effect of early disease onset on survival has been previously reported, not only in Leigh syndrome, but in mitochondrial disorders in general [9, 101]. In our study, respiratory complications were found to be the leading cause of death. In view of the significant correlation between the presence of brainstem lesions on neuroimaging and poor survival, we believe that brainstem involvement may also be responsible for the respiratory complications seen in patients with Leigh syndrome [102].

The presence of pathological signs at birth and a history of epileptic seizures were associated with higher risk of acute exacerbations and/or relapses. The relatively high incidence of pathological signs at birth, even if not fully expressing Leigh syndrome, suggests that an ongoing energy failure linked to mitochondrial dysfunction may already have started in utero, and as a consequence, these patients might be more susceptible to external stress factors, such as infection, poor nutrition and dehydration, that may lead to acute exacerbations later in the disease course.

The presence of increased levels of lactate in CSF was significantly correlated to a more severe disease course in our patients, characterized by early disease onset before six months of age, acute exacerbations and/or relapses, as well as brainstem involvement. Therefore, we suggest that the presence of increased lactate levels in CSF may be used as a prognostic factor of disease severity in Leigh syndrome.

5.6 The differential diagnosis of Leigh syndrome

As previously discussed, Leigh syndrome encompasses an increasing number of phenotypically and genetically variable mitochondrial disorders and as such, this syndrome should be differentiated from other mitochondrial encephalopathies with similar phenotypic spectrum, as well as other disorders with degeneration of the deep gray matter structures.

Leigh syndrome shares phenotypical similarities with Alpers syndrome, as both syndromes are typically characterized by early-onset encephalopathy, abnormal muscle tone, psychomotor delay, epilepsy and involvement of other organs of high-energy demands. The neuropathology in both syndromes results from mitochondrial energy failure, but the lesion sites, often delineated first on neuroimaging, differ; in Leigh syndrome, the lesions affect predominantly the deep gray matter and/or brainstem, while in Alpers syndrome, the cerebral cortex is primarily affected. This site specificity may weaken with disease duration, often leading to diffuse, generalized atrophy in Alpers syndrome. However, the cerebral cortex in Leigh syndrome is usually better preserved [99].

Leigh syndrome should be differentiated from other diseases associated with degeneration of the basal ganglia in the pediatric population, such as carbon monoxide intoxication, Wilson's disease, juvenile Huntington's chorea, neurodegeneration with brain iron accumulation, familial degeneration of the striatum, non-familial infantile bilateral striatal necrosis (IBSN), and GAMT (guanidinoacetate methyltransferase) deficiency. These diseases can generally be ruled out on the basis of laboratory tests, history of exposure to toxins and family history.

5.7 The role of neuroimaging in identifying mitochondrial encephalopathies

Neuroimaging of the brain is a pivotal examination when suspecting a mitochondrial encephalopathy. Our study shows that a combination of the conventional MRI sequences with DWI and MRS provide a good indicator of the underlying neuropathology, which can be missed in conventional sequences alone. This is particularly important at early stages of disease, where the lesions may be captured only on DWI.

We have previously discussed the role of brain MRI in the differential diagnosis between Alpers syndrome and Leigh syndrome (see paragraph 5.6). Neuroimaging helps differentiate not only among mitochondrial encephalopathies, but also from other metabolic, inflammatory, vascular or toxic disorders with similar features. Furthermore, MRS is an important adjuvant diagnostic tool to traditional brain MRI, as it provides useful diagnostic information especially in the context of metabolic white matter disorders.

6 CONCLUSIONS

POLG1 mutations only explain the mitochondrial respiratory chain deficiency in some patients with Alpers syndrome, a disorder better known as Alpers-Huttenlocher syndrome. Alpers syndrome not associated to *POLG1* mutations presents phenotypical differences from Alpers-Huttenlocher syndrome.

With the help of whole exome sequencing, we have identified mutations in *NARS2* and *PARS2* in two of our patients with Alpers syndrome. Mutations in these genes have not previously been associated with human disease or Alpers syndrome. We suggest that these are candidate genes for Alpers syndrome not associated to *POLG1* mutations.

Leigh syndrome is an umbrella term used to describe mitochondrial disorders of progressive, neurodegenerative nature resulting from affected deep gray matter structures and/or the brainstem. Disease onset before six months of age, failure to thrive, brainstem lesions on neuroimaging and intensive care treatment are associated with poorer survival of patients with Leigh syndrome. The presence of pathological signs at birth and a history of epileptic seizures increase the risk for acute exacerbations and/or relapses. Increased lactate in CSF may be used as a predictor of disease severity.

If a mitochondrial disorder is suspected, neuroimaging should include conventional MRI sequences in combination with DWI, ADC and MRS. When a mitochondrial disorder is suspected on clinical or neuroimaging grounds, mitochondrial investigations should be performed. A follow-up brain MRI should be considered, particularly if the first MRI investigation has been normal.

7 FUTURE PROSPECTS

Some of our results from the study on Leigh syndrome have been reported and discussed in this thesis, mainly pertaining to the natural history and predictors of long-term outcomes. Upon completion of our work, we hope to show specific associations between genotype and phenotype in Leigh syndrome.

Moreover, through our collaboration with MCRN, we aim to study the brain MRIs of our patients with Leigh syndrome and further develop MRI-based pattern recognition algorithms.

The genetic causes of Alpers and Leigh syndrome in some of our patients still remain unknown. Future projects will include the application of WES to uncover the underlying genetic causes and study potential relationships to the respective phenotypes.

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