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Tuomas Komulainen

DISTURBANCES IN MITOCHONDRIAL DNA MAINTENANCE IN NEUROMUSCULAR DISORDERS AND VALPROATE-INDUCED LIVER TOXICITY

UNIVERSITY OF OULU GRADUATE SCHOOL;
UNIVERSITY OF OULU,
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NUFFIELD DEPARTMENT OF OBSTETRICS AND GYNAECOLOGY

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Abstract

Mitochondrial DNA depletion and deletions are related to mutations in the nuclear genes responsible for replication and maintenance of mitochondrial DNA (mtDNA). The *POLG1* gene encodes the enzyme responsible for replication of mtDNA. A particular feature of the *POLG1* mutations is an increased risk of acute liver failure (ALF) upon exposure to sodium valproate (VPA), but the pathomechanism is not resolved.

The present work studies the molecular genetic aetiology and clinical phenotypes associated with mtDNA depletion and deletion. Another objective was an investigation of clinical phenotypes in *POLG1* mutations and disentangling the pathomechanism of VPA-induced ALF in *POLG1* mutations. Mitochondrial toxicity of VPA was examined using HepG2 cells as an experimental in vitro model.

In this work, mtDNA depletion was associated with severe neonatal-onset encephalopathy. Furthermore, mtDNA depletion was found in muscle dystrophy as a secondary finding to muscle degradation. Multiple mitochondrial DNA deletions were found in two patients with Kearns-Sayre syndrome suggesting a genetic origin of the disease. *POLG1* p.R722H mutation has been previously reported as a neutral polymorphism, but we found evidence suggesting that *POLG1* p.R722H could be a pathogenic mutation in a homozygous or compound heterozygous state.

We identified retrospectively five patients, who required liver transplant after VPA-induced ALF. All five patients harboured *POLG1* mutations supporting the evidence of *POLG1* mutations as a risk factor for VPA-induced ALF. Previously, patients with *POLG1* mutations have been considered unsuitable for liver transplantation, but we found that homozygous *POLG1* mutations and adolescent or adult-onset disease predicted a good outcome following liver transplantation. *In vitro* studies on HepG2 cells showed that VPA disturbs mitochondrial respiration.

Our results expand the phenotypes and molecular genetic features in mitochondrial DNA depletion and deletion syndromes. We found evidence that *POLG1* mutations are not a contraindication for liver transplantation; rather, mutation status and age at onset affect survival. This finding should be taken in consideration in the treatment of VPA-induced ALF. Furthermore, our findings indicate that sodium valproate is toxic to mitochondria and should be avoided in patients with mitochondrial disease.

Keywords: drug toxicity, liver failure, mitochondrial DNA, mitochondrial DNA deletions, mitochondrial DNA depletion syndrome, neuromuscular disorders, POLG1 gene, sodium valproate

Komulainen, Tuomas, Mitokondrion DNA:n ylläpidon häiriöt neuromuskulaaritaudeissa ja valproaatin maksatoksisuudessa.

Oulun yliopiston tutkijakoulu; Oulun yliopisto, Lääketieteellinen tiedekunta, Kliinisen lääketieteen laitos, Lastentaudit; Neurologia; Oulun yliopistollinen sairaala; Medical Research Center Oulu; Valtakunnallinen kliininen tutkijakoulu; University of Oxford, John Radcliffe Hospital, Women's Centre, Nuffield Department of Obstetrics and Gynaecology

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Tiivistelmä

Mitokondrion DNA:n (mtDNA) kahdentumisesta ja ylläpidosta vastaavien tuman geenien mutaatiot voivat johtaa mtDNA:n määrän vähenemiseen (depleetioon) ja katkoksiin (deleetioihin). MtDNA:n kahdentumisesta vastaavaa entsyymiä koodaa tuman *POLG1*-geeni. *POLG1*-mutaatioihin liittyy kohonnut riski sairastua natriumvalproaatin (VPA) aiheuttamaan akuuttiin maksavaurioon.

Tutkimuksen tavoitteena oli tutkia mtDNA:n depleetion ja deleetioiden molekyyligeneettistä etiologiaa ja kliinisiä taudinkuvia. Tutkimuksessa selvitettiin myös *POLG1*-mutaatioihin liittyviä taudinkuvia ja *POLG1*-mutaatioihin liittyvän akuutin maksavaurion patomekanismia. VPA:n vaikutusta mitokondrioiden toimintaan tutkittiin in vitro HepG2-solumallissa.

Tutkimuksessa todettiin mtDNA:n depleetion liittyvän vaikeaan varhain alkavaan aivosairauteen. Depleetio todettiin myös sekundaarisena merosiini-negatiivisessa lihasdystrofiassa. Kahdella Kearns-Sayren syndroomaa sairastavalla potilaalla todettiin multippelit mtDNA:n deleetiot, mikä viittaa syndrooman geneettisen alkuperään. *POLG1* p.R722H-mutaatiota on aiemmin pidetty neutraalina polymorfiana, mutta tutkimuksen tulokset viittasivat siihen, että homotsygoottisena tai yhdistelmäheterotsygoottisena mutaatio on tautia aiheuttava.

Helsingin yliopistollisen sairaalan elinsiirtorekisteristä tunnistettiin retrospektiivisesti viisi potilasta, jotka olivat saaneet maksansiirteen VPA:n aiheuttaman maksavaurion vuoksi. Kaikilla viidellä potilaalla todettiin *POLG1*-geenin mutaatio, mikä vahvistaa käsitystä geenin yhteydestä VPA:n aiheuttamaan maksavaurioon. *POLG1*-mutaatioita on pidetty vasta-aiheena maksansiirrolle, mutta tutkimuksessa todettiin homotsygoottisena esiintyvän *POLG1*-mutaation ja nuoruusiällä tai varhaisella aikuisiällä alkaneen taudin liittyvän parempaan maksansiirron jälkeiseen ennusteeseen. HepG2-solumallilla tehdyt tutkimukset osoittivat VPA:n haittaavan mitokondrioiden soluhengitystä.

Tutkimuksen tulokset tuovat lisätietoa mtDNA:n depleetioon ja deleetioihin liittyvistä taudinkuvista ja molekyyligeneettisestä taustasta. *POLG1*-mutaatiot eivät ole ehdoton vasta-aihe maksansiirrolle; potilaan geneettinen status ja ikä taudin alkamishetkellä vaikuttavat ennusteeseen, mikä tulisi huomioida potilaiden hoidossa. Tulokset myös osoittivat VPA:n olevan mitokondriotoksinen lääke, jonka käyttöä tulisi välttää mitokondriotautipotilaiden hoidossa.

Asiasanat: lääketoksisuus, mitokondrio-DNA, mitokondrio-DNA:n deleetio, mitokondrio-DNA:n depleetiosyndrooma, mitokondriotaudit, neuromuskulaariset sairaudet, POLG1-geeni, vaikea maksan vajaatoiminta, valproaatti

To my family

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Kajaani, December 2014 Tuomas Komulainen

Abbreviations

ad	autosomal dominant
ADP	adenosine diphosphate
AHS	Alpers-Huttenlocher syndrome
ALF	acute liver failure
AMP	adenosine monophosphate
ANT1	the gene encoding adenine nucleotide translocator 1
ar	autosomal recessive
ATP	adenosine triphosphate
bp	base pair
C10orf2	the gene encoding mitochondrial DNA helicase (Twinkle)
CK	creatine kinase
CNS	central nervous system
CoA	coenzyme A
CoQ	ubiquinone (coenzyme Q)
CoQH2	ubiquinol
COX	cytochrome c oxidase
CPEO	chronic progressive external ophthalmoplegia
CPT	carnitine palmitoyl transferase
CSF	cerebrospinal fluid
CT	computed tomography
Da	dalton
dCK	deoxycytidine kinase
dGK	deoxyguanosine kinase
DGUOK	the gene encoding deoxyguanosine kinase
DILI	drug-induced liver injury
DLD	dihydrolipoyl dehydrogenase
DNA2	the gene encoding DNA replication helicase/nuclease 2
dNTP	deoxyribonucleotide
DRP	dynamin-related proteins
EEG	electroencephalography
ENMG	electroneuromyography
ER	endoplasmic reticulum
FAD^+	flavin adenine dinucleotide
$FADH_2$	reduced flavin adenine dinucleotide
FAO	fatty acid oxidation

FDG-PET	^{18}F-deoxyglucose positrone emission tomography
FFA	free fatty acid
FGF-21	fibroblast growth factor 21
FI	fluorescence intensity
GPX	glutathione peroxidase
IMM	inner mitochondrial membrane
IMS	intermembrane space
IOSCA	infantile-onset spinocerebellar ataxia
KSS	Kearns-Sayre syndrome
LDH	lactate dehydrogenase
LHON	Leber hereditary optic neuropathy
LNA	locked nucleic acid
LS	Leigh syndrome
LT	liver transplantation
MDC1A	merosine-deficient muscular dystrophy
MDDS	mitochondrial DNA depletion syndrome
MELAS	mitochondrial myopathy, encephalopathy, lactic acidosis and stroke-like episodes
MERRF	myoclonic epilepsy and ragged-red fibers
MFN1	the gene encoding mitofusin 1
MFN2	the gene encoding mitofusin 2
MIRAS	mitochondrial recessive ataxia syndrome
MM	mitochondrial matrix
MNGIE	mitochondrial neurogastrointestinal encephalopathy
MOMP	mitochondrial outer membrane permeabilization
MPT	mitochondrial permeability transition
MPTP	mitochondrial permeability transition pore
MPV17	the gene encoding mitochondrial inner membrane protein Mpv17
MRI	magnetic resonance imaging
MSCAE	mitochondrial spinocerebellar ataxia with epilepsy
mtDNA	mitochondrial DNA
MTT	(3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide
NAD^+	nicotinamide adenine dinucleotide
NADH	reduced nicotinamide adenine dinucleotide
NARP	neuropathy, ataxia and retinitis pigmentosa
nDNA	nuclear DNA
nt	nucleotide

OCR	oxygen consumption rate
OGDC	2-oxoglutarate dehydrogenase complex
OGDH	2-oxoglutarate dehydrogenase
OMM	outer mitochondrial membrane
OPA1	the gene encoding dynamin-related GTPase protein
OXPHOS	oxidative phosphorylation
PCIAA	phenol-chloroform-isoamyl alcohol extraction
PCR	polymerase chain reaction
PDC	pyruvate dehydrogenase complex
PDHG	pyruvate dehydrogenase
PEO	progressive external ophthalmoplegia
PI	propidium iodine
pol γA	mitochondrial DNA polymerase γ subunit A
pol γB	mitochondrial DNA polymerase γ subunit B
pol γ	mitochondrial DNA polymerase γ
POLG	the gene encoding mitochondrial DNA polymerase γ subunit A
POLG2	the gene encoding mitochondrial DNA polymerase γ subunit B
PS	Pearson syndrome
qRT-PCR	quantitative real-time polymerase chain reaction
RFLP	restriction fragment length polymorphism
RIPA	radioimmuneprecipitation assay
RITOLS	RNA incorporation during mtDNA replication
RNR	ribonucleoreductase
ROS	reactive oxygen species
RRF	ragged-red fiber
RRM2B	the gene encoding the small R2 subunit of p53-inducible ribonucleoreductase (p53R2)
rRNA	ribosomal RNA
SANDO	sensory ataxic neuropathy, dysarthria and ophthalmoparesis
SOD	superoxide dismutase
SUCL	succinyl-CoA synthase
SUCLA2	the gene encoding the ATP-forming β subunit of succinyl-CoA synthase
SUCLG1	the gene encoding the α subunit of succinyl-CoA synthase
TCA cycle	tricarboxylic acid cycle
TFAM	mitochondrial transcription factor A
TFMB	mitochondrial transcription factor B

TK2	the gene encoding thymidine kinase 2
TP	the gene encoding thymidine phosphorylase
TR-F	time-resolved fluorescence
tRNA	transfer RNA
VDAC	voltage-dependent anion channel
VPA	sodium valproate
XL-PCR	long-range polymerase chain reaction
Ψ_m	mitochondrial membrane potential

α	alpha
β	beta
Δ	delta
γ	gamma
Ψ	psi

List of original publications

This thesis is based on the following publications, which are referred to in the text by their Roman numerals:

I Komulainen T, Hautakangas MR, Hinttala R, Pakanen S, Vähäsarja V, Lehenkari P, Olsen P, Vieira P, Saarenpää-Heikkilä O, Palmio J, Tuominen H, Kinnunen P, Majamaa K, Rantala H & Uusimaa J (2014) Mitochondrial DNA depletion and deletions in paediatric patients with neuromuscular diseases – novel phenotypes. Manuscript.

II Komulainen T, Hinttala R, Kärppä M, Pajunen L, Finnilä S, Tuominen H, Rantala H, Hassinen I, Majamaa K & Uusimaa J (2010) *POLG1* p.R722H mutation associated with multiple mtDNA deletions and a neurological phenotype. BMC Neurol 10: 29.

III Hynynen J*, Komulainen T*, Tukiainen E, Nordin A, Arola J, Kälviäinen R, Jutila L, Röyttä M, Hinttala R, Majamaa K, Mäkisalo H & Uusimaa J (2014) Acute liver failure after valproate exposure in patients with *POLG1* mutations and the prognosis after liver transplantation. Liver Transpl 20(11): 1402–1412.

IV Komulainen T, Lodge TA, Hinttala R, Bolszak M, Pietilä M, Koivunen P, Hakkola J, Poulton J, Morten KJ & Uusimaa J (2014) Sodium valproate induces mitochondrial respiration dysfunction in HepG2 *in vitro* liver model. Manuscript.

* = Equal contribution

Contents

1 Introduction

The mitochondrion is the cell organelle responsible for the energy metabolism of the eukaryotic cell. Each cell contains hundreds to thousands of mitochondria depending on the energetic demands of the cell. An important function of the mitochondria is producing high-energy compound adenosine triphosphate (ATP). A four-complex mitochondrial respiratory chain (OXPHOS chain) and ATP synthase in the inner mitochondrial are responsible for ATP production via electron transportation and oxidative phosphorylation. Oxidative phosphorylation consumes oxygen, and therefore, it is often referred to as mitochondrial respiration.

Mitochondria have their own genome. Mitochondrial DNA (mtDNA) is a circular DNA molecule of 16 569 base pairs (bp) located in the mitochondrial matrix, which encodes the subunits of OXPHOS chain complexes, mitochondrial transfer RNAs (tRNA) and mitochondrial ribosomes. Each mitochondria contains several copies of the mtDNA molecules, and therefore, each cell may contain 1,000 to 10 000 copies of the mtDNA molecules. Assembly of the mitochondria is directed by both mtDNA and nuclear DNA (nDNA). In addition, replication and maintenance of mtDNA is carried out entirely by nDNA-encoded proteins.

Mutations in nDNA-encoded mtDNA replication and maintenance proteins can lead to point mutations, deletions and depletion of mtDNA, which may compromise the mitochondrial structure and impair the mitochondrial function. This process can give rise to mitochondrial diseases, a clinically heterogeneous group of disorders, which most often affect tissues that have high energy demand, e.g., the nervous system, muscle, heart, and liver. To date 14 genes have been associated with mtDNA replication and maintenance. *POLG1* gene encodes the catalytic subunit A of mitochondrial DNA polymerase γ, the enzyme responsible for the synthesis and repair of mtDNA.

Over 160 pathogenic *POLG1* mutations have been described (Human DNA polymerase gamma Mutation Database, http://tools.niehs.nih.gov/polg/). Alpers syndrome is a severe, often fatal childhood-onset hepatoencephalopathy caused by *POLG1* mutations and mtDNA depletion. Typically patients with Alpers disease present with epilepsy with various types of seizures. Mitochondrial recessive ataxia syndrome (MIRAS) is a *POLG1*-related disease, which often presents with epilepsy. MIRAS is common especially in Finland and other Nordic countries. Previous studies have shown that *POLG1* mutations increase the risk for acute liver failure (ALF) induced by sodium valproate (VPA), a widely used

anticonvulsant drug. However, the pathomechanism of VPA-induced ALF is still unclear. In addition, often patients with *POLG1*-related disease require a liver transplant due to VPA-induced ALF, but the evidence on the prognosis of liver transplantation is very contradictory.

The objective of the present work was to study the clinical phenotypes and molecular aetiology associated with mtDNA deletions and depletion. In addition, the aim is to evaluate the clinical phenotypes caused by *POLG1* mutations. A specific aim is to study the pathomechanism of VPA-induced ALF that is related to *POLG1* mutations. Furthermore, the effect of VPA on the mitochondrial function is studied *in vitro* using HepG2 cells as an experimental model.

2 Review of the literature

2.1 Mitochondrion: Structure and function

Mitochondrion is an intracellular organelle essential for cellular function and survival. Mitochondria take part in numerous intracellular functions. The main purpose of mitochondria is to provide energy for cellular metabolism through oxidative respiration. In addition, mitochondria have shown to play an important role in other cellular functions, including intracellular signalling, apoptosis, and Ca^{2+} homeostasis. The citric acid cycle (also known as the tricarboxylic acid cycle, TCA cycle, or Krebs cycle), β-oxidation and pathways for lipid and cholesterol synthesis are a few of the important metabolic routes in mitochondria (Schapira 2006, Venditti *et al.* 2013).

2.1.1 The structure of mitochondrion

Traditionally, mitochondria are described as isolated, double-membrane- bound intracellular organelles with a diameter of 1-2 μm. However, according to the most recent view, mitochondria form a complex and dynamic tubular network, which is constantly altering its organization through division and fusion. The mitochondrial structure and position varies by cellular type, developmental stage, and physiological conditions (Logan 2006, Schapira 2006, Lackner 2013). The division, fusion, and motility of the mitochondrial network govern the organellar function and, therefore, mitochondria are essential for cellular functions (Picard *et al.* 2011). Interestingly, recent experimental data shows that tubules of endoplasmic reticulum (ER) are found in the division sites of mitochondria in both yeast and mammalian cells. This finding suggests that ER plays an important role in the dynamics of the mitochondrial network (Friedman *et al.* 2011, Kornmann 2013).

Mitochondria are composed of two membranes: The outer mitochondrial membrane (OMM) and the inner mitochondrial membrane (IMM). Between these membranes is intermembrane space (IMS). OMM is the limiting structure of the mitochondria with fairly simple topology. The composition of OMM is fairly homogenous with a low protein and phospholipid ratio and a low proportion of cardiolipin. The essential structures of OMM are voltage dependent anion channels (VDAC), also called porins. VDACs are highly conserved protein

structures that make OMM permeable to ions and small molecules up to 5 kDa in size. IMM has a high ratio of proteins and phospholipids and is rich in cardiolipin. Only lipophilic molecules can be transported freely through IMM, while all other molecules essential for mitochondrial metabolism, including nucleotides and substrates for mitochondrial energy metabolism, need specific transporters to cross IMM. In addition, the composition of IMM makes it impermeable to H^+ ions, which are essential for oxidative phosphorylation and ATP production (Navarro & Boveris 2007, Walther & Rapaport 2009, Lemasters *et al.* 2012). IMM folds into internal compartments called *cristae* ("crests"). Previously these structures were thought to be simple in-folds of IMM, but recent studies have shown IMM to form cristae through active invagination as a reaction to a changing physiological condition. This process refers to regulations of mitochondrial functions through changes in the topology of IMM, e.g. changing the diffusion of metabolites between the mitochondrial compartments (Mannella 2006). The complexes of the mitochondrial respiration chain (OXPHOS chain) and ATP synthase are embedded in IMM. IMM confines the protein-rich mitochondrial matrix (MM), which contains pools of important metabolites, such as NAD^+, NADH, ADP, and ATP, and many important metabolic pathways, including mitochondrial protein synthesis, pyruvate dehydrogenase complex (PDC), TCA cycle, and β-oxidation (Logan 2006). A schematic illustration of mitochondrial structure is presented in Fig. 1 (Logan 2006 and Navarro & Boveris 2007, Davis & Williams 2012).

Mitochondria have their own genome and protein synthesis, but the majority of mitochondrial structures are encoded by the nuclear genome and synthesized on cytosolic ribosomes (Spinazzola 2011, Davis & Williams 2012). Over 1000 mitochondrial structural proteins have been identified (MitoCarta, https://www.broadinstitute.org/pubs/MitoCarta, Calvo & Mootha 2010, Calvo *et al.* 2012), and only 13 – all of which are subunits of the respiratory chain complexes and ATP synthase – are encoded by the mitochondrial genome. Succinate dehydrogenase, a membrane-bound enzyme of the TCA cycle and complex II of the respiratory chain, is completely encoded by the nuclear genome (Spinazzola 2011, Davis & Williams 2012). Nuclear-encoded mitochondrial precursor proteins are imported into mitochondria according to a specific signal presequence that is usually located in the N-terminus of the protein. Protein transportation between cytosol and the mitochondria is controlled by a group of mitochondrial membrane proteins called translocase of the outer membrane (TOM), sorting and assembly machinery (SAM), translocase of the inner

membrane (TIM22 and TIM23) and a presequence translocase of the inner membrane (PAM), all of which work in conjunction with chaperons and supporting proteins to serve as a complex transportation machinery (Becker *et al.* 2012, Kulawiak *et al.* 2013).

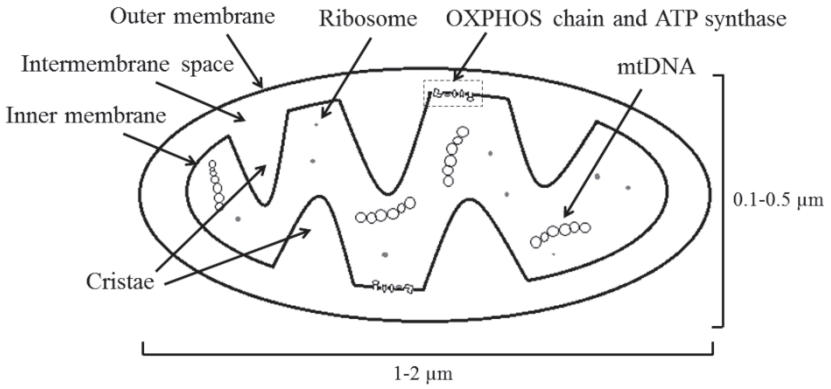

Fig. 1. Structure of the mitochondrion. Adapted from Logan 2006, Navarro & Boveris 2007 and Davis & Williams 2012.

2.1.2 Mitochondrial bioenergetics, OXPHOS chain, and the production of ATP

The main purpose of mitochondria is to provide energy for cellular metabolism. Dietary nutritients are metabolised and used as substrates of mitochondrial oxidative respiration to produce energy in the form of the high-energy phosphate compound adenosine triphosphate (ATP). Carbohydrates, fats, and proteins are broken down in the digestive system and metabolised to acetyl-CoA, which is then fed into the first step of oxidative metabolism: the TCA cycle in the mitochondrial matrix. Dietary carbohydrates are digested and absorbed mainly as glucose. Glucose is catabolised by glycolysis in the cytosol to pyruvate, which is transported to the mitochondrial matrix. Under anaerobic conditions, pyruvate is transformed into lactate in the cytosol by lactate dehydrogenase (LDH), which subsequently oxidizes NADH to NAD^+. After transportation through IMM, pyruvate is transformed into acetyl-CoA in parallel to the reduction of NAD^+ to NADH in a reaction catalysed by the pyruvate dehydrogenase complex (PDC). This enzyme complex consists of three subunits: pyruvate dehydrogenase

(PDHG; subunit E_1), dihydrolipoyl transacetylase (DHTA; subunit E_2), and dihydrolipoyl dehydrogenase (DLD; subunit E_3). The reaction catalysed by PDC is physiologically unidirectional, thus forming a rate-limiting and irreversible step in the oxidative metabolism of glucose. Free fatty acids (FFA) are transported into mitochondrial matrix by the carnitine-mediated shuttle formed by carnitine palmitoyltransferase I (CPT I) on OMM and CPT II on IMM and then catabolised to acetyl-CoA by β-oxidation. Specific enzymes metabolise amino acids by transamination to pyruvate, which is transformed to acetyl-CoA by the PDC complex. Furthermore, amino acids can be converted to substrates of TCA cycle enzymes (Murray *et al.* 2003, Osellame *et al.* 2012).

The TCA cycle is a series of enzymatic steps located in the mitochondrial matrix, which oxidizes acetyl residues and also reduces co-enzymes that are coupled to the formation of ATP through re-oxidation by the respiratory chain. The reaction cycle starts when acetyl-CoA is transferred to the four-carbon oxaloacetate by citrate synthase, thereby forming a six-carbon citrate. Subsequent series of reactions catabolize citrate to oxaloacetate, which is again ready to be transformed to citrate. The 2-oxoglutarate dehydrogenase complex (OGDC) is a three-subunit enzyme complex of the TCA cycle analogous to PDC complex; the subunit E3 (DLD) is structurally and functionally the same in both enzyme complexes. The OGDC complex converts 2-oxoglutarate into succinyl-CoA and reduces NAD^+ to NADH. This reaction is physiologically unidirectional and serves as a rate-limiting step in the TCA cycle. Each turn of the TCA cycle reduces three molecules of NAD^+ to NADH and one molecule of FAD^+ to $FADH_2$, which are then transported to the respiratory chain (Murray *et al.* 2003, Osellame *et al.* 2012). An overview of mitochondrial bioenergetics is presented in Fig. 2 (Murray *et al.* 2003).

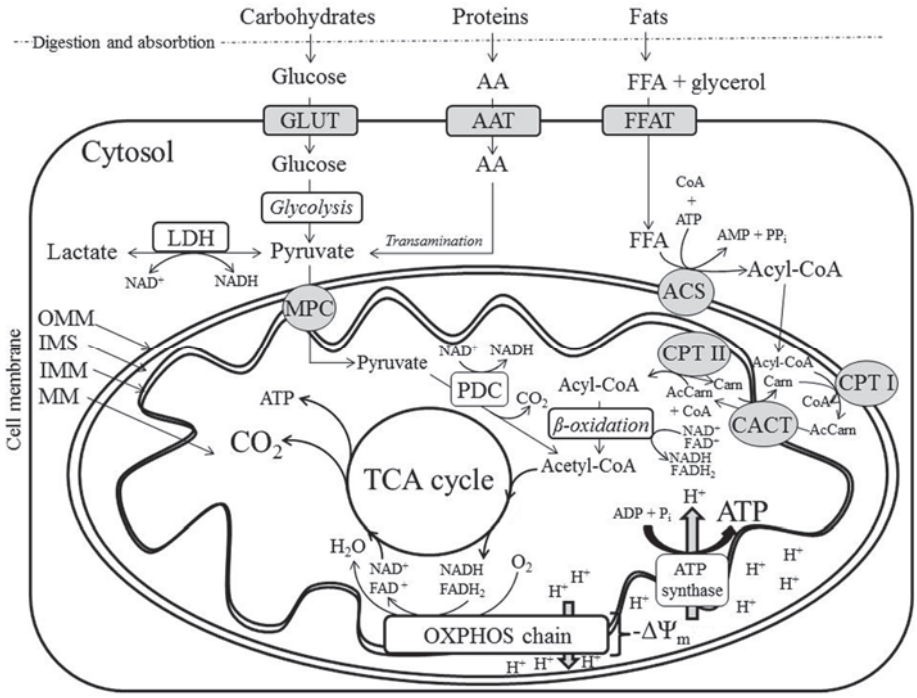

Fig. 2. Mitochondrial bioenergetics. Dietary nutrients are digested to simple sugars (mainly glucose), amino acids, and free fatty acids. Nutrients are catabolised in oxidation reactions, and NADH, FADH2 and acetyl-CoA are produced. Acetyl-CoA is used as a substrate in the tricarboxylic acid cycle, which produces the reduced coenzymes, NADH and FADH2. Reduced coenzymes are oxidized by the mitochondrial respiratory chain in order to create H+ gradient over the inner mitochondrial membrane. H+ gradient is consumed by ATP synthase to phosphorylate ADP to ATP. AA = amino acid, AAT = amino acid transporter, AcCarn = acylcarnitine, ACS = acyl-CoA synthetase, ADP = adenosine diphosphate, AMP = adenosine monophosphate, ATP = adenosine triphosphate, CACT = carnitine acylcarnitine translocase, Carn = carnitine, CoA = coenzyme A, CPT I/II= carnitine palmitoyltransferase I/II, FFA = free fatty acid, FFAT = transporter, GLUT = glucose transporter, IMM = inner mitochondrial membrane, IMS = intermembrane space, LDH = lactate dehydrogenase, MPC = mitochondrial pyruvate transporter, MM = mitochondrial matrix, OMM = outer mitochondrial membrane, OXPHOS chain = mitochondrial respiratory chain, PDC = pyruvate dehydrogenase complex, TCA cycle = tricarboxylic acid cycle, $-\Delta\Psi_m$ = mitochondrial membrane potential, and the mitochondrial matrix is negatively charged relative to intermembrane space. Adapted from Murray *et al.* 2003.

The mitochondrial respiratory chain (OXPHOS chain) is formed by four multi-subunit protein complexes (complex I-IV) embedded in IMM that function as electron carriers and redox pairs. The complexes of the respiratory chain are not separate, but rather form bigger supercomplexes, depending on the respiratory state of the cell. Chemiosmotic theory (Mitchell 1961) explains the mechanism of oxidative phosphorylation as a coupling of H^+ gradient over IMM to the phosphorylation of adenosine diphosphate (ADP), thus producing adenosine triphosphate (ATP). The OXPHOS chain transfers electrons from the donors NADH and $FADH_2$, following the oxidation potential of the complexes ultimately to the acceptor O_2. The difference in oxidation potential provides energy for the respiratory complexes I, III, and IV to function as H^+ pumps in a series with respect to electron flux. H^+ ions are transferred from MM to IMS. As IMM is impermeable to H^+ ions, the respiratory chain creates an H^+ gradient over IMM, thus producing an electrical voltage (mitochondrial membrane potential, Ψ_m) of -200 millivolts, where the mitochondrial matrix has negative charge relative to IMS. The H^+ gradient provides the energy for the synthesis of ATP, the final step of oxidative phosphorylation, by generating a re-entry flux of H^+ ions through ATP synthase (complex V, F_OF_1 ATPase) (Murray *et al.* 2003, Navarro & Boveris 2007, Osellame *et al.* 2012). A schematic illustration of the OXPHOS chain and ATP synthase is presented in Fig. 3 (Murray *et al*. 2003, Navarro & Boveris 2007, Davis & Williams 2012, Osellame *et al.* 2012).

Complex I (NADH-ubiquinone oxidoreducatase; NADH dehydrogenase) is constructed of 45 subunits and includes flavin mononucleotide (FMN) containing flavoprotein and six iron-sulphur (Fe-S) clusters. NADH (reduced by the TCA cycle) is oxidized by complex I and donates two electrons to the FMN-flavoprotein. Subsequently the electrons are transferred through complex I by a series of Fe-S clusters to redox carrier ubiquinone, which then transfers the electrons to complex III. Transportation of the electrons causes a conformational change of complex I, allowing transfer of 4 H^+ ions through IMM to (IMS) (Murray *et al.* 2003, Navarro & Boveris 2007, Osellame *et al.* 2012).

Complex II (succinate dehydrogenase) is a 123 kDA component of the TCA cycle embedded in IMM, which also functions as a part of the OXPHOS chain and contains a covalently bound FAD and Fe-S clusters. Complex II reduces FAD to $FADH_2$, which in turn is oxidized, and the donated electrons are transferred to *b* heme and ubiquinone by Fe-S clusters. Complex II does not transfer H^+ ions through IMM (Murray *et al.* 2003, Navarro & Boveris 2007, Osellame *et al.* 2012).

Ubiquinone (coenzyme Q, CoQ) is a lipid-soluble benzoquinone, which can diffuse freely through IMM and acts as a redox carrier. Once ubiquinone (CoQ) is reduced by either complex I or complex II to ubiquinol ($CoQH_2$), it shuttles the electrons to complex III (cytochrome bc_1 complex; ubiquinol-cytochrome *c* oxidoreductase). Complex III is composed of 9-10 polypeptides and contains three redox centers: the cytochromes b_L, b_H, and c_1. Complex III oxidizes QH_2 and with the aid of the redox centers and Fe-S clusters transfers the electrons ultimately to cytochrome *c*. The redox reaction enables complex III to transfer four H^+ ions to IMS (Murray *et al.* 2003, Navarro & Boveris 2007, Osellame *et al.* 2012).

Reduced cytochorome *c* transfers the electrons to complex IV (cytochrome *c* oxidase; cytochrome *c*-O_2 oxidoreducatase), which is the final redox complex in the OXPHOS chain. Complex IV contains an active binuclear copper center (Cu_A), which accepts the electrons upon oxidation of cytochrome *c*. The electrons are then passed via the mononuclear copper center (Cu_B) and hemes *a* and a_3 to dioxygen (O_2), which is ultimately reduced to water (H_2O); thus, the functional OXPHOS chain consumes oxygen, hence the name respiratory chain. At the same time, four H^+ ions are pumped to IMS by complex IV (Murray *et al.* 2003, Navarro & Boveris 2007, Osellame *et al.* 2012).

The ATP synthase is formed by two subdomains: subdomain F_O is embedded in IMM and serves as a transmembrane channel for H^+ influx to the mitochondrial matrix. Subdomain F_1 is a peripheral membrane protein on the side of the mitochondrial matrix, which forms an asymmetric stalk linked to the stationary subunit F_O. The H^+ flow causes the F_1 subunit to rotate, which in turn rotates the stalk in the stationary subunit F_O leading to a conformational change in subunit F_O. This process activates the catalytic reaction and binds ADP and phosphate to form ATP (Murray *et al.* 2003, Navarro & Boveris 2007, Osellame *et al.* 2012).

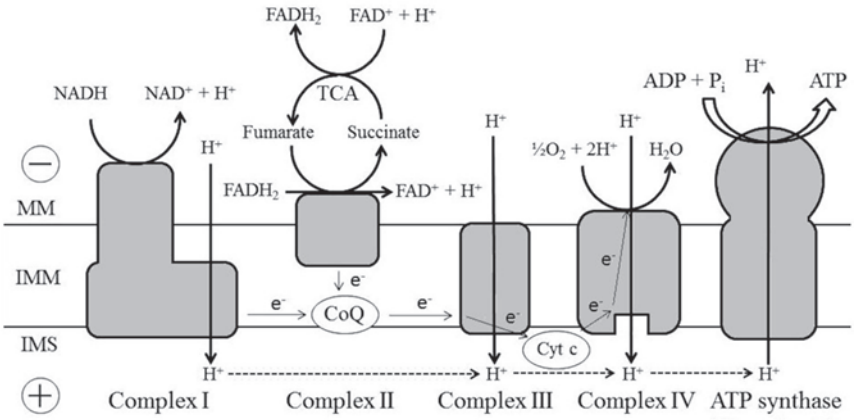

Fig. 3. Schematic presentation of mitochondrial respiratory chain, ATP synthase, and oxidative phosphorylation. H+ and electron flux is presented as arrows. MM = mitochondrial matrix, IMM = inner mitochondrial membrane, IMS = intermembrane space, Cyt c = cytochrome c, CoQ = coenzyme Q, ADP = adenosine diphosphate, ATP = adenosine triphosphate. Adapted from Murray *et al.* 2003, Navarro & Boveris 2007, Davis & Williams 2012 and Osellame *et al.* 2012.

2.1.3 The production of mitochondrial reactive oxygen species

Even if oxygen is a requirement for aerobic metabolism and cell survival, oxygen may also have toxic effects in a cell. Normal cell metabolism produces a reactive oxygen species (ROS) as by-products of cellular functions. As mitochondrion is the organelle responsible for cellular respiration and usage of oxygen, it is the main source of intracellular ROS.

The primary ROS superoxide anion ($O_2^{\bullet -}$) is formed by a one electron reduction of molecular oxygen, thus creating a relatively stable oxygen intermediate, which can generate more reactive secondary ROS. $O_2^{\bullet -}$ is converted into hydrogen peroxide (H_2O_2) and O_2 by metalloenzymes called superoxide dismutases (SOD): cytosolic and extracellular superoxide dismutase Cu-ZnSOD and mitochondrial superoxide dismutase Mn-SOD. H_2O_2 can be transformed by the Fenton reaction into highly reactive hydroxyl radicals with a short half-life ($^{\bullet}OH$ and OH^-) in the presence of reduced transition metals, such as Fe^{2+} and Cu^+. Mitochondria can remove both internally and externally produced ROS via an antioxidant defence system. H_2O_2 is transformed into H_2O by glutathione

peroxidase (GPX) and α-tocopherol (vitamin E), CoQ, and cytochrome *c* function as ROS scavengers (Orrenius *et al.* 2007, Murphy 2009, Venditti *et al.* 2013).

During normal steady-state oxidative respiration, approximately 1-2% of consumed O_2 is converted into oxygen radicals. In this situation, mitochondrial ROS are mainly produced by minor electron leakage from complex I and complex III, and the oxidation of NADH and reduction of CoQ by complex I produces most of the cellular $O_2^{\bullet-}$ (Orrenius *et al.* 2007, Murphy 2009, Webster 2012, Venditti *et al.* 2013). $O_2^{\bullet-}$ production is high, when either the NADH/NAD^+ ratio or the $CoQH_2$/CoQ ratio is high during damaged oxidative respiration, during release of cytochrome *c* (e.g. in apoptosis), low ATP demand, high Ψ_m or a slow respiration rate. These factors lead to reverse electron transportation in complex I, thus causing increased production of $O_2^{\bullet-}$. Also, factors outside mitochondria, including ischemia, ischemia-reperfusion, and muscle exercise, may induce mitochondrial ROS production: under oxygen depletion, the OXPHOS chain lacks the final electron acceptor, which leads to an accumulation of $CoQH_2$ and reverse electron transportation toward complex I (Murphy 2009, Webster 2012).

When production of ROS exceeds the removal by antioxidants, oxidative stress is generated. As highly reactive compounds, ROS can damage various components of the cell and generate double-strand breaks and alterations in the base sequence in a DNA molecule. Especially, mitochondrial DNA molecules are susceptible to ROS-induced damage due to their close proximity to the OXPHOS chain and ROS production sites. Oxidative stress also induces the release of Ca^{2+} and cytochrome *c*, which initiates an intrinsic apoptotic pathway. Considering the detrimental effects ROS have in various cellular structures and functions, ROS have been suggested to play a role in various conditions, including cancer, cardiovascular disease, neurodegeneration, and aging (Orrenius *et al.* 2007, Roy Chowdhury *et al.* 2010, Webster 2012, Venditti *et al.* 2013).

2.1.4 Mitochondria in cell death

Mitochondria are involved in both accidental cell death (necrosis) and programmed cell death (apoptosis). Necrosis is mainly a passive process caused by the metabolic failure of the cell, whereas apoptosis is a coordinated and active process that requires energy. The purpose of apoptosis is to remove unwanted cells in early development and senescent cells in mature tissues (Osellame *et al.* 2012, Webster 2012). Apoptosis can be triggered by two different pathways: extrinsic or intrinsic. Mitochondria are involved in both pathways, as they are the

target of the extrinsic pathway and the initiator of the intrinsic pathway (Galluzzi *et al.* 2012, Giorgi *et al.* 2012, Webster 2012, Bravo-Sagua *et al.* 2013).

The extrinsic apoptosis pathway is commonly associated with the inflammatory process and is activated by extracellular pro-inflammatory ligands, such as the tumour necrosis factor α (TNFα), Fas ligand (FasL), and TNF-related apoptosis-inducing ligand (TRAIL). Extracellular stimuli activate cell death receptors on a plasma membrane, e.g. tumor necrosis factor α receptor 1 (TNFR1), which initiates formation of a death-inducing signal complex (DISC). This process eventually leads to activation of caspases 7, 6, and 3, which serve as executioners of apoptosis (Webster 2012). The extrinsic pathway may also require amplification by the intrinsic pathway (Galluzzi *et al.* 2012, Webster 2012).

The intrinsic apoptosis pathway is triggered by intracellular stimuli, such as disrupted Ψ_m, oxidative stress, DNA damage, or high cytosolic Ca^{2+}. The intrinsic pathway involves the release of pro-apoptotic factors, such as cytochrome *c*, the apoptosis-inducing factor (AIP), and the second mitochondria-derived activator of caspaces (Smac), from IMS to cytosol. The initiation of the intrinsic apoptosis has been suggested to be preceded by VDAC-related mitochondrial outer membrane permeabilization (MOMP), opening of the mitochondrial permeability transition pore (MPTP) leading to mitochondrial permeability transition (MPT) and loss of Ψ_m (Galluzzi *et al.* 2012, Webster 2012). MOMP and MPT have been reported to be regulated by the B-cell lymphoma 2 (Bcl-2) protein family, which includes both pro-apoptotic and anti-apoptotic regulator proteins; for instance, BAK and BAX bind to VDACs and alter the conformation of IMM proteins (e.g. adenine nucleotide translocase 1, ANT1) to form transition pores, thus regulating the permeability of mitochondrial membranes and enabling release of pro-apoptotic factors. Also, VDACs and MPTPs are suggested to generate a common pore through OMM and IMM to create an open channel between cytosol and MM (Tsujimoto 2003, Orrenius *et al.* 2007, Duchen 2012, Galuzzi *et al.* 2012, Webster 2012, Bravo-Sagua *et al.* 2013). High Ca^{2+} concentration has been related to conformational changes in VDACs and opening of MPTPs, thus activating the initiating steps of the intrinsic apoptosis pathway (Orrenius *et al.* 2007, Duchen 2012, Webster 2012). The previous reports associating VDACs and MOMP with MPTPs are opposed by the findings, which relate mitochondrial permeability transition (MPT) to IMM (Hunter & Haworth, 1979). In addition, genetic ablation studies have shown that VDACs are not essential to apoptosis (Krauskopf *et al*. 2006, Baines *et al*. 2007) and data from the ANT1 knock-out mouse model shows that MPTPs are active despite inactivated ANT1 (Kokoszka *et al.* 2004), which

puts the role of VDACs and ANT1 in apoptosis under debate. Although the exact molecular nature of MPTP is yet to be resolved, the pores are likely formed by dimers of ATP synthase (Bernardi *et al.* 2013, Giorgio *et al*. 2013). Ultimately, the apoptotic cascade leads to activation of caspase 9 and caspase 3, which are the activators of apoptosis (Galuzzi *et al.* 2012). Activation of the caspase pathway leads to the degradation Na^+/K^+-ATPase, which is responsible for maintaining the plasma membrane potential, thus coupling MPT to plasma membrane depolarization (Dussmann *et al.* 2003). During the following stages of apoptosis, cell structures and organelles are fragmented and dismantled in an organized manner (Haddad 2004, Galluzzi *et al.* 2012).

In necrosis, loss of oxygen stops the function of the OXPHOS chain, disrupts mitochondrial membrane potential (Ψ_m), and creates a depletion of ATP. As ionic pumps of plasma membrane require ATP to maintain plasma membrane potential, Na^+ and Ca^{2+} accumulate in cytosol with ATP depletion. This activity causes swelling of the cell, mitochondria, and other organelles leading to disruption of membrane potential, structural disorganization, and degeneration of organelles and dissolution of the cell. Disruption of cellular plasma membrane integrity and the release of intracellular toxins launch an inflammatory response in the surrounding cells ultimately leading to the cascade that is cell death (Webster 2012). Although traditionally necrosis is thought of as a passive process due to the metabolic failure of the cell leading to random and violent cell death, there is also evidence of a programmed necrosis, which involves signal pathways very similar to extrinsic apoptosis. This activity can be apparent in ischemia-reperfusion injury that is initially caused by ischemic insult to the tissue leading to dissolution of cells and the release of pro-inflammatory ligands (Galluzzi *et al.* 2012, Webster 2012). In addition, necrosis can be initiated by an intrinsic factor of the cell, e.g. a reactive oxygen species in the same manner as in intrinsic apoptosis (Davis & Williams 2012).

2.1.5 Ca^{2+} regulation

Mitochondrion is a key regulator of intracellular Ca^{2+} signalling. Ca^{2+} is a co-factor and a regulator in many cellular metabolic pathways and is a fundamental signalling mediator between cytosol and mitochondrion. Mitochondrion has different pathways for Ca^{2+} uptake and efflux: Ca^{2+} uniporters (CaUP) embedded in IMM are responsible for mitochondrial Ca^{2+} uptake, whereas Na^+/Ca^{2+} and H^+/Ca^{2+} antiporters control the efflux. OMM is relatively permeable to Ca^{2+} due to

VDACs, which are controlled by chaperones, the NADH level and Bcl-2 proteins (Csordas *et al.* 2012, Bravo-Sagua *et al.* 2013). Separate pathways allow mitochondria to function as Ca^{2+} storage and actively alter the dynamics of intracellular Ca^{2+} concentration (Griffiths & Rutter 2009, Walsh *et al.* 2009). Intramitochondrial Ca^{2+} ($[Ca^{2+}]_m$) is vital for mitochondrial ATP production, as it activates several dehydrogenases along the route of oxidative phosphorylation, including PDHG and 2-oxoglutarate dehydrogenase (OGDH) of the TCA cycle, which increases production of NADH and likely activates complex V directly (Denton 2009, Griffiths & Rutter 2009).

As a response to cell stimulus, a concentration of cytosolic Ca^{2+} ($[Ca^{2+}]_c$) increases, which promote activation of cellular functions and increase ATP demand. Subsequently increased cytosolic Ca^{2+} is driven by a concentration gradient into the mitochondrial matrix through CaUPs, thus increasing $[Ca^{2+}]_m$. In the mitochondrial matrix, Ca^{2+} activates dehydrogenases and the OXPHOS chain (Denton 2009, Gellerich *et al.* 2010). Being an important regulator of cellular energy metabolism, Ca^{2+} is essential for all the cellular functions that require energy, including contraction, secretion, and maintenance of plasma membrane potential (Osellame *et al.* 2012).

Ca^{2+} has also an important role to play in cell death pathways (Duchen 2012, Giorgi *et al.* 2012, Osellame *et al.* 2012, Smaili *et al.* 2013). Release of Ca^{2+} from intracellular storage compartments, such as endoplasmic reticulum (ER), lead to a sudden increase in $[Ca^{2+}]_c$ and activation of apoptosis (Giorgi *et al.* 2012, Smaili *et al.* 2012, Bravo-Sagua *et al.* 2013). Furthermore, if the mitochondrial Ca^{2+} influx via CaUPs exceeds the efflux through Na^+/Ca^{2+} and H^+/Ca^{2+} antiporters, a mitochondrial Ca^{2+} overload may lead to swelling of mitochondria and disruption of OMM integrity further leading to a release of pro-apoptotic factors to cytosol (Giorgi *et al.*, 2012).

2.1.6 Mitophagy and mitochondrial quality control

Mitochondria may be damaged under pathological conditions or even during normal mitochondrial function, and that damage can be induced, for instance, by ROS. Structural quality control of damaged mitochondria can be achieved via degradation and removal of the entire mitochondrion by autophagy. In general, autophagy refers to the removal of damaged organelles or cytosolic components, but may also occur under starvation to redistribute the cellular structures.

Autophagy is specifically called mitophagy in the instance of mitochondrial degradation (Ashrafi & Schwarz 2013).

Autophagy and mitophagy are characterized by five stages: Initiation of the isolation membrane, elongation, closure of the membrane to form an autophagosome, fusion to lysosome to form an autolysosome, and finally lysosomal degradation. This series of actions is directly regulated by the mammalian target of rapamycin complex 1 (mTORC1) and the ATG protein family. Also, in case of cellular stress response, chaperon proteins, such as heat shock protein 90 (Hsp90), take part in regulation of autophagy and mitophagy. Microtubule-associated, light-chain protein LC3 is transformed into LC3-I in an ubiquitin-like reaction by the ATG4B protein and further processed by phosphatidyl ethanolamine (PE) to LC3-II, which is located in the isolation membrane and is essential for the elongation phase. (Ashrafi & Schwarz 2013). PTEN-induced kinase 1 (PINK1) and Parkin are proteins associated with the pathogenesis of Parkinson's disease (PD). Both take part in the regulation of mitophagy: PINK1 detects damaged mitochondria that have lost their membrane potential and recruits Parkin, which initiates mitophagy by the ubiquitylation of OMM proteins (Jin & Youle 2012). Studies with ATG7 knock-out mice, which lack the ability to form autophagosomes and autolysosomes, have shown that autophagy is a crucial process in the maintenance of normal cellular function, because inhibited autophagy leads to an accumulation of damaged mitochondria and severe organ damage (Komatsu *et al.* 2005). Interestingly, hematopoietic-specific ATG7 knock-out mice develop severe anaemia, which suggests that mitophagy is important also in normal maturation and for the removal of mitochondria in red blood cells (Mortensen *et al.* 2010).

Based on a finding that autophagosomes include mitochondria, which have lost the membrane potential, mitophagy was initially suggested to remove the damaged mitochondria (Lemasters *et al.* 1998). Still, it is unclear why all damaged mitochondria or mitochondria harbouring mitochondrial (mtDNA) mutations are not removed by mitophagy, as these conditions may lead to mitochondrial disease. The experimental data have shown that loss of membrane potential alone is not a sufficient signal to initiate mitophagy; it also requires recruitment of mTORC1 - even in the case of mitochondrial DNA mutations (Gilkerson *et al.* 2012). Furthermore, an accumulation of unfolded proteins in the mitochondrial matrix leads to PINK1/Parkin-mediated mitophagy of energetically healthy mitochondria, thus suggesting that unfolded proteins provide a signal to induce mitophagy and not the energetic balance of the cell alone (Jin & Youle

2013). In all, the pathways regulating mitophagy seem to have an essential role in mitochondrial pathology.

2.2 Mitochondrial DNA and mitochondrial genetics

Mitochondria are mainly constructed by nuclear-encoded genes (Spinazzola 2011, Davis & Williams 2012). In addition, mitochondria contain their own genetic material called mitochondrial DNA (mtDNA), which has been suggested to originate from when mitochondria were once free organisms before forming a symbiotic relation with eukaryotes (Yang *et al.* 1985). MtDNA is essential for normal mitochondrial function, and any rearrangements may lead to mitochondrial disease (McFarland *et al.* 2002, Chen & Butow 2005, Falkenberg *et al.* 2007).

2.2.1 Structure and organization of mitochondrial DNA

MtDNA is a double-stranded circular DNA molecule of 16 569 base pairs (Anderson *et al.* 1981), The two strands are called heavy (H) and light (L) strand according to their base composition, which causes different densities of alkaline chloride gradients. MtDNA encodes 37 genes: 13 proteins, all essential subunits of the OXPHOS chain complexes and ATP synthase, with two ribosomal RNAs (rRNA) and 22 transfer RNAs (tRNA) required for mitochondrial translation. Seven of the mtDNA genes (*MT-ND1* to *MT-ND6* and *MT-ND4L*) encode subunits of complex I, one gene (*Cyt b*) encodes part of complex III, three genes (*MT-COI-III*) are related to the catalytic subunit of complex IV, and *MT-ATP6* and *MT-ATP8* take part in complex V. The mtDNA is very compact and, unlike nuclear DNA (nDNA), does not contain any introns; rather the protein and rRNA coding regions of mtDNA are flanked by tRNA genes instead. In addition, mtDNA encloses a 1 kb non-coding region called a displacement loop (D-loop), which is located between the *tRNA(Phe)* and *tRNA(Pro)* genes. The D-loop includes one promoter for transcription of the H-strand (HSP_1), the promoter for transcription of the L-strand (LSP) and the origin of replication for the H-strand (O_H). In addition, another initiation site for H-strand replication (HSP_2) is located in the *tRNA(Phe)* gene adjacent to the D-loop. A shorter non-coding region of 30 nucleotides is located inside a tRNA cluster, approximately two thirds of the mtDNA molecule length from O_H. The non-coding region contains the origin of the replication of L-strand (O_L) (McFarland *et al.* 2002, Schapira 2006, Mao &

Holt 2009, Larsson 2010, Davis & Williams 2012). The structure and organization of the mtDNA molecule is illustrated in Fig. 4 (Mao & Holt 2009, Larsson 2010).

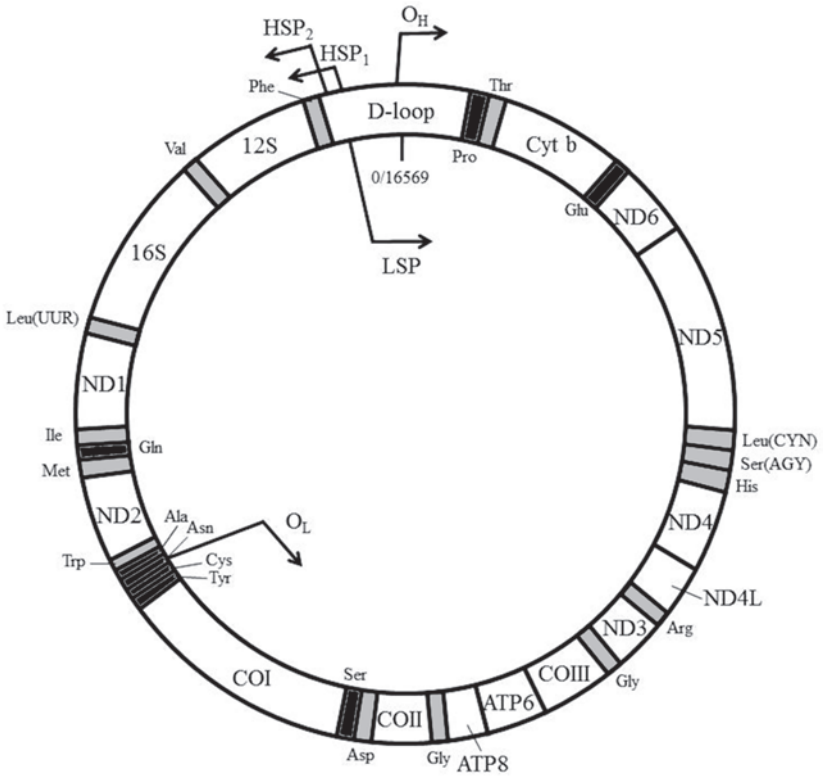

Fig. 4. The structure of human mitochondrial DNA. Mitochondrial DNA encodes seven subunits of complex I (ND), three subunits and the cytochrome *b* subunit of complex III, two subunits of ATP synthase, ribosomal RNAs 12S and 16S and 22 mitochondrial transfer RNAs. ND6 is encoded by the L-strand, but all other proteins are encoded by the H-strand. tRNA genes encoded by the H-strand are marked with grey and tRNA genes encoded by the L-strand are marked with black. Outer circle = heavy (H) strand, inner circle = light (L) strand, HSP = promoter for transcription of the H-strand, LSP = the promoter for transcription of the L-strand, O_H = origin of replication of H-strand, O_L = origin of replication of L-strand. Adapted from Mao & Holt 2009 and Larsson 2010.

Each mitochondrion contains 2-10 mtDNA molecules. As each cell can have hundreds of mitochondria, a single cell can contain hundreds up to thousands of copies of mtDNA molecule (polyploidy), whereas the nuclear genome has two

alleles, paternal and maternal, of each gene. The copy number of mtDNA correlates to the type and energetic needs of the cell (Copeland 2008, Spinazzola 2011). MtDNA molecules are packed into protein-DNA complexes called nucleoids, which are in close connection to IMM. Some of the proteins associated with mtDNA packaging, e.g. the mitochondrial transcription factor A (TFAM) and the mitochondrial single-strand DNA binding protein (mtSSB), are essential for the transcription and replication of mtDNA. Furthermore, nucleoids take part in mitochondrial fusion and fission and mtDNA segregation during cell division, therefore, affecting the distribution of mtDNA in the mitochondrial network and the daughter cells (Chen & Butow 2005, He *et al.* 2007, Holt *et al.* 2007).

2.2.2 Mitochondrial DNA replication and maintenance

The mtDNA is replicated in the mitochondrial matrix independently from the cell cycle and nDNA replication (Bogenhagen & Clayton 1977). The exact mechanism of mtDNA replication is still unclear. Three different models of mtDNA replication have been suggested (Holt & Reyes 2012). In the asynchronous (strand-displacement) model, mtDNA replication is initiated by transcription. A primary RNA for H-strand replication is synthetized at LSP. A replisome is formed by the mtDNA replication proteins, while mitochondrial DNA polymerase γ (pol γ) begins the DNA synthesis by extending the RNA primer; RNA as a primer is degraded by the RNAase enzyme. When approximately 70% of the H-strand is synthetized, the replisome reaches O_L. Displacement of the H-strand from the L-strand at O_L allows for an initiation of L-strand synthesis, which is carried out in the opposite direction than the replication of the H-strand. Finally, two newly synthetized mtDNA molecules are replicated, but the O_H-initiated molecules are finished first. Hence, in this model the newly synthetized H-strand is called the leading strand, and the L-strand is the lagging strand (Fernandez-Silva *et al.* 2003, Graziewicz *et al.* 2006, Mao & Holt 2009, Holt & Reyes 2012). Mitochondrial replisome is illustrated in Fig. 5 (Graziewicz *et al.* 2006, Hudson & Chinnery 2006, Krishnan *et al*. 2008).

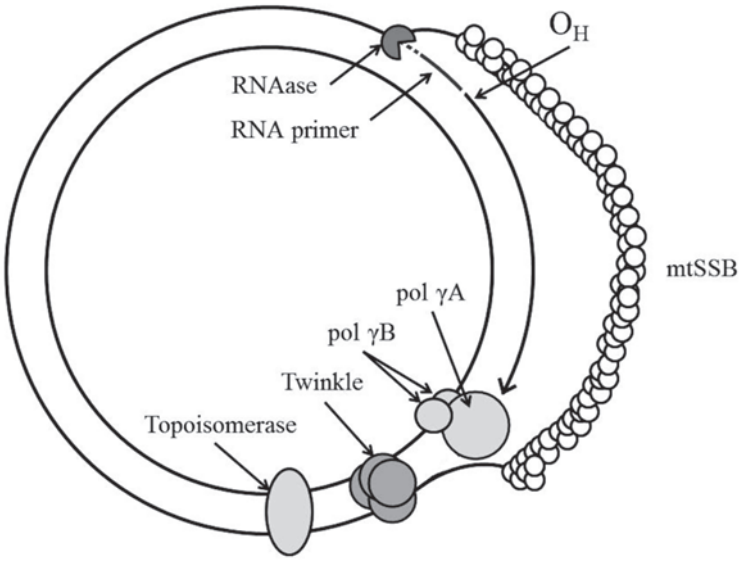

Fig. 5. Mitochondrial replisome. Topoisomerase enzyme maintains the topology of the DNA molecule suitable for replication and allows the Twinkle helicase to unwind the double-stranded DNA. Mitochondrial single-strand protein (mtSSB) stabilizes the single-strand DNA during DNA replication. Mitochondrial DNA polymerase γ, a heterotrimer enzyme, formed by the catalytic subunit pol γA and two accessory pol γB subunits, synthetizes the mitochondrial DNA. A RNA primer is formed by transcription, and after pol γ initiates the DNA synthesis, primary RNA is degraded by the RNAase enzyme. O_H = origin of replication of H-strand, pol γA = DNA polymerase γ subunit A, pol γB = DNA polymerase γ subunit B, Twinkle = mitochondrial DNA helicase, mtSSB = mitochondrial single strand protein. Adapted from Graziewicz *et al.* 2006, Hudson & Chinnery 2006 and Krishnan *et al*. 2008.

According to synchronous (strand-coupled) model, the mtDNA replication of both H and L-strand are replicated by mtDNA replication machinery at the same time and bidirectionally. Experimental data suggests that this model involves a reduced number of mtDNA molecules and is active in cells with depleted mtDNA (Holt *et al.* 2000). Strand-coupled replication can start at any site of mtDNA, but mainly replication is initiated at the zone of replication (O_Z), which is a region of several kilobases (kb) adjacent to the D-loop (Bowmaker *et al.* 2003). The third model, RNA incorporation during mtDNA replication (RITOLS), is similar to the strand-displacement model. The replication of the leading strand is initiated at O_H, but RNA is incorporated into lagging strand during the synthesis of the leading strand. When the replication fork reaches O_L and initiates a lagging strand

synthesis, RNA is either replaced or converted to DNA by pol γ (Holt & Reyes 2012).

The mutation frequency of mtDNA is 10 to 20-fold compared to nDNA (Brown *et al.* 1979). Unlike nDNA, mtDNA molecules are not covered by protective histones. In addition, mtDNA is bound by IMM and is in close proximity to the OXPHOS chain, the main intracellular source of ROS (Bohr *et al.* 2002, Bogenhagen *et al.* 2003); these factors make mtDNA very vulnerable to mutations. Mitochondrial replication machinery plays an important role in mtDNA repair and maintenance. In addition to the polymerase function, pol γ has exonuclease and proofreading activity and, therefore, plays an important role in mtDNA maintenance (Graziewicz *et al.* 2006, Hudson & Chinnery 2006). Furthermore, the mitochondria lack *de novo* synthesis of nucleotides (dNTP) and, therefore, dNTPs have to be transported into the mitochondria by the specific transporters, thymidine kinase 2 (TK2) and deoxyguanosinekinase (dGK), to maintain a normal nucleotide pool. Imbalance in dNTPs due to transporter malfunction can lead to either error-prone or prematurely halted mtDNA replication (Spinazzola & Zeviani 2005, Mao & Holt 2009).

2.2.3 Mitochondrial DNA transcription

Transcription of mtDNA initiates at three different points: One initiation site for the L-strand (L) and two initiation sites for the H-strands (H_1 and H_2). The activity of H_1 is 20-fold higher than H_2, and is also associated with transcription termination (Fernandez-Silva *et al.* 2003, Martin *et al.* 2005). On the other hand, *in vitro* promotor analysis has shown no significant initiation activity at H_2 suggesting there is only one major initiation site (H_1) for transcription of H-strand (Clayton 1992). Both of these sites have a two-part structure: promotor elements surround the initiation site and upstream from the initiation site is an enhancer element that binds TFAM to initiate transcription. H_2, which is suggested as being the second initiation site for H-strand transcription, apparently does not have a TFAM binding elements. Functionally HSP_1 and LSP are independent (Fernandez-Silva *et al.* 2003).

Transcription of both strands starts with the formation of a polycistronic primary RNA molecule composed of eight tRNAs and mRNA of *ND6* (Attardi & Schatz 1988). TFAM and mitochondrial transcription factor B (TFMB) are required to initiate the transcription. The RNA synthesis and elongation of the primary RNA molecule is carried out by mitochondrial RNA polymerase

(POLMRT) (Fernandez-Silva *et al.* 2003, Falkenberg *et al.* 2007, Mao & Holt 2009). The size of the primary molecules can vary, but especially primary molecules produced from H_2 and L are typically almost at a genomic length and are cleaved to mature RNA molecules after transcription, whereas H_1 mainly produces RNA primers or transcripts for mitochondrial ribosomes (mitoribosomes), which are necessary for mitochondrial translation (Silva & Larsson 2002, Fernandez-Silva *et al.* 2003, Martin *et al.* 2005, Bonawitz *et al.* 2006). The tRNA sequences are located in between each of the rRNA and mRNA sequences in the primary RNA molecule. The secondary cloverleaf structure of tRNA has been suggested as functioning as a signal for the endonucleolytic cleavage of tRNAs sequences, which yield fully processed tRNA, rRNA and mRNA (Ojala *et al.* 1981). Still, as in steady-state conditions rRNA levels are much higher than mRNA levels, most likely there are separate mitochondrial transcription mechanisms for rRNA and mRNA (Fernandez-Silva *et al.* 2003, Mao & Holt 2009).

2.2.4 Mitochondrial translation

All proteins produced by mitochondrial translation are mtDNA-encoded subunits of the OXPHOS chain complexes I, III, and IV and ATP synthase (Taanman 1999). Transcription of mtDNA produces nine monocistronic and two dicistronic mRNA molecules that are translated to proteins on mitoribosomes (Christian & Spremulli 2012). The mitoribosome is formed by two subunits: A small (SSU or 28S) and large (LSU or 39S) subunit, which together form the 55S ribosomal unit. The two subunits are formed by two mitochondrial rRNAs (12S and 16S) and 81 mitochondrial ribosomal proteins (MRP) (Smits *et al.* 2010, Christian & Spremulli 2012).

The mitochondrial genetic code differs from the universal genetic code, e.g., AUA codes methionine instead of isoleucine, and UGA codes tryptophan instead of a stop codon, whereas the universal codons AGA and AGG for arginine function as a stop codon. The different coding system allows the translation of all codons with only 22 tRNAs instead of normal 31. Mitochondrial mRNAs also have a few unique features as they have no or only a few non-coding nucleotides in 5'-end, are uncapped, and contain a poly-A tail after the stop codon (Watanabe 2010, Smits *et al.* 2010, Diodato *et al.* 2014). Mitochondrial translation machinery involves numerous nuclear-encoded proteins, such as mitochondrial initiation (mtIF), elongation (mtEF), releasing (mtRF), and ribosomal recycling

(mtRRF) factors, subunits and the assembly factors of mitoribosomes and the tRNA modification factors TMRU and PUS1. Before the actual translation can take place, the polycistronic mtDNA transcripts are cleaved from the 5'- and 3'-ternini of the tRNA sequences by RNase P (5'-end endonuclease) and tRNase Z (3'-end endonuclease) (Shutt & Shadel 2010). This action releases tRNA, mRNA and rRNA transcripts for post-transcriptional modification (Ojala *et al.* 1981), which enables the correct folding of tRNAs and the stabilisation of mRNA (Pearce *et al.* 2013). rRNA and mRNA are polyadenylated by mitochondrial poly(A) polymerase (mtPAP), thus creating stop codons for the mRNA transcripts and the unique poly-A tail after the stop codon in mitochondrial mRNAs (Smits *et al.* 2010, Diodato *et al.* 2014).

The actual translation process can be divided into four phases: initiation, elongation, termination, and recycling of transcription factors (Pearce *et al.* 2013). In the initiation phase, the 55S mitoribosome is broken in the two separate subunits, 28S and 39S, which allow for the binding of mRNA. Formylmethionyl-tRNA (fMet-tRNA) is the first amino acid residue in all mitochondrial proteins; fMet-tRNA binds to the start codon and forms the first protein site (P site) on the mitoribosome. The 39S subunit binds to the mRNA:28S complex and moves the mitoribosome further at the mRNA molecule and allows for the elongation of the polypeptide chain. During the elongation phase, the next aminoacyl-tRNA enters the acceptor site (A site) of the mitoribosome, according to specific codon-anticodon pairing, and forms a peptidyl bond with the aminoacyl-tRNA at the P site. The tRNA molecule at the P site and mRNA are then released, and the mitoribosome binds again further down the mRNA molecule to form new A and P sites, thus allowing the attachment of a new aminoacyl-tRNA and the sequential elongation of the polypeptide chain. The elongation cycle is repeated until a stop codon is encountered. The peptidyl-tRNA bond is hydrolysed, and the mature polypeptide chain is released from the tRNA in the last P site. The process is finished by recycling factors, which induce the release of mRNA, tRNA, and the mitoribosome, allowing them to be used in yet another translation process (Smits *et al.* 2010, Christian & Spremulli 2012, Pearce *et al.* 2013, Diodato *et al.* 2014).

2.2.5 Characteristic features of mitochondrial genetics

Heteroplasmy and the threshold effect

Each cell can have several hundred copies of the mtDNA molecule, i.e., the cells are polyploidic with respect to mtDNA (Copeland 2008, Spinazzola 2011). Under normal conditions, there are no mutations present in mtDNA (wild-type mtDNA). If all the mtDNA molecules are identical – wild-type or mutated - that condition is called *homoplasmy*. Normal wild-type and mutated mtDNA molecules can exist in the same cell. A situation where multiple mtDNA genotypes are present in the same cell is called *heteroplasmy* (Lightowlers *et al.* 1997, Fernandez-Silva *et al.* 2003). Threshold effect means that mtDNA mutation does not have any biochemical effect on the cellular function unless the ratio between mutated and wild-type mtDNA is high enough to prevent the co-existing wild-type mtDNA from compensating the mutation (Zeviani & Di Donato 2004). The energetic demands of the tissue determine the critical threshold rate. Usually a heteroplasmy rate that is higher than 60-80% is required for the mutation to have biochemical effects (Sciacco *et al.* 1994, Larsson & Clayton 1995, Lightowlers *et al.* 1997).

Maternal inheritance

Unlike the nuclear genome, which is inherited from both parents, the mitochondrial genome is maternally inherited. Only the mtDNA from the oocyte of the mother, including possible mtDNA mutations - are passed to the offspring upon fertilization. This effect also means that only daughters transfer the mtDNA to the next generation (Birky 1995, Ankel-Simons & Cummins 1996). Few mechanisms are yet proposed to explain the maternal inheritance of mtDNA. A mature oocyte can have over 100 000 copies of mtDNA, whereas a sperm cell holds in its mid-piece only 50-75 mitochondria with only one copy of the mtDNA molecule (Hecht *et al.* 1984, Piko & Taylor 1987). Therefore, initially it has been suggested that maternal inheritance is explained by this 1 000-fold difference in the copy number of maternal and paternal mtDNA and with only a minor contribution of paternal mtDNA (Hecht *et al.* 1984). The risk of transferring mtDNA mutations to the offspring has been shown to follow the heteroplasmy rate of the mother, thus implying that there is no selection against oocytes carrying mtDNA mutations. This finding suggests that the high number of

mitochondria in oocytes is a mechanism that ensures the distribution of mtDNA in the next generation (Shoubridge & Wai 2007).

Another suggestion is that, upon fertilization, only the paternal nuclear genome carried in the head of the sperm cell can enter the zygote, and the mid-piece of the sperm cell, including the paternal mitochondria, are left out and discarded (Ankel-Simons & Cummins 1996). Later experimental data has shown that paternal mitochondria also enter the zygote, but they are actively eliminated by the ubiquitin-mediated mechanism (Sutovsky *et al.* 1999). In addition, paternal mtDNA has not been detected in infants born after intracytoplasmic sperm injection used as treatment for infertility, which also suggests the active elimination of paternal mitochondria in the zygote (Houshmand *et al.* 1997). Still, few rare cases related to mitochondrial disease have actually shown paternal inheritance of mtDNA in relevant proportions (Schwartz & Vissing 2002).

Genetic bottleneck and random genetic drift

Due to the heteroplasmy and threshold effect, a single mutated mtDNA molecule is not expected to cause impairment of the mitochondrial function, and two or more different mtDNA clones can coexist in the same cell. During cell division and oogenesis in the maternal germ cell line, only a small proportion of mtDNA molecules from the cell are randomly passed to each primordial germ cell and amplified during cell maturation. The fast reduction in the number of transferred mtDNA molecules creates a genetic bottleneck that can lead to random genetic drift and an accumulation of mtDNA mutations in a single cell line from very low levels of mutated mtDNA. The sequence variants of mtDNA shift to homoplasmic very rapidly in the pedigree, even after one generation (Jenuth *et al.* 1996, Lightowlers *et al.* 1997, Elson *et al.* 2001, Zeviani & Di Donato 2004, Durham *et al.* 2006, Shoubridge & Wai 2007, Larsson 2010). Random genetic drift and fast segregation of mtDNA sequence variants between generations may contribute to the diversity of clinical phenotypes that are associated with mtDNA mutations (DiMauro 2004, Lombes *et al.* 2014).

Clonal expansion

MtDNA is replicated independently of the cell cycle, and mtDNA replication is active also in postmitotic tissues (Clayton 1982). Because of the relaxed replication of mtDNA, there is no mechanism to ensure that every mtDNA

molecule in the cell is replicated. Highly active mitochondrial metabolism in the cell causes oxidative stress, which can give raise to DNA point mutations and double strand breaks, which in turn can cause error in mtDNA replication and repair (Krishnan *et al.* 2008, Reeve *et al.* 2008). Random genetic drift can take place after cell division in the post-mitotic tissues, which may lead to an accumulation of mtDNA mutations from very low levels of mutated mtDNA. This effect may produce cell lines, which are heteroplasmic with respect to mitochondrial genome (Jenuth *et al.* 1996, Elson *et al.* 2001, Durham *et al.* 2006, Larsson 2010). Accumulation of mtDNA mutations have been shown to be tissue specific and especially tissues with low mitotic activity, but high mitochondrial metabolism, (heart, neurons and skeletal muscle) can present with mtDNA mutations (Cortopassi *et al.* 1992). The overall amount of mutated mtDNA in the tissue may be low, but individual cells may present with a high number of single mutated clone (Brierley *et al.* 1998). There is evidence of mosaic distribution of cytochrome c oxidase-negative (COX-negative) muscle fibres with a high load of mtDNA deletions and point mutations in an aged heart and muscle, which suggests that random genetic drift and clonal expansion of mtDNA mutations in post-mitotic tissues are part of the aging process (Khrapko *et al.* 1999, Fayet *et al.* 2002, Bua *et al.* 2006).

2.3 Mitochondrial diseases

A consensus for the exact definition of a mitochondrial disease has as yet not been achieved. Often mitochondrial diseases are strictly defined as clinical syndromes caused by dysfunction in mitochondrial oxidative phosphorylation (DiMauro 2004). In the broader view, mitochondrial diseases can be defined as dysfunction in any of the metabolic or structural components of mitochondria (Koopman *et al.* 2012, DiMauro *et al.* 2013). In addition, mitochondrial dysfunction can be secondary to other metabolic diseases that affect e.g. fatty acid oxidation or amino acid metabolism (Haas *et al.* 2007) or drug toxicity (Cohen 2010, Finsterer & Segall 2010), e.g. sodium valproate (Silva *et al.* 2008). Biochemically, mitochondrial diseases can be classified according to five major metabolic steps: Substrate transport, substrate utilization, TCA cycle, OXPHOS chain, and oxidation-phosphorylation coupling (DiMauro *et al.* 1985).

2.3.1 Common clinical features in mitochondrial diseases

Due to the double-genome control of mitochondria and the complexity of mitochondrial metabolism, the clinical spectrum of mitochondrial diseases is very diverse (DiMauro 2004, Koopman *et al.* 2012, DiMauro *et al.* 2013, Lombes *et al.* 2014). The first clinical phenotype associated with the biochemical dysfunction of the mitochondrion is Luft syndrome, i.e., non-thyroidal hypermetabolism (Luft *et al.* 1962), but since, the clinical spectrum of mitochondrial diseases has greatly expanded. Most often, tissues with high energy demand and high density of mitochondria (nervous tissue, skeletal and cardiac muscle, smooth muscle of the bowel liver, and kidneys) are affected. The clinical features are determined by the affected tissue and may also present as a multiorgan disease (DiMauro *et al.* 2013, Greaves *et al.* 2012, Schapira 2006).

Common symptoms in mitochondrial diseases include, e.g., epilepsy, ataxia, cognitive impairment, ophthalmoplegia, retinopathy, muscle weakness, exercise intolerance, cardiomyopathy, and endocrinopathies (Zeviani & Di Donato 2004, Greaves *et al.* 2012, DiMauro *et al.* 2013, Lombes *et al.* 2014). Age at onset is also very variable and first signs of a mitochondrial disease can present at any age (McFarland *et al.* 2010). Paediatric patients with mitochondrial diseases often present with fatal multiorgan disease, encephalomyopathies, or isolated myopathies with seizures, psychomotor delay, hypotonia of skeletal muscle, lactic acidosis, and cardiorespiratory failure as common clinical symptoms (Zeviani & Carelli 2003, Zeviani & Di Donato 2004, Taylor & Turnbull 2005). In adult-onset mitochondrial diseases, myopathy with muscle weakness or exercise intolerance is a common clinical presentation, and it may be accompanied by involvement of the nervous system with seizures, ataxia, hearing loss, pigmentary retinopathy, polyneuropathy, dementia, and rarely, movement disorders (Zeviani & Carelli 2003, Zeviani & Di Donato 2004, Taylor & Turnbull 2005). Clinical symptoms in mitochondrial diseases are usually progressive and disabling (Reeve *et al.* 2008).

2.3.2 Epidemiology and prevalence

Due to the clinical and molecular heterogeneity and variable penetrance of pathological mutations, reliable epidemiological assessments on mitochondrial diseases are difficult to make (Greaves *et al.* 2012). A study carried out at Oulu University hospital showed that mitochondrial pathology is relatively common in paediatric patients with encephalomyopathy and myopathy (Uusimaa *et al.* 2000).

A study on Australian population revealed inherited dysfunction of OXPHOS chain in 1 out of 5,000 births (Skladal *et al.* 2003). An analysis for 10 common pathogenic mtDNA point mutations in neonatal cord-blood samples collected in North Cumbria, England, revealed 1 pathogenic mutation in every 200 live births (Elliott *et al.* 2008). A study carried out in Northeastern England reported the prevalence of clinically determined mitochondrial diseases to be 9.2/100 000 in the general population and 16.5 per 100 000 people were at risk of developing a mitochondrial disease (Schaefer *et al.* 2008). Studies on the population of Northern Ostrobothnia in Finland estimated the prevalence of MELAS m.3243A>G mutation to be 16.3 per 100,000 in adults (Majamaa *et al.* 1998) and 18.4 per 100,000 in children (Uusimaa *et al.* 2007). In addition, carriers of pathogenic mutations in nuclear genes are fairly common. *POLG1* A467T mutation is present in 36% of *POLG1*-related diseases (Ferrari *et al.* 2005, Horvath *et al.* 2006, Nguyen *et al.* 2006, de Vries *et al.* 2007, Wong *et al.* 2008). The carrier frequency of *POLG1* A467T mutation is 1:167 in Belgian (Van Goethem *et al.* 2001), 1:145 in British (Horvath *et al.* 2006), 1:100 in Norwegian (Winterthun *et al.* 2005) and 1:500 in Finnish (Luoma *et al.* 2005) control populations. In Finnish population, *POLG1* p.W748S and Q1236H mutations are very common with carrier frequencies of 1:125 (Hakonen *et al.* 2005) and 1:68 (Luoma *et al.* 2005), respectively. These findings suggest that mutations associated with mitochondrial diseases are relatively common also in the general population.

2.3.3 Diagnostics

The clinical variety of signs and symptoms creates a challenge for the identification of mitochondrial diseases and the range of differential diagnostics is very broad. Therefore, setting the diagnosis of a mitochondrial disease requires the synthesis of a wide spectrum of clinical, biochemical, and radiological investigations. The diagnostic protocol typically starts with less invasive investigations, including general clinical evaluation, radiological imaging, and metabolic screening tests, followed by more specific histological, biochemical, and genetic studies, which often require invasive procedures, e.g., a tissue biopsy (Haas *et al.* 2007, Haas *et al.* 2008, McFarland *et al.* 2010, Milone & Wong 2013). A diagnostic protocol for mitochondrial diseases is presented in Fig. 6.

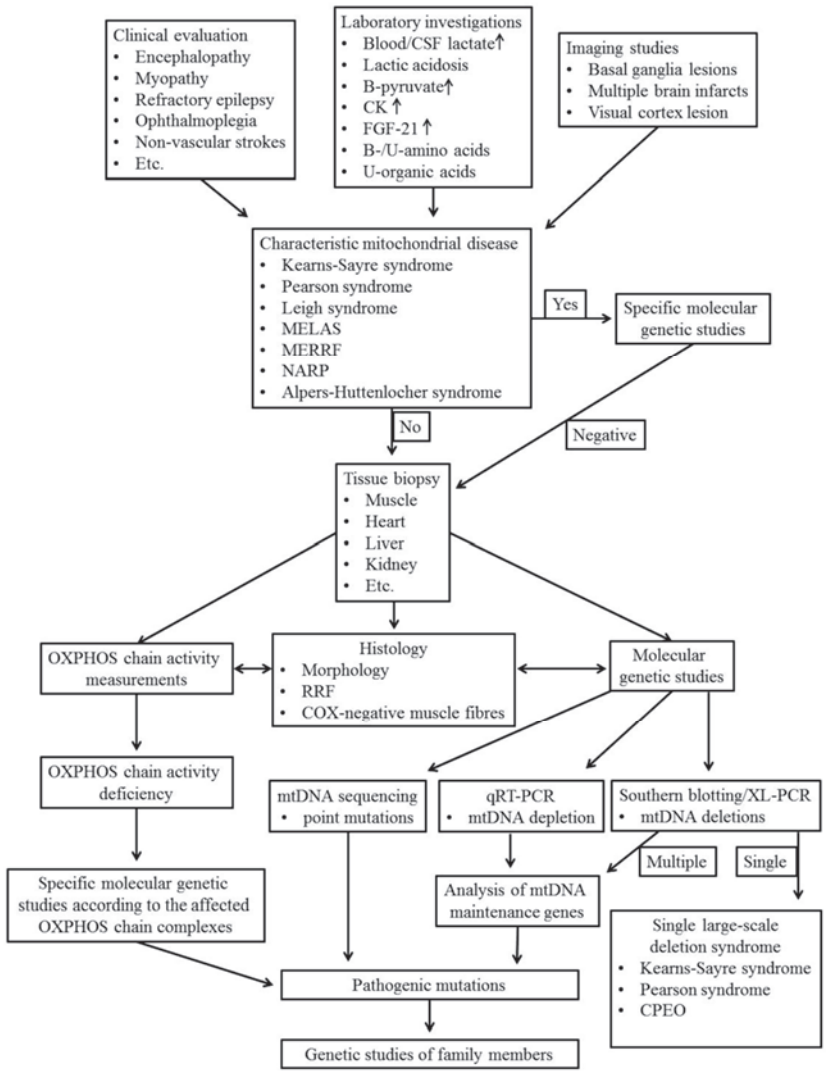

Fig. 6. Diagnostic protocol for mitochondrial diseases. CSF = cerebrospinal fluid, MELAS = mitochondrial myopathy, encephalopathy, lactic acidosis and stroke-like episodes, RRF = ragged-red fibres, qRT-PCR = quantitative real-time PCR, XL-PCR = long-range PCR, MERRF = myoclonic epilepsy and ragged-red fibers, NARP = neuropathy, ataxia and retinitis pigmentosa, CPEO = chronic progressive external ophthalmoplegia. Adapted from Haas *et al.* 2007, Haas *et al.* 2008, McFarland *et al.* 2010 and Milone & Wong 2013.

Clinical evaluation

The first signs and symptoms of mitochondrial diseases can present at any age and in any organ (Haas *et al.* 2007, McFarland *et al.* 2010). Mitochondrial diseases may present with an uncommon combination of signs originating from multiple organs, which often is the case with the classic mitochondrial syndromes (McFarland *et al.* 2010). As a guiding rule, involvement of three or more organ systems without a unifying diagnosis is suggestive of a mitochondrial disease (Haas *et al.* 2007). A wide spectrum of neurologic, ophthalmologic, cardiologic, or gastroenterological signs can refer to a mitochondrial disease, e.g., non-vascular cerebral stroke-like lesions, refractory epileptic seizures, ophthalmoplegia, myopathy, ataxia, and an unexplained hypotonia, failure to thrive and/or (lactic) acidosis of a newborn (Haas *et al.* 2007). Considering the genetic origin of mitochondrial diseases, a detailed family history is essential for mitochondrial diagnostics (McFarland *et al.* 2010).

Laboratory investigations

Typically, laboratory investigations for mitochondrial diseases include metabolic analysis of blood, cerebrospinal fluid (CSF) and urine (Haas *et al.* 2007, McFarland *et al.* 2010). Required tests often include lactate and pyruvate analysis, creatine kinase (CK) levels in the blood, quantitative analysis of amino acids, quantitative analysis of organic acids in urine, and measurement of blood acylcarnitines (Haas *et al.* 2007).

Increased lactate and pyruvate in blood or CSF and lactic acidosis typically are signs of increased anaerobic metabolism and can indicate a mitochondrial disease. Determination of the lactate/pyruvate ratio indirectly reflects the cytoplasmic redox state. Increase of both lactate and pyruvate can be found in various other clinical conditions, and therefore, they are not specific signs of mitochondrial pathology, although increased levels of pyruvate often do refer to a deficiency of mitochondrial oxidative phosphorylation (Haas *et al.* 2007, Haas *et al.* 2008). Levels of lactate and pyruvate may sometimes be present only during metabolic decompensation and may be increased only in CSF in patients with an isolated mitochondrial disease of the central nervous system. (Haas *et al.* 2008). Blood CK levels are often mildly elevated in patients presenting with myopathy, and in high quantities, they signify rhabdomyolysis, although CK is not a specific marker for mitochondrial disease (Milone & Wong 2013). Fibroblast growth

factor 21 (FGF-21) is a regulator of energy homeostasis in the body (Kim & Lee 2014). FGF-21 is elevated in the serum in primary mitochondrial diseases, and therefore offers a sensitive biomarker for identification of primary respiratory chain deficiencies (Suomalainen *et al.* 2011, Davis *et al.* 2013, Salehi *et al.* 2013).

Amino acids in blood and urine can be analysed for mitochondrial diagnostics (Haas *et al.* 2008). Generalized aminoaciduria can be a renal manifestation of a mitochondrial disorder with mtDNA deletions (Haas *et al.* 2008), but may also be present in mitochondrial depletion syndromes (Bornstein *et al.* 2008, Dimmock *et al.* 2008, Uusimaa *et al.* 2014). An amino acid profile in blood can offer a clue of the mitochondrial origin of the disease. Increased level of alanine may be a sign of a long-term increase in pyruvate levels, and elevated levels of proline, glycine and sarcosine may be detected in mitochondrial dysfunction. Increase in blood tyrosine is met in early-onset liver dysfunction caused by mtDNA depletion (Haas *et al.* 2008).

Organic acid analysis is preferably performed from a urine sample, but can be done on blood and CSF in selected cases. Organic acid analysis is most often required in infancy-onset encephalopathies in order to diagnose and differentiate organic acidopathies from mitochondrial diseases. Organic acid analysis can also help identify mitochondrial fatty acid oxidation disorders, and the intermediates of TCA cycle can be met in mitochondrial diseases. Carnitine functions as a mediator between acetyl-CoA and free fatty acids. Quantification of total and free carnitine and acylcarnitine may be useful in the diagnosis of fatty acid oxidation disorders, but can also be useful in detection of aminoacidemias and organic acidemias (Haas et al 2008).

Imaging studies

Most patients with a mitochondrial disease of the central nervous system (CNS) involvement present with abnormal findings in a brain MRI (Bricout *et al.* 2014). Some of the findings in radiographs can be specific for certain mitochondrial syndromes: symmetrical basal ganglia lesions are often present in Leigh syndrome (LS) (Baertling *et al.* 2014), whereas transient or non-vascular pattern brain infarct lesions are seen in mitochondrial myopathy, encephalopathy, lactic acidosis, and stroke-like episodes (MELAS) (Pauli *et al.* 2013), and lesions of the visual cortex are often present in Alpers syndrome (Saneto *et al.* 2013). Brain proton magnetic resonance spectroscopy (MRS) can measure brain and CSF lactate noninvasively. In patients with mitochondrial myopathy without CNS

involvement, the brain MRI is typically normal. ^{31}-phosphorous MRS (^{31}-P MRS) can be used to evaluate mitochondrial metabolism by following phosphocreatine, ATP, and inorganic phosphate kinetics in the muscle tissue (Anglin *et al.* 2013, Milone & Wong 2013).

Electrophysiological studies

Mitochondrial disease with CNS involvement often present with epileptic seizures, which can be further studied by electroencephalography (EEG). Electroneuromyography (ENMG) can show unspecific signs of myopathy and also may identify neuropathies, which can be a sign of a systemic disease with mitochondrial origin (Milone & Wong 2013).

Tissue biopsies; biochemical, and histological studies

Biopsies of the most affected tissue may be required for more specific biochemical and histological studies. Skeletal muscle is often affected in mitochondrial diseases, and therefore, it is often used in mitochondrial diagnostics (Haas *et al.* 2008). Light and electron microscopy and immunohistochemistry can be used in the assessment of the structure and organisation of the muscle fibres, accumulation of fibrin and collagen and structure and distribution of the mitochondria. A mosaic or global pattern of COX-deficient muscle fibres and ragged-red fibres (RRF), a sign of mitochondrial proliferation, are considered to be findings suggesting a mitochondrial disorder. In some cases, RRFs are missing in paediatric patients with mitochondrial diseases as RRF accumulate over time. Muscle biopsy specimens can also be used in OXPHOS chain activity measurements, which offer essential information on mitochondrial function (Haas *et al.* 2008, McFarland *et al.* 2010, Milone & Wong 2013). A muscle biopsy specimen can also be used to quantify CoQ and identify primary CoQ deficiency (Haas *et al.* 2008). As muscle tissue can present with normal histological and biochemical findings, liver and cardiac muscle biopsies may be required for more specific investigations when these organs are affected. Muscle and skin biopsies can also be acquired to obtain myoblasts and fibroblasts for functional and metabolic studies (Haas *et al.* 2008).

Molecular genetic studies

As mitochondria are under the control of mitochondrial and nuclear genomes, analysis of both of these genomes may be required to identify the causative genetic factor behind the mitochondrial disease (Haas *et al.* 2008, McFarland *et al.* 2010). The primary rearrangements of mtDNA associated with mitochondrial diseases are point mutations and single deletions, which can be detected by Southern blotting, long-range PCR (XL-PCR), and direct sequencing (Greaves *et al.* 2012). The nuclear genes responsible for mtDNA maintenance can lead to secondary defects of mtDNA including multiple deletions and depletion. In addition, mutations in the nuclear genes encoding structural proteins of mitochondria or affecting the mitochondrial dynamics can lead to mitochondrial disease (DiMauro 2004). Point mutations of mtDNA are maternally inherited, whereas mutations in nuclear genes follow Mendelian inheritance (Spinazzola & Zeviani 2005).

In the case of a classic mitochondrial syndrome, analyses for specific mtDNA or nDNA point mutations can be performed (McFarland *et al.* 2010). In addition, genetic studies can be directed, based on previous knowledge regarding the association of certain clinical features with specific genes, e.g., *POLG1, DGUOK*, and *MPV17* genes in liver involvement (Haas *et al.* 2008). Exome sequencing is a new molecular genetic method that enhances the identification of new pathogenic mutations in nuclear genes leading to mitochondrial diseases (Calvo *et al.* 2012, Carroll *et al.* 2014, Taylor *et al.* 2014). DNA extracted from tissue biopsy specimens can be analysed by Southern blotting, long-range PCR, and quantitative PCR for tissue-specific mtDNA rearrangements including mtDNA deletions and depletion (Haas *et al.* 2008, McFarland *et al.* 2010).

2.3.4 Treatment

Treatment of mitochondrial diseases is mainly supportive (McFarland *et al.* 2010). Studies to find specific pharmaceutical agents to treat mitochondrial diseases have included attempts to induce mitochondrial function by e.g., increasing the availability of OXPHOS chain substrates, enhancing the electron transfer in the OXPHOS chain or augmenting OXPHOS chain complexes; however, there is no clear evidence available to support the benefits of these treatments (Pfeffer *et al.* 2012, Pfeffer & Chinnery 2013). Positive outcomes have been achieved by high-dose CoQ therapy in primary CoQ deficiency related to

ETFDH gene mutations (Gempel 2007), and mitochondrial diseases caused by error in CoQ biosynthesis are thought to be treatable with CoQ supplementation (Rahman 2012). In addition, Leigh syndrome caused by mutations in *SLC19A3* gene can be successfully treated with thiamine (vitamin B_1) (Fassone *et al.* 2013, Distelmaier *et al.* 2014). Riboflavin (vitamin B_2), which is the precursor of FAD in OXPHOS chain complexes I and II, has been used with good response in myopathy related to complex I deficiency (Bernsen *et al.* 1993) and Brown-Vialetto-Van Laere syndrome that is related to dysfunction in riboflavin transporter RFVT2 (Foley *et al.* 2014). Enhancement of cerebral circulation via nitric oxide-mediated vasodilatation has been achieved by treating MELAS patients with L-arginine, a nitric acid precursor, which has improved the outcome of the stroke-like episodes related to MELAS (Koga *et al.* 2005).

A high-fat and low-carbohydrate ketogenic diet (KD) has been studied as a potential therapy for mitochondrial diseases, and the preclinical and clinical data suggest that KD enhances the mitochondrial function by increasing fatty oxidation and decreasing glycolysis (Gano *et al.* 2014). *In vitro* experimental data from cultured cells with heteroplasmic mtDNA deletions suggests that a ketone-rich environment leads to a reduction of deleted mtDNA molecules by favouring wild-type mtDNA and, therefore, may restore mitochondrial function (Santra *et al.* 2004). KD has also improved the features of late-onset myopathy in a mutant mouse model by inducing mitochondrial biogenesis and restoring lipid levels (Ahola-Erkkila *et al.* 2010). Aerobic exercise has been shown to improve mitochondrial respiration and tissue perfusion in patients with mitochondrial myopathies with a short-term response (Jeppesen *et al.* 2006, Taivassalo *et al.* 1998, Taivassalo *et al.* 2006). Resistance training recruits satellite cells in the muscles, which decreases the proportion of mtDNA deletions and COX-negative fibers and improve muscle strength and function in mitochondrial myopathies (Murphy *et al.* 2008).

2.4 Mitochondrial diseases related to primary mtDNA rearrangements

2.4.1 Large-scale mtDNA rearrangements: Deletions and duplications

Deletions are qualitative defects of the mitochondrial genome referring to fragmentary loss of mtDNA molecules (Krishnan *et al.* 2008). Single large-scale mtDNA deletions are most often the primary rearrangements associated with mitochondrial diseases. So far, more than 120 different mtDNA deletions have been described (Greaves *et al.* 2012). Primary single deletions of mtDNA are thought to be of sporadic origin, occurring in the germ line and not genetically transmitted (Chinnery *et al.* 2004, Russell & Turnbull 2014). The prevalence of mtDNA deletions in the adult Finnish population has been estimated to be 1.6 per 100, 000 (Remes *et al.* 2005).

The size of deletions can vary from a single nucleotide up to several kb, and they can be located in any part of the mtDNA molecule (Schapira 2006). The majority of mtDNA deletions are located in the arc between mtDNA replication initiation sites (Bua *et al.* 2006). The most common single large-scale mtDNA deletion is 4977 base pairs (bp) of size spanning between the genes for cytochrome B (*CytB*) and cytochrome c oxidase subunit II (*COXII*) (Remes *et al.* 2005, Schapira 2006, Krishnan *et al.* 2007).

Deletions always lead to the loss of one or more mitochondrial tRNA genes and, therefore, affect the translation of all mtDNA genes. Without exception, single deletions are heteroplasmic (Spinazzola 2011). As deletions create a loss of several genes, a relatively low heteroplasmy rate of >60% can lead to mitochondrial dysfunction (Sciacco *et al.* 1994). The clinical phenotypes caused by deletions are highly variable, and the age at onset of the disease can vary from birth to late adult age (Mao & Holt 2009). Single large-scale deletions are typically associated with chronic progressive external ophthalmoplegia (CPEO), Kearns-Sayre syndrome (KSS) and Pearson syndrome (PS) (Zeviani *et al.* 1988, Moraes *et al.* 1989, Fischel-Ghodsian *et al.* 1992, DiMauro 2004, Remes *et al.* 2005, Schapira 2006, Greaves *et al.* 2012). Still, mtDNA deletions are not restricted to only these clinical phenotypes, but are thought to be present in mitochondrial encephalomyopathies (Schapira 2006). Still, it must be noted that the increasing prevalence of mtDNA deletions in aging tissues - especially in tissues with low mitotic activity, but high mitochondrial metabolism (heart,

neurons and skeletal muscle) - has been previously observed both in humans and in animal models (Piko & Taylor 1987, Zhang *et al.* 1992, Cortopassi *et al.* 1992, Corral-Debrinski *et al.* 1992, Brierley *et al.* 1998, Fayet *et al.* 2002, Pak *et al.* 2005, Bua *et al.* 2006). This phenomenon poses a great challenge for any evaluation of mtDNA deletions in relation to the clinical phenotype.

Furthermore, a complete or partial duplication of the mtDNA molecule can be present in mitochondria, altering the mitochondrial function, and may present alone or together with deletions (DiMauro 2004). Initially, duplications were described in patients with mitochondrial myopathy and multiorgan affision similar to KSS (Poulton *et al.* 1989) and PS (Rotig *et al.* 1990). Later studies have shown that duplications are frequent in patients with KSS and may be a particular feature of this disease (Poulton *et al.* 1994, Poulton *et al.* 1995b, Odoardi *et al.* 2003). Although duplications themselves do not lead to loss of mtDNA genes, they may interfere with mitochondrial function with abnormal transcripts or proteins of rearranged genes, excess protein transcripts of the duplicated region, or through a homologous recombination of mtDNA (Poulton 1992, Odoardi *et al.* 2003). Duplications have not been shown to interfere with OXPHOS chain activity (Odoardi *et al.* 2003).

Chronic external ophthalmoplegia

Chronic external ophthalmoplegia (CPEO; #609286) is a mitochondrial disease characterized by paralysis of external eye muscles, which leads to ptosis and weakness of eye movements. In addition, exercise intolerance may be present referring to skeletal myopathy. CPEO may present as a single entity or as a part of other mitochondrial disease, and possible additional clinical features can be found according to the present clinical syndrome.

Kearns-Sayre syndrome and Pearson syndrome

Kearns-Sayre syndrome (KSS; OMIM #530000) was first described by Kearns and Sayre in 1958. The syndrome is characterized by a clinical triad of chronic external ophthalmoplegia, pigmentary retinopathy and cardiac conduction block. Additional clinical features may include myopathy, sensorineural hearing loss, ataxia, and endocrinopathies. In some contexts, KSS has been considered to be a more severe variant of CPEO. RFFs are typically seen in muscle histology. Typical onset of the disease is before 20 years. Pearson syndrome (Pearson

marrow-pancreas syndrome, PS; OMIM #557000) presents with early-onset sideroblastic anaemia and failure of the exocrine pancreas (Pearson et al 1979). Often, various neurological symptoms are present, and other features may include e.g., insulin-deficient diabetes and Addison's syndrome. The clinical course of PS is often fatal, but some patients, who have survived the initial phase of the disease, have developed progressive KSS.

2.4.2 mtDNA point mutations

Considering the high mutation rate of mtDNA, distinguishing pathogenic point mutations from population-specific neutral polymorphisms is challenging. Four features are usually present in pathogenic point mutations: high conservation of the nucleotide or amino acid or loss of function of the gene product; segregation with the clinical phenotype; correlation between the heteroplasmy rate and the clinical phenotype; and identification of the mutation in ethnically distinctive families (Zeviani & Carelli 2003). Point mutations affecting tRNA genes may affect overall mitochondrial protein synthesis, whereas mutations in genes encoding subunits of respiratory complexes specifically impair the corresponding respiratory complex (Mariotti *et al.* 1994).

Over 250 pathogenic mtDNA point mutations have been described (Taylor & Turnbull 2005). Pathogenic mtDNA point mutations follow maternal inheritance (see Chapter 2.2.5), but both genders can be affected. Pathogenic mutations have been found in tRNA, rRNA, and protein-encoding genes (Russell & Turnbull 2014). Although tRNA genes comprise only 5% of the mitochondrial genome, over half of the pathogenic mtDNA point mutations affect tRNA genes. Compared to deletions, point mutations affecting a single gene usually require a higher heteroplasmy (see Chapter 2.2.5) rate of 80-90% to cause mitochondrial dysfunction (Mariotti *et al.* 1994, Lombes *et al.* 2014). Pathogenic mtDNA point mutations are most often heteroplasmic, but mutations associated with certain mitochondrial diseases, e.g., Leber's hereditary optic atrophy, are homoplasmic (McFarland *et al.* 2007). In general, homoplasmic mtDNA point mutations affect single tissue as opposed to multiorgan involvement in diseases caused by heteroplasmic mutations (Zeviani & Di Donato 2004). Penetrance of homoplasmic mutations is not complete, suggesting involvement of other regulating factors, e.g., additional mtDNA polymorphisms, nuclear gene mutations, or environmental factors, in the development of homoplasmic mtDNA

disease (Howell & Mackey 1998). Below are the descriptions of a few "classic" mitochondrial cytopathies associated with mtDNA point mutations.

Heteroplasmic mtDNA diseases

Mitochondrial myopathy, encephalopathy, lactic acidosis, and stroke-like episodes (MELAS; #OMIM 540000) were described by Pavlikis et al in. 1984. MELAS syndrome mainly affects CNS and skeletal muscle tissue. Typical clinical presentations include seizures, hemiparesis, hemianopia, cortical blindness, and episodic vomiting. Most patients suffer from stroke-like episodes before age 40, and a brain MRI often discloses lesions similar to brain infarcts. Recurrent stroke-like episodes usually lead to progressive loss of vision, movement disorders, and dementia. Mitochondrial dysfunction often induces over-production of lactate leading to lactic acidosis. MELAS is caused by point mutations in the mitochondrial tRNA genes. Over 30 pathogenic mutations have been associated with MELAS, but typically the syndrome is caused by m.3243A>G transition in the mitochondrial *tRNA(Leu)* gene. Hearing loss and diabetes are the most common clinical features associated with m.3243A>G mutation. Maternally inherited diabetes and deafness (MIDD) and PEO are commonly associated with m.3243A>G mutation, but the use of these terms has been critisiced by Mancuso *et al.* (2014) as these syndromes may present with systemic complications typical to MELAS.

Myoclonic epilepsy and ragged-red fibers (MERRF; #545000), first described by Fukuhara et al 1980, is a very rare mitochondrial disease presenting with progressive myoclonus, epilepsy, muscle weakness, and muscle spasticity. Characteristic RRFs are seen in the muscle histology. Other clinical features may include ataxia, peripheral neuropathy, and lipomatosis. In the majority of cases, MERRF is caused by m.8344A>G transition affecting the *tRNA(Lys)* gene.

Neuropathy, ataxia, and retinitis pigmentosa (NARP; #OMIM 551500) were first described by Holt et al in 1990 in a family having various clinical features related to the nervous system. Often NARP presents in young adults. Characteristic features of NARP include numbness and limb weakness caused by sensomotoric neuropathy, ataxia, and poor night vision or even visual loss caused by retinitis pigmentosa. Additional clinical features may include seizures, hearing loss, and cardiac conduction block. Learning difficulties and developmental delay are often seen in children affected by NARP. Lactic acidosis is a rare feature in NARP. Typically for mtDNA-related disease, NARP is maternally inherited and

often associated with m.8993T>G or m.8993T>C mutation in the *MT-ATP6* gene. If either of the mutations is present in heteroplasmy rate 70-90%, the patient typically develops NARP, whereas heteroplasmy rate over 90% leads to onset of more severe Leigh syndrome.

Homoplasmic mtDNA diseases

Leber optic atrophy and dystonia (LHON; OMIM# 500001) were initially described by Theodor Leber, who identified a familial syndrome characterized by loss of vision in 1871. LHON affects ganglion cells of the retina and causes atrophy of the optic nerve, which leads to painless central scotoma and loss of vision. Often the first signs are unilateral, and a few weeks later the other eye is also affected. Typically, the first symptoms emerge at midlife with acute or subacute onset. Traditionally, LHON is considered to affect only the retina and optic nerve, and LHON with additional clinical signs and symptoms is often referred to as "LHON plus". Further, 95% of LHON cases are caused by mtDNA point mutations m.3460G>A (*MT-ND1*), m.11778G>A (*MT-ND4*) and m.14484T>C (*MT-ND6*).

2.5 Nuclear genes encoding mitochondrial structure proteins in mitochondrial diseases

The nuclear genome constitutes the majority of mitochondrial structures (Calvo & Mootha 2010, Calvo *et al.* 2012), and therefore, mutations in nuclear genes play a major role in the molecular genetic aetiology of mitochondrial diseases (DiMauro 2004, McFarland et al 2010). Nuclear-encoded genes are involved in the five major steps of mitochondrial function: assembly of OXPHOS chain complexes and ATP synthase; mtDNA replication, translation and maintenance; transportation of nuclear-encoded protein into mitochondria; formation of the lipid bilayer of IMM; and mitochondrial network dynamics, i.e., the fusion and fission of mitochondria (DiMauro 2004, Haas et al 2007).

2.5.1 OXPHOS chain subunits and assembly proteins

Out of the approximately 85 OXPHOS chain subunits recognized so far, 13 are encoded by mtDNA. Thus, over 70 proteins encoded by the nuclear genome are necessary in the construction of mitochondrial respiratory chain complexes

(Zeviani & Di Donato 2004, DiMauro *et al.* 2013). Mutations directly affecting the subunits have been located in all of the OXPHOS chain complexes and ATP synthase (McFarland et al 2010, DiMauro *et al.* 2013).

Complex I is most often affected, and pathogenic mutations have been identified in 16 out of 45 of the subunits of complex I. Direct mutations of the 11 nuclear-encoded subunits of complex IV seem to be rare, as thus far, mutations have been detected only in two. The clinical phenotype associated with defects in the OXPHOS chain subunits is most often early-onset progressive encephalopathy with lactic acidosis, very often compatible with autosomal recessive LS (Zeviani & Di Donato 2004, DiMauro *et al.* 2013). Interestingly, mutations in the subunits of complex II are associated with potentially malignant tumours, paragangliomas, and pheochromocytoma (Vicha *et al.* 2014). The majority of pathogenic mutations of the OXPHOS chain subunits are autosomal recessive (Zeviani & Di Donato 2004).

In addition to subunit-encoding genes, a vast number of nuclear-encoded chaperon proteins are needed to target the synthetized subunits to IMM and the synchronised assembly of the OXPHOS chain complexes (McFarland *et al.* 2010, DiMauro *et al.* 2013). A common presentation of assembly protein mutation is encephalopathy with multiorgan affision, including cardiomyopathy, nephropathy, or hepatopathy (Papadopoulou *et al.* 1999, Valnot *et al.* 1999, Valnot *et al.* 2000). Other distinguishable mitochondrial diseases associated with assembly proteins include growth retardation, aminoaciduria, cholestasis, iron overload, lactic acidosis, and early death (GRACILE) with mutations in *BCS1L* affecting complex III (Visapää *et al.* 2002), leukoencephalopathy with brainstem and spinal cord involvement and high brain lactate (LBSL) with mutation in *DARS2* (van der Knaap *et al.* 2003). Furthermore, mutations in the genes responsible for the biogenesis of CoQ can cause CoQ deficiency and lead to mitochondrial diseases with myopathy, myoglobinuria, and encephalopathy with seizures, ataxia, cerebellar atrophy, and mental retardation (Ogasahara *et al.* 1989, Lamperti *et al.* 2003). The diagnosis of primary CoQ deficiencies is important, because these patients can respond to CoQ supplementation (Hirano *et al.* 2012).

Leigh syndrome

Leigh syndrome (LS; OMIM #256000) is one of the “classic” mitochondrial diseases first described by Leigh in 1951. LS is a rare, severe neurodegenerative disorder characterised by focal necrotic lesions in CNS. Typically, basal ganglia,

thalamus, cerebellum, and the spinal cord are affected. The signs and symptoms are variable and are defined by the affected region in CNS. Often lactic acidosis is present. Typically, the first signs and symptoms are present by the age of 2 years and very rarely appear in adolescence or adult age. The disease is often fatal and leads to death in childhood, but LS caused by mutations in *SLC19A3* gene can be managed with thiamine substitution. The molecular genetic aetiology of LS is very heterogeneous, but it is considered to be most commonly associated with mutations in the OXPHOS chain complex I subunits.

2.5.2 Mitochondrial translation

Mutations in nuclear genes encoding various proteins of the mitochondrial translation machinery are one of the most recent findings in mitochondrial molecular pathology (Boczonadi & Horvath 2014). A typical biochemical defect associated with mutations in this group of genes is a combined deficiency of multiple OXPHOS chain complexes, but with no signs of disorder in mtDNA maintenance (Kemp *et al.* 2011). Clinically, these disorders present with severe neurological syndrome, such as LS, leukodystrophy or recessive ataxia, hepatocerebral syndrome, cardiomyopathy and lactic acidosis (DiMauro *et al.* 2013, Boczonadi & Horvath 2014). In addition to mitochondrial pathology, dysfunctional mitochondrial tRNA modification is associated with various human pathology, including neurodegeneration disorders, cancer, and type 2 diabetes mellitus (Torres *et al.* 2014). Interestingly, the clinical course of a liver failure caused by a mutation in the gene encoding mitochondrial specific tRNA modification enzyme tRNA 5-methylaminomethyl-2-thiouridylate methyltransferase (*TRMU*) can be reversible (Schara *et al.* 2011), and a patient with reversible COX-deficient myopathy caused by *TRMU* gene mutation has been described (Uusimaa *et al.* 2011). TRMU is required in 2-thiouriylation of mitochondrial tRNA(Glu), tRNA(Lys) and tRNA(Gln) and downregulation of TRMU leads to impaired mitochondrial protein translation. The actual mechanism of the spontaneous recovery in *TRMU*-related myopathy is yet unresolved, but the function of TRMU can be restored with L-cysteine supplementation – a finding that may offer a new therapy for mitochondrial protein translation deficincies (Boczonadi *et al.* 2013).

2.5.3 Mitochondrial protein transportation

Mitochondrial proteins synthesized in cytosol need complicated machinery composed of docking proteins, chaperonins, proteases, and transmembrane transporters to facilitate their transportation into mitochondria (Okamoto *et al.* 2002). Only a few pathogenic mutations have been detected in the mitochondrial transportation system, as most likely these mutations are lethal already at the embryonic state (Fenton 1995). Mohr-Tranebjaerg deafness-dystonia syndrome is an X-chromosome linked disease presenting with sensorineural hearing loss, dystonia, cortical blindness, and psychiatric symptoms. It is associated with mutations in the *TIMM8A* gene encoding deafness-dystonia protein (DDP1), an intermembrane component of the mitochondrial transport machinery (Roesch *et al.* 2002). Another clinical entity is autosomal dominant hereditary spastic paraplegia caused by a mutation in chaperonin heat-shock protein 60 (HSP60) (Hansen *et al.* 2002).

2.5.4 Inner mitochondrial membrane and lipid bilayer

IMM is an essential structure for normal mitochondrial function. OXPHOS chain complexes are embedded in IMM and the impermeability of IMM to H^+ is important for mitochondrial energy production and bioenergetics (Logan 2006, Navarro & Boveris 2007). IMM is an active structure that alters its conformation according to the metabolic state of the cell (Mannella 2006). Increasing evidence shows an association between the lipid structure of IMM and mitochondrial pathology (DiMauro et al 2013).

Cardiolipin is a characteristic phospholipid in the mitochondrial membranes, and especially, IMM is rich in cardiolipin (Walther & Rapaport 2009). Cardiolipin plays many roles in the organization and function of IMM (Schlame & Ren 2009), and it is the major phospholipid component of IMM (Valianpour *et al.* 2002). Reduced concentration of cardiolipin and an altered cardiolipin-composition of IMM is associated with Barth syndrome, X-chromosome linked mitochondrial myopathy, and cardiomyopathy with neutropenia, growth disorder, and 3-methylglutaconic aciduria (Vreken *et al.* 2000, Schlame *et al.* 2002). Clinical features of Sengers syndrome include congenital cataracts with myopathy and cardiomyopathy and lactic acidosis (Sengers *et al.* 1975). The molecular aetiology of Sengers syndrome has been tracked to the mutation of the

acylglyserol kinase gene (*AGK*), which takes part in the regulation of the phospholipid milieu of IMM (Mayr *et al.* 2012).

The mitochondria-associated endoplasmic reticulum membrane (MAM) facilitates the connection between mitochondria and endoplasmic reticulum (ER). MAM is involved in the regulation of the mitochondrial function and takes part in processing and import of mitochondrial lipids (Vance 2014). An example of MAM-associated disease is 3-methylglutaconic aciduria, with deafness, encephalopathy and Leigh-like syndrome (MEGDEL). This syndrome is associated with mutations in the *SERAC1* gene, which encodes a phospholipid transporter protein in MAM (Wortmann *et al.* 2012).

2.5.5 Mitochondrial network dynamics

Mitochondria form an active network that transforms its conformation and location by fusion and fission as a reaction to the metabolic conditions of the cell (Picard *et al.* 2011). Dynamin-related proteins (DRP) form the mitochondrial fusion and fission machinery and regulate the mitochondrial network dynamics through GTP-dependent assembly and GTP hydrolysis-mediated conformational changes and remodelling of the mitochondrial membranes. Mitofusins 1 and 2 (*MFN1* and *MFN2*) are important proteins in the fusion of OMM, and OPA1 takes part in the fusion of IMM (Lackner 2013).

Mutations in genes regulating the mitochondrial network dynamics are recognized as a cause of neurological disorders, e.g., *MFN2* mutations cause Charcot-Marie-Tooth neuropathy 2A (Zuchner *et al.* 2004). *OPA1* mutations can be found in autosomal dominant optic atrophy with multiple mtDNA deletions and defect in cytochrome *c* oxidase (COX) (Hudson *et al.* 2008). In addition, *MFN2* mutations are associated with multiorgan phenotype with myopathy - similar to optic atrophy caused by *OPA1* mutations - and multiple deletions and depletion of mtDNA (Rouzier *et al.* 2012, Vielhaber *et al.* 2013). These findings suggest that the mitochondrial network dynamics is important also in the maintenance of mtDNA.

2.6 Nuclear-encoded mitochondrial DNA maintenance apparatus in mitochondrial diseases: Intergenomic signalling in mitochondrial pathology

All the proteins responsible for mtDNA replication and maintenance are encoded by the nuclear genome. Therefore, mutations in these genes can compromise the mitochondrial replication machinery and lead to an instability of mtDNA, presenting either as qualitative (deletions and site-specific point mutations) or quantitative defects (depletion). These mtDNA rearrangements are secondary to nuclear gene mutations and are not themselves genetically transmitted, but follow Mendelian inheritance of the nuclear gene mutations (Spinazzola & Zeviani 2005, DiMauro *et al.* 2013). Typically, deletions caused by mtDNA maintenance gene mutations are autosomal dominant or recessive *multiple* deletions, in comparison to sporadic primary mtDNA deletions, which present as single deletions (DiMauro 2004). Initially, multiple mtDNA deletions and depletion were thought to be present with certain characteristic clinical phenotypes and associated with a specific nuclear mtDNA maintenance gene, but next-generation sequencing has shown that these two conditions may co-exist, and mutations in the same genes can lead to either multiple deletions or depletions (DiMauro *et al.* 2013).

POLG1 and *C10orf2* are the key genes of mtDNA replication, encoding mitochondrial DNA polymerase γ (pol γ) and mtDNA helicase (Twinkle), respectively (Hudson & Chinnery 2006). In addition, a group of nuclear-encoded proteins that are responsible for mitochondrial nucleotide transport, salvage, and synthesis are essential for mtDNA replication and maintenance (Alberio *et al.* 2007, Copeland 2008, Poulton *et al.* 2009a, Spinazzola *et al.* 2009, Suomalainen & Isohanni 2010). Moreover, there is increasing evidence of involvement of mitochondrial network dynamics and fusion of both OMM and IMM in mtDNA instability (Hudson et al 2008, Rouzier et al 2012, Vielhaber et al 2013). Nuclear genes associated with mtDNA maintenance and the typical molecular genetic and clinical features related to mutations in each gene are presented in Table 1.

Table 1. Nuclear genes related to mitochondrial DNA maintenance.

Gene	Protein	Function	Depletion	Multiple Deletions	Clinical Phenotypes
POLG1	pol γA	mtDNA synthesis	Yes	Yes	AHS ad/arPEO, MIRAS
POLG2	pol γB	mtDNA synthesis	No	Yes	adPEO
C10orf2	Twinkle helicase	mtDNA helicase	Rarely	Yes	adPEO Hepatoencephalopathy IOSCA
DNA2	DNA replication helicase/nuclease 2	RNA primer synthesis	No	Yes	Myopathy
DGUOK	Deoxyguanosine kinase	Nucleotide salvage pathway	Yes	No	Hepatoencephalopathy
TK2	Thymidine kinase 2	Nucleotide salvage pathway	Yes	No	Myopathy
SUCLA2	Succinyl-CoA synthase, β (ATP) subunit	dNTP pool regulation	Yes	No	Encephalomyopathy
SUCLG1	Succinyl-CoA synthase, α subunit	dNTP pool regulation	Yes	No	Encephalomyopathy
RRM2B	small R2 subunit of p53-inducible ribonucleoreductase	dNTP pool regulation	Yes	Yes	Encephalomyopathy adPEO
TP	Thymidine phopshorylase	Nucleotide salvage pathway	Yes	Yes	MNGIE
TFAM	Mitochondrial transcription factor A	mtDNA transcription and copynumber regulation	Yes	No	OXPHOS chain dysfunction, adPEO
ANT1	Adenine nucleotide translocator 1	ADP/ATP transporation	Yes	Yes	adPEO
OPA1	Dynamin-related GTPase	Mitochondrial network dynamics	No	Yes	adOPA
MFN2	Mitofusin 2	Mitochondrial network dynamics	Yes	Yes	Optic atrophy, myopathy, Charcot-Marie-Tooth
MPV17	Mpv17	Inner mitochondrial membrane protein	Yes	No	Hepatoencephalopathy, renal disease

mtDNA = mitochondrial DNA, AHS = Alpers-Huttenlocher syndrome, PEO = progressive external ophthalmoplegia, ad = autosomal dominant, ar = autosomal recessive, MIRAS = mitochondrial recessive ataxia syndrome, IOSCA = infantile-onset spinocerebellar ataxia, MNGIE = mitochondrial neurogastrointestinal encephalopathy, adOPA = autosomal dominant optic atrophy.

Mitochondrial DNA polymerase γ

DNA polymerase γ (pol γ) is the only DNA polymerase located in mammalian mitochondria thus far (Kaguni 2004). It is responsible for replication, recombination, and repair of mtDNA (Graziewicz *et al.* 2006). Structurally pol γ is a 195 kDa heterotrimer consisting of one 140 kDa catalytic subunit (pol γA) and two identical 55 kDa accessory subunits (pol γB) (Yakubovskaya *et al.* 2006). pol γA and pol γB are encoded by nuclear genes *POLG1* (on chromosome locus 15q25) and *POLG2* (on chromosome locus 17q23-24), respectively (Chan & Copeland 2009, Copeland 2008).

The catalytic subunit pol γA is formed by three functionally different domains; N-terminal *exo* domain, C-terminal *pol* domain and a highly conserved linker region situated in between the *exo* and *pol* domains (Ropp & Copeland 1996, Kaguni 2004). The *exo* domain has 3'-5' exonuclease activity and is responsible for proofreading of mtDNA, whereas *pol* domain with 5'-3' DNA polymerase activity is responsible for DNA synthesis (Graziewicz *et al.* 2006). In addition, pol γA has 5'-deoxyribose phosphate lyase activity, which serves in the base excision repair of mtDNA (Longley *et al.* 1998). The linker region mediates the focal contact with the dimeric pol γB accessory subunit, which enhances the binding of pol γ to a DNA strand and is important to the processivity of pol γ (Yakubovskaya *et al.* 2006).

Over 160 pathogenic *POLG1* mutations have been identified by this current date (Human DNA polymerase gamma Mutation Database, http://tools.niehs.nih.gov/polg/). Especially *POLG1* p.A467T and p.W748S mutations are known to be common in Finland and other Nordic countries (Hakonen *et al.* 2005, Winterthun *et al.* 2005, Kollberg *et al.* 2006). *POLG1* mutations may cause multiple mtDNA deletions and depletions (Wanrooij *et al.* 2007). The secondary mtDNA rearrangements may vary by the mutated pol γ domain. Mutations in the polymerase domain reduce the processivity of pol γ, thus leading to mtDNA depletion (Wong *et al.* 2008, Chan & Copeland 2009), but experimental data does show that polymerase domain mutations can compromise the initiation of mtDNA synthesis and lead to mtDNA deletions (Roos *et al.* 2013). Mutations in the exonuclease domain cause infidelity of mtDNA replication and proofreading errors, which can cause the accumulation of mtDNA point mutations and deletions (Del Bo *et al.* 2003). Mutations in the linker region compromise the association of pol γA and pol γB and are likely to decrease the polymerase activity of pol γ (Chan *et al.* 2005, Luoma *et al.* 2005). Therefore,

linker region mutations can lead to both mtDNA deletions and depletion by decreasing the processivity of pol γ (Cohen & Naviaux 2010). Linker domain mutations often present as compound heterozygous mutations with a polymerase domain mutation, which is a prominent cause of mtDNA depletion (Suomalainen & Isohanni 2010). Farnum *et al*. (2014) have proposed a model that divides pol γ into functional clusters according to protein and functional analysis and crystal structure. This model may aid to find correlations between *POLG1* mutations and clinical phenotypes and predict the pathogenity of novel *POLG1* mutations. Clustering model suggests that compound heterozygous mutations in multiple different functional regions of pol γ cause more severe clinical phenotype with earlier onset compared to mutations only in one cluster (Farnum *et al.* 2014).

The clinical phenotypes in *POLG1* mutations are extremely heterogeneous (Cohen & Naviaux 2010, Milone & Massie 2010). Due to the heterogeneity of *POLG1* mutations phenotypes, it is difficult to make clear genotype-phenotype correlations, but in general, early-onset *POLG1* diseases present with mutations in the polymerase and liker region, whereas late-onset diseases often have mutations in the exonuclease domain (DiMauro *et al.* 2006). Typically, *POLG1* mutations cause various neurological symptoms, including epilepsy, ataxia, neuropathy, and myopathy in association with the disorder of another organ system, e.g., cardiomyopathy or liver failure (Horvath *et al.* 2006, Blok *et al.* 2009). *POLG1* mutations are associated with various clinical syndromes, including autosomal dominant and recessive external ophthalmoplegia (Van Goethem *et al.* 2001, Lamantea *et al.* 2002, Agostino *et al.* 2003, Filosto *et al.* 2003), Alpers syndrome (Naviaux *et al.* 1999, Naviaux & Nguyen 2004), sensory ataxic neuropathy, dysarthria and ophthalmoparesis (SANDO) (Fadic *et al.* 1997, Van Goethem *et al.* 2003), also called mitochondrial recessive ataxia syndrome (MIRAS) (Hakonen *et al.* 2005, Winterthun *et al.* 2005). Moreover, *POLG1* mutations are linked to Parkinsonism, premature menopause and male subfertility (Luoma *et al.* 2004, Schapira 2006, Luoma *et al.* 2007). Dominant *POLG1* mutations often lead to late-onset myopathies and encephalopathies, such as adPEO. In addition, families with dominant *POLG1* mutations may present with psychiatric disorders, Parkinsonism, and hypogonadism. Recessive *POLG1* mutations typically cause early-onset disease and are an important cause of Alpers syndrome, an early-onset fatal hepatoencephalomyopathy with mtDNA depletion (Hudson & Chinnery 2006, Cohen & Naviaux 2010, Milone & Massie 2010). Interestingly, transgenic *POLG1* knock-out mouse models have shown that recessive *POLG1* mutations

can cause embryonic lethality and premature aging, which emphasises the importance of a functional pol γ in cell survival (Trifunovic *et al.* 2004).

Although the great majority of pol γ related pathology is caused by *POLG1* mutations, mutations in *POLG2* are also associated with autosomal dominant PEO. Mutations in *POLG2* may cause defective binding of the accessory subunit pol γB to the catalytic polymerase, thus leading to stalled mtDNA replication and an accumulation of mtDNA deletions (Longley *et al.* 2006).

Twinkle helicase

Initially, Twinkle helicase protein was found to have a similarity to phage protein T7 gp 4 helicase-primase, but was lacking the primase domain, suggesting this protein functions as mtDNA helicase (Spelbrink *et al.* 2001). Later studies have shown that Twinkle may also act as a mtDNA primase (Shutt & Gray 2006). It is a 77 kDa protein encoded by the *C10orf2* gene, also known as *PEO1* and *TWINKLE* (located on the chromosome locus 10q24) (Spelbrink *et al.* 2001). Twinkle is a 5'-3' helicase and unwinds the double-stranded mtDNA molecule, thus facilitating the formation of a replication fork (Korhonen *et al.* 2003). Being an important protein that facilitates mtDNA replication, Twinkle is essential in the regulation of the mtDNA copy number (Tyynismaa *et al.* 2004). *C10orf2* mutations are associated with the autosomal dominant PEO with multiple mtDNA deletions (Spellbrink *et al.* 2001). Infantile-onset spinocerebellar ataxia (IOSCA) is caused by *C10orf2* mutations and is most common in the Finnish population (Hakonen *et al.* 2007, Lönnqvist *et al.* 2009). Rarely, *C10orf2* mutations may cause an early-onset Alpers-like hepatocerebral syndrome with mtDNA depletion (Hakonen *et al.* 2007, Sarzi *et al.* 2007b).

DNA2

DNA replication helicase/nuclease 2 (DNA2) is a conserved protein localized in both the nucleus and the mitochondria (Zheng *et al.* 2008, Duxin *et al.* 2009). It is encoded by the *DNA2* gene localized on chromosome 10q21.3-q22.1. DNA2 has both helicase and nuclease activity, and the main function of DNA2 is to process RNA primer intermediates and Okazaki fragments during DNA replication (Duxin *et al.* 2012). In the mitochondria, it is co-localized with pol γ (Zhang *et al.* 2008) and Twinkle helicase (Duxin *et al.* 2009) and is essential in the processivity and fidelity of mtDNA replication and repair (Zheng *et al.* 2008, Duxin *et al.*

2009, Karanja *et al.* 2012). In addition, DNA2 is important in maintaining the integrity of telomerases and tumor suppression (Chai *et al.* 2013, Lin *et al.* 2013). Clinically, *DNA2* mutations are associated with myopathy and multiple mtDNA deletions (Ronchi *et al.* 2013).

2.6.1 Mitochondrial dNTP pool maintenance

DGUOK & TK2

As mtDNA is replicated independently of the cell cycle, the integrity of mtDNA depends on a constant supply of deoxyribonucleoside triphosphates (dNTPs) (Villarroya *et al.* 2011). Deoxyguanosine kinase (dGK) and thymidine kinase 2 (TK2) are proteins involved in the mitochondrial nucleotide salvage pathway (Copeland 2008), in which deoxynucleosides are phosphorylated step-wise to form deoxyribonucleoside dNTP (Arner & Eriksson 1995). Further, dGK and TK2 are the enzymes responsible for the first and rate-limiting step of the mitochondrial nucleotide salvage pathway (Jullig & Eriksson 2000).

The dGK is a 58 kDa dimer deoxyribonucleoside kinase, which phosphorylates purine deoxyribonucleosides deoxyguanosine, deoxyinosine and deoxyadenosine to nucleotide monophosphates (Arner & Erikson 1995). It is encoded by the *DGUOK* gene located on chromosome 2p13 (Johansson *et al.* 1996, Mandel *et al.* 2001). dGK is located in the mitochondrial matrix (Jullig & Eriksson 2000). TK2, a 29 kDa monomer protein, also located in the mitochondrial matrix, is responsible for the phosphorylation of deoxythymidine, deoxycytidine and deoxyuridine (Arner & Erikson 1995, Suomalainen & Isohanni 2010) and is encoded by the nuclear *TK2* gene located on chromosome 16q22 (Copeland 2008).

Primarily, *DGUOK* gene mutations have been associated with neonatal or childhood-onset hepatoencephalopathies with mtDNA depletion (Mandel *et al.* 2001, Dimmock *et al.* 2008), but they are also associated with adult-onset myopathy, presenting with multiple mtDNA deletions (Ronchi *et al.* 2012). *TK2* mutations are typically associated with myopathy and mtDNA depletion (Galbiati *et al.* 2006, Blakely *et al.* 2008, Gotz *et al.* 2008), but features of encephalopathy may also be present (Lesko *et al.* 2010). In addition, *TK2* mutations have been reported in PEO with multiple mtDNA deletions (Tyynismaa *et al.* 2012). Although both dGK and TK2 take part in the regulation of the nucleotide salvage

pathway, differences in tissue-specific expression could explain the difference in the clinical phenotypes related to mutations in these two genes (Alberio *et al.* 2007). In addition, deoxycytidine kinase (dCK) may compensate for the lack of dGK function in muscle, but it is not present in the liver – thus, the dysfunction of dGK leads to liver disease (Saada 2004).

SUCLA2 & SUCLG1

Succinyl-CoA synthase (SUCL) is an enzyme of the TCA cycle in the mitochondrial matrix, catalysing a synthesis of succinyl-CoA and ADP to form succinate and ATP/GTP. *SUCLA2* (on chromosome 13q12.2-q13.3) encode the β subunit of succinyl-CoA synthase, which is responsible for the formation of ATP, whereas *SUCLG2* encodes the GTP-forming β subunit. *SUGLG1* (chromosome locus 2p11.2) encodes the invariable α subunit, which forms a heterodimer with either of the β subunits (Copeland 2008, Suomalainen & Isohanni 2010). Dysfunction in SUCL has been associated with mtDNA instability and encephalomyopathy, but the actual pathomechanism is still unclear. Elevated levels of methylmalonic acid in urine or serum may refer to dysfunction in SUCL (Elpeleg *et al.* 2005, Carrozzo *et al.* 2007, Ostergaard *et al.* 2007a, Ostergaard *et al.* 2007b, Lamperti *et al.* 2003, El-Hattab & Scaglia 2013). It has been suggested that SUCL forms a complex with mitochondrial nucleoside diphosphonate kinase (NDPK), an enzyme responsible for phosphorylation dNDPs to dNTP in mitochondria. Malfunction in SUCL disrupts the kinase activity, thus leading to dNTP pool instability (Elpeleg *et al.* 2005, Ostergaard *et al.* 2007a, Ostergaard *et al.* 2007b).

RRMB2

RRM2B (located on chromosome 8q23.1) is a gene that encodes the small R2 subunit of p53-inducible ribonucleoreductase (p53R2). Ribonucleoreductase (RNR) is a 53 kDa heterotetrameric enzyme in the cytosol, which is formed by two homodimeric subunits R1 and R2 (Bourdon *et al.* 2007). RNR takes part in *de novo* synthesis of deoxyribonucleoside 5'-diphosphate from ribonucleoside 5'-diphosphate, which is essential for DNA replication and repair (Nordlund & Reichard 2006). RNR is a key regulator of the cytoplasmic dNTP pool during the S phase of the cell cycle (Bourdon *et al.* 2007), and the R2 subunit is likely to play an important role in the maintenance of mitochondrial dNTP for mtDNA

replication in post-mitotic cells (Pontarin *et al.* 2008). In addition to regulation by the cell cycle, a homologous p53R2 protein has been identified as related to tumour suppressor p53, which is essential for a basal level of DNA repair and mtDNA synthesis in non-proliferating cells (Tanaka *et al.* 2000). *RRM2B* mutations were initially reported in early-onset encephalomyopathy with mtDNA depletion (Bornstein *et al.* 2008, Acham-Roschitz *et al.* 2009, Kollberg *et al.* 2009); however, they are also associated with PEO with multiple mtDNA deletions (Tyynismaa *et al.* 2009, Fratter *et al.* 2011) and mitochondrial neurogastrointestinal encephalopathy (MNGIE) (Shaibani *et al.* 2009).

TP

Thymidine phosphorylase (TP) is a 47 kDA homodimer protein located in the cytosol. The function of TP is to phosphorylate thymidine in reaction which produces thymine and 2-deoxy-D-ribose (Brown & Bicknell 1998). TP is involved in mitochondrial nucleotide salvage pathway indirectly: thymine is translocated into mitochondria and processed further to form thymidine deoxycytidine, which is used to form deoxythymidine monophosphate (dTMP) (Copeland 2008, Hirano *et al.* 2005). TP is encoded by the *TP* gene (*TYMP*, *THYM, ECGF1*) located on chromosome 22q13.32 (Nishino *et al.* 1999). Mutations in the TP gene are commonly associated with MNGIE and mtDNA deletions or depletion (Hirano *et al.* 1994, Papadimitriou *et al.* 1998, Nishino *et al.* 2000).

2.6.2 Other genes related to mitochondrial DNA maintenance

TFAM

Human mitochondrial transcription factor A is encoded by the nuclear *TFAM* gene (on chromosome 10q21). The size of the protein is 25 kDa, and structurally, it consists of the N-terminal high-mobility-group (HMG) domain, a linker region followed by a second HMG domain and a carboxyterminal tail (Parisi & Clayton 1991, Dairaghi *et al.* 1995). TFAM is known to be essential for mtDNA transcription, as it promotes transcription by binding upstream of the L and H-strand promoter sites (Fernandez-Silva *et al.* 2003, Falkenberg *et al.* 2007, Mao & Holt 2009). Because replication of the leading strand is dependent on the RNA

primer produced from the L-strand promoter, TFAM has also been suggested as regulating mtDNA replication (Parisi *et al.* 1991, Dairaghi *et al.* 1995). This hypothesis is supported by experimental data from transgenic mouse models showing that TFAM is a key regulator of the mtDNA copy number, and dysfunction of TFAM leads to mtDNA depletion, OXPHOS chain dysfunction, and also possible disruption of embryogenesis (Larsson *et al.* 1998, Li *et al.* 2000, Ekstrand *et al.* 2004).

ANT1

Adenine nucleotide translocator 1 is a homodimer protein composed of two 30 kDa subunits encoded by the *ANT1* gene on chromosome locus 4q34-35 (Kaukonen *et al.* 2000, Copeland 2008). ANT1 is an isoform of adenine nucleotide translocator, which is predominantly expressed in post-mitotic tissues heart, skeletal muscle, and brain (Dolce *et al.* 2005). ANT1 is located in IMM and is the most abundant protein of IMM (Kaukonen *et al.* 2000, Copeland 2008). It mainly serves as an inner transmembrane protein, which transports ADP into MM and in exchange transports, ATP out of MM (Kaukonen *et al.* 2000, Copeland 2008). Previously, ANT1 has been described as a structural element of MPTP (Zoratti & Szabo 1995, Halestrap & Pasdois 2009) and was suggested to play an important role in the regulation of apoptosis (Marzo *et al.* 1998). ANT1 was thought to interact with VDACs located on OMM and form a permeable conduit through both mitochondrial membranes, thus regulating apoptosis (Leung & Halestrap 2008), but further studies have refuted the role of ANT1 and VDACs in formation of MPTP and apoptosis as described in Chapter 2.1.4. *ANT1* mutations are associated with adPEO with multiple deletions (Kaukonen *et al.* 2000, Deschauer *et al.* 2005). The protein content of mitochondrial ANT1 is reduced in Sengers syndrome with mtDNA depletion, although actual mutations in *ANT1* gene are not found Sengers syndrome. Instead, low ANT1 content in Sengers syndrome is probably secondary to mutations in *AGK* gene (Jordens *et al.* 2002, Mayr *et al.* 2012, Calvo *et al.* 2012).

OPA1

OPA1 gene (on chromosome 3q28-29) encodes a dynamin-related GTPase protein located on IMM (Delettre *et al.* 2000, Delettre *et al.* 2001). OPA1 protein takes part in mitochondrial network dynamics and is essential for mitochondrial fusion

(Cho *et al.* 2010). Dysfunction of OPA1 leads to disturbance of IMM, fragmentation of mitochondria, and apoptosis (Olichon *et al.* 2003, Olichon *et al.* 2007). Mutations in the *OPA1* gene are associated with autosomal dominant optic atrophy, multiple mtDNA deletions, and defect in COX, suggesting it also has a role in mtDNA maintenance (Hudson *et al.* 2008). It has been suggested that OPA1 interacts with mtDNA nucleoids, thus promoting mtDNA replication and regulating mtDNA copy number (Elachouri *et al.* 2011).

MFN2

The *MFN2* gene (located on chromosome 1p36.22) encodes a mitochondrial fusion protein mitofusin 2 (Lackner 2013). Mitofusin 2 is a large dynamin-like GTPase situated in OMM, and it promotes mitochondrial membrane fusion (Koshiba *et al.* 2004). In addition, mitofusin 2 is involved in mitochondrial energy metabolism and the coupling of Ψ_m to OXPHOS chain activity (Loiseau *et al.* 2007). *MFN2*, previously associated with Charcot-Marie-Tooth neuropathy 2A (Zuchner *et al.* 2004, Loiseau *et al.* 2007), is the latest addition to the mtDNA maintenance genes. *MFN2* mutations have been found in patients with myopathy and optic atrophy with multiple mtDNA deletions and depletion in muscle (Rouzier *et al.* 2012, Vielhaber *et al.* 2013). The exact mechanism, namely, how *MFN2* mutation leads to mtDNA instability, is still unclear. Deletions related to *MFN2* mutations may be secondary to increased mitochondrial damage, inhibition of mtDNA repair, failed clearance of damaged mitochondria, or alteration in the mitochondrial proteome (Rouzier *et al.* 2012). The inhibition of mtDNA replication as in *OPA1* mutations has been suggested as the cause of mtDNA depletion (Vielhaber *et al.* 2013).

MPV17

Mutations in the *MPV17* gene on chromosome locus 2p21-23 have been associated with the depletion of mtDNA, OXPHOS chain malfunction, and hepatocerebral syndrome (Spinazzola *et al.* 2006, Uusimaa *et al.* 2014). Human Mpv17 protein is an ortholog of mouse kidney disease protein Mpv17 (Spinazzola *et al.* 2006). Initially, Mpv17 was associated with peroxisomes and the metabolism of ROS (Zwacka *et al.* 1994, Binder *et al.* 1999, Iida *et al.* 2006). Later studies have shown it to localize on IMM; however, the exact function of the protein is still unclear (Spinazzola *et al.* 2006, Viscomi *et al.* 2009). Loss of

function in the *MPV17* knock-out mouse model leads to renal glomerulosclerosis and severe kidney dysfunction, age-dependent hearing loss (Meyer zum Gottesberge *et al.* 1996, O'Bryan *et al.* 2000, Meyer zum Gottesberge & Felix 2005) and a decrease in mtDNA copy number in the liver, skeletal muscle, brain, and kidney (Spinazzola *et al.* 2006).

2.6.3 Multiple mtDNA deletions

Multiple mtDNA deletions are typically present in late-onset diseases caused by mutations in mtDNA maintenance genes (DiMauro 2004). A typical clinical presentation related to multiple mtDNA deletions is adult-onset progressive external ophthalmoplegia (PEO) (Moraes *et al.* 1989, Zeviani *et al.* 1989, Zeviani *et al.* 1990, Servidei *et al.* 1991, Spelbrink *et al.* 2001, Van Goethem *et al.* 2001), which can be either autosomal dominantly or recessively inherited (adPEO and arPEO). Clinical features associated with multiple mtDNA deletions include e.g., exercise intolerance, myopathy, cardiomyopathy, diabetes, neuropathy, ataxia, deafness, and Parkinsonism (Kiechl *et al.* 2004, Luoma *et al.* 2004, Wong *et al.* 2008, Stumpf & Copeland 2011). Multiple mtDNA deletions are also associated with mitochondrial neurogastrointestinal encephalopathy (MNGIE), and mitochondrial recessive ataxia syndrome (MIRAS) (Spinazzola & Zeviani 2005). The increasing prevalence of mtDNA deletions in aging post-mitotic tissue (Piko & Taylor 1987, Zhang *et al.* 1992, Cortopassi *et al.* 1992, Corral-Debrinski *et al.* 1992, Brierley *et al.* 1998, Fayet *et al.* 2002, Pak *et al.* 2005, Bua *et al.* 2006) has to be considered when evaluating the role of deletions in the clinical phenotype.

adPEO and arPEO

The main clinical presentation of autosomal dominant, progressive external ophthalmoplegia (adPEO; OMIM #157460, #609283, #609286) and autosomal recessive PEO (arPEO; OMIM #258450) is very similar to CPEO: paralysis of the external eye muscles, which leads to bilateral ptosis and weak eye movements. In adPEO and arPEO, patients often have other clinical manifestations, e.g., neuropathy, ataxia, sensorineural hearing impairment, myopathy, exercise intolerance, or cardiomyopathy. Typically, the clinical presentation in the recessive form is worse. CPEO is associated with sporadic, single large-scale mtDNA deletions, whereas adPEO and arPEO present with multiple mtDNA deletions. adPEO and arPEO are hereditary: adPEO is related to

heterozygous mutations in the *C10orf2*, *POLG1*, *POLG2* and *ANT1* genes, but arPEO typically presents with compound heterozygous *POLG1* mutations.

MNGIE

Mitochondrial neurogastrointestinal encephalopathy (MNGIE; #OMIM 603041) is characterized by both neurological and gastrointestinal symptoms, including PEO, diffuse encephalopathy, neuropathy with gastrointestinal dysmotility, gastroparesis, and pseudo-obstruction often leading to cachexia. The onset of the disease is often at young adult age or middle age. MNGIE can present with multiple mtDNA deletions, mtDNA depletion, and point mutations. Because of the presence of mtDNA depletion, MNGIE is often recognized to be a type of mtDNA depletion syndrome (MDDS). MNGIE follows recessive inheritance and is associated with mutations in the *TP* gene, which encodes thymidine phosphorylase, a protein involved in the mitochondrial nucleotide salvage pathway.

Mitochondrial ataxia-neuropathy spectrum

Mitochondrial ataxia-neuropathy spectrum is a group of similar *POLG1*-related ataxia disorders that include mitochondrial recessive ataxia syndrome (MIRAS; #607459), sensory ataxic neuropathy dysarthria and ophthalmoparesis (SANDO) and mitochondrial spinocerebellar ataxia with epilepsy (MSCAE). In practise, these different acronyms refer to the same clinical syndrome. Characteristic features include sensory, motor or mixed-type ataxia, and dysarthria. Most patients also present with encephalopathy and epilepsy. Additional clinical features may include PEO, myoclonus, liver disease, and psychiatric symptoms. MIRAS can be divided in three subgroups based the clinical presentation and the age at onset: 1) childhood-onset hepatoencephalopathy; 2) adolescent-onset refractory epilepsy and migraine-like headache; and 3) adult-onset ataxia and neuropathy with additional features, e.g., psychiatric symptoms (Hakonen *et al.* 2010). MIRAS epilepsy can be life-threatening and requires active treatment. Patients with MIRAS are very vulnerable to sodium valproate-induced ALF requiring liver transplantation, which has to be taken in consideration when treating MIRAS epilepsy (Hakonen *et al.* 2010). MIRAS is common in the Nordic countries and is the most common hereditary adult-onset ataxia in Finland. MIRAS is typically associated with *POLG1* p.A467T and especially the

homozygous p.W748S mutation. Molecular genetic studies often disclose multiple mtDNA deletions and possibly mtDNA depletion.

2.6.4 mtDNA depletion syndromes

Mitochondrial DNA depletion syndromes (MDDS) are clinically and genetically a heterogeneous group of diseases characterized by severe reduction in mtDNA in the affected organ (Alberio *et al.* 2007, Rotig & Poulton 2009, Spinazzola *et al.* 2009, Suomalainen & Isohanni 2010). Based on the affected tissues and their mtDNA content, the clinical presentations of MDDS can be classified as hepatocerebral, encephalomyopathic and myopathic forms (Suomalainen & Isohanni 2010). MNGIE syndrome can present with both mtDNA deletions and depletion and is suggested to be a neurogastrointestinal form of MDDS (El-Hattab & Scaglia 2013). MDDS was initially described by Moraes *et al.* (Moraes *et al.* 1991) and since then, numerous pathogenic mutations have been found in nuclear genes encoding proteins vital to mtDNA maintenance (Poulton *et al.* 2009b, Spinazzola *et al.* 2009, El-Hattab & Scaglia 2013). The amount of mtDNA varies by age and tissue (Poulton *et al.* 1995c, Dimmock *et al.* 2010). In the liver, mtDNA content is doubled during the first year of life (Morten *et al.* 2007). Therefore, depletion analysis should be done in relation to age-matched controls. MtDNA copy number per cell affects the rate of translation and is thought to be the first step in the regulation of OXPHOS activity (Fernandez-Vizarra *et al.* 2011).

Approximately 50% of respiratory chain deficiencies in childhood are estimated to be caused by MDDS (Sarzi *et al.* 2007a). For diagnostic purposes, mtDNA depletion has been defined as mtDNA content lower than 30% relative to age-matched controls. In MDDS, the levels of mtDNA are often below 10-30% of age-matched controls and sometimes as low as 1-2%, especially in hepatocerebral forms of the disease (Rahman & Poulton 2009). However, measuring intracellular mtDNA content is technically challenging because of age- and tissue-related variation (Poulton *et al.* 1995c, Morten *et al.* 2007, Dimmock *et al.* 2010). Liver and muscle have been shown to be the most sensitive and thus reliable tissues in MDDS diagnostics (Dimmock *et al.* 2010).

Hepatoencephalopathic MDDS

Hepatoencephalopathy is thought to be the most common variant of MDDS (Alberio *et al.* 2007). Most often, the genetic aetiology is mutations in *POLG1*, *C10orf2*, *DGUOK,* or *MPV17*. Typically, the clinical features consist of neonatal or juvenile-onset hepatic dysfunction with severe neurological symptoms, including psychomotor delay, epilepsy, hypotonia, and stroke-like episodes with cerebral lesions in neuroimaging studies (Alberio *et al.* 2007, Spinazzola *et al.* 2009, Suomalainen & Isohanni 2010, El-Hattab & Scaglia 2013,).

Alpers syndrome (progressive infantile poliodystrophy) is a rare progressive encephalopathy of childhood and adolescence first described by Alpers (1931). The neurological symptoms are usually associated with liver dysfunction (Huttenlocher *et al.* 1976), and the combination of characteristic encephalopathy and liver disease is often referred to as Alpers-Huttenlocher syndrome (AHS). Patients with AHS are normal at birth and develop normally for the first week to first few years of their lives. The signs and symptoms of the disease emerge often secondary to a febrile disease at the age of 2 to 4 years, but the age of onset can vary from 3 months to 36 years (Simonati *et al.* 2003, Cohen & Naviaux 2010). The typical clinical signs include refractory seizures (*epilepsia partialis continua*), psychomothoric regression, and cortical blindness with loss of central visual functions (Harding *et al.* 1995, Simonati *et al.* 2003, Naviaux & Nguyen 2004, Davidzon *et al.* 2005, Nguyen *et al.* 2006). Some patients present with developmental delay, hypotonia, ataxia, hemiparesis, and neuropathy feeding difficulties, vomiting, and failure to thrive before the onset of epileptic seizures (Simonati *et al.* 2003, Gordon 2006, Lee & Sokol 2007, Cohen & Naviaux 2010). Liver dysfunction may emerge in parallel to neurological symptoms, or it may be induced by sodium valproate if this drug is admitted as a treatment for seizures (Milone & Massie 2010). The neuropathological findings consist of spongiform degeneration and neuronal loss accompanied by astrocytosis of the visual cortex. Liver histology typically discloses liver steatosis, fibrosis, and micronodular cirrhosis (Simonati *et al.* 2003, Naviaux & Nguyen 2004, Davidzon *et al.* 2005, Nguyen at al 2006). AHS is a progressive disease typically leading to death in childhood. Alpers syndrome is associated with recessive *POLG1* mutations and mitochondrial DNA depletion (Naviaux *et al.* 1999, Naviaux & Nguyen 2004). The most common *POLG1* gene mutations in Alpers syndrome are A467T and W748S as a homozygous or as compound heterozygous mutation (Tzoulis *et al.* 2006, Alberio *et al.* 2007, Uusimaa *et al.* 2008).

C10orf2 mutations most commonly lead to PEO with multiple mtDNA deletions, but rare cases of hepatoencephalopathic MDDS have been reported (Hakonen *et al.* 2007, Sarzi *et al.* 2007b). *DGUOK* mutations are primarily associated with isolated liver disease or neonatal-onset hepatic failure and encephalomyopathy with lactic acidosis and hypoglycaemia. These patients often present with aminoaciduria and proteinuria, suggesting renal involvement (Mandel *et al.* 2001, Dimmock *et al.* 2008, Suomalainen & Isohanni 2010, El-Hattab & Scaglia 2013). Mutations in the *MPV17* gene are associated with severe infantile-onset hepatoencephalopathy with renal involvement and mtDNA depletion, eventually leading to liver failure (Wong *et al.* 2007, Suomalainen & Isohanni 2010, El-Hattab & Scaglia 2013, Uusimaa *et al.* 2014). *MPV17* mutations are also recognized as the cause of Navajo neurohepatopathy (Karadimas *et al.* 2006).

Encephalomyopathic MDDS

Encephalomyopathic MDDS is most commonly associated with mutations in the *SUCLA2*, *SUCLG1* and *RRM2B* genes (Suomalainen & Isohanni 2010, El-Hattab & Scaglia 2013). Typically, patients with *SUCLA2* and *SUCLG1* mutations present with early-onset hypotonia, and that clinical presentation progresses with muscle atrophy and involvement of CNS (psychomotor regression, dystonia, movement disorders and epilepsy). Often lactic acidosis and elevated plasma and urine methylmalonic acid are present. Fiber variability, an increased number of mitochondria and fat accumulation can be seen in the muscle histology and combined deficiency of complex I, III, and IV with normal complex II activity being present (Elpeleg *et al.* 2005, Carrozzo *et al.* 2007, Ostergaard *et al.* 2007a, Ostergaard *et al.* 2007b, Lamperti *et al.* 2012, El-Hattab & Scaglia 2013). Typical clinical presentation in *RRM2B* mutations is a fatal infantile-onset multiorgan disease with psychomotor delay, microcephaly, hypotonia, lactic acidosis and renal disease with mtDNA depletion in the muscles (Bornstein *et al.* 2008, Acham-Roschitz *et al.* 2009, Kollberg *et al.* 2009). MNGIE, caused by mutations in the *TP* gene, is commonly associated with mtDNA deletions, but may also be related to mtDNA depletion. Therefore, it can be classified as encephalopathic or gastrointestinal form of MDDS (Suomalainen & Isohanni 2010, El-Hattab & Scaglia 2013). MNGIE is described in Chapter 2.4.6.

Myopathic MDDS

The majority of patients with myopathic MDDS harbour *TK2* mutations (Alberio *et al.* 2007, Spinazzola *et al.* 2009). The clinical phenotype can vary, but it is often composed of early-onset hypotonia, muscle fatigue, proximal muscle, and bulbar weakness leading to facial muscle weakness, dysphagia, feeding difficulty, and dysarthria (Galbiati *et al.* 2006, Blakely *et al.* 2008, Gotz *et al.* 2008, Suomalainen & Isohanni 2010, El-Hattab & Scaglia 2013). Cognitive dysfunction is not typically present, but encephalopathy can be a rare presentation in *TK2* mutations (Lesko 2010). Muscle histology discloses ragged red fibres, variation in muscle fibre size, sarcoplasmic vacuoles, and fibrosis, and OXPHOS activity measurements show a combined dysfunction of the complexes I, III, and IV. (Galbiati *et al.* 2006, Blakely *et al.* 2008, Gotz *et al.* 2008, Suomalainen & Isohanni 2010, El-Hattab & Scaglia 2013). Although the clinical onset in *TK2*-related diseases is before the age of 2 years, adult-onset myopathies have been associated with *TK2* mutations (Behin *et al.* 2012). The clinical course of early-onset myopathy progresses rapidly, and in the majority of cases leads to death due to respiratory failure or respiratory infection within a few years of onset (El-Hattab & Scaglia 2013).

2.7 Mitochondrial drug toxicity

2.7.1 General features of drug-induced liver injury

Over 1000 pharmacological agents are known to cause drug-induced liver injury (DILI) (Biour *et al.* 2004). Based on their clinical and histological features, DILI is categorized as hepatocellular, cholestatic, or mixed type injury (Fontana *et al.* 2010, Padda *et al.* 2011). Several risk factors for development of DILI are recognized, including age, gender, ethnic background, alcohol use, underlying liver disease, and polypharmacy (Fontana *et al.* 2010). Young age is an important risk factor for sodium valproate-induced idiosyncratic liver failure as a majority of these cases are present in childhood (Dreifuss *et al.* 1987, Bryant & Dreifuss 1996, Koenig *et al.* 2006, Schmid *et al.* 2013).

Six different mechanisms have been connected to DILI. A drug can interrupt intracellular Ca^{2+} homeostasis, which causes a disruption of actin fibrils, thus leading to loss of cell membrane integrity and cell lysis. If the disruption of actin filaments involves the canaliculi, then the structures responsible for bile excretion

in hepatocytes, transportation of bilirubin and other toxic cellular compounds is disturbed, causing cholestatic hepatic injury. The cytochrome P450 system is essential for detoxification of xenobiotics in the liver, but some drug molecules may form a non-functional adduct with cytochrome P450 enzymes through a covalent bond. These newly formed adducts migrate to the cell membrane and function as antigens to the T cell-mediated cytolytic immune reaction. Some drugs can trigger apoptotic cascade by activating TNF-α receptors. Toxic metabolites excreted to the bile may be harmful for the epithelium of the bile ducts. In addition to these mechanisms, mitochondria play a key role in the pathogenesis of DILI (Lee 2003, Grattagliano *et al.* 2009, Tujios & Fontana 2011).

2.7.2 Mitochondrial dysfunction in drug-induced hepatotoxicity

Mitochondrion is a key regulator of intracellular energy metabolism and homeostasis and is, therefore, an important target in drug-induced hepatotoxicity (Grattagliano *et al.* 2009). The mechanisms behind mitochondrial drug toxicity include uncoupling of the OXPHOS chain, direct inhibition of OXPHOS chain complexes, inhibition of mitochondrial fatty acid oxidation (FAO), damage and/or depletion of mtDNA, and MPTP opening (Begriche *et al.* 2011).

Drugs can cause dysfunction of the OXPHOS chain by uncoupling the Ψ_m from ATP production, by directly inhibiting the activity of OXPHOS chain complexes, or by uncoupling Ψ_m while inhibiting the OXPHOS chain complexes (Begriche *et al.* 2011). Drugs, which are protonized into cationic compounds in IMS, are driven into MM by Ψ_m. As a by-product, this process releases H^+ ions into MM independently of ATP synthase and, therefore, impairs ATP production (Berson *et al.* 1996, Berson *et al.* 2006). Direct inhibition of the OXPHOS chain complexes prevents electron transport and cellular respiration, which leads to a decrease in Ψ_m and compromises ATP production.

In all, ATP depletion caused by the OXPHOS chain uncoupling and dysfunction leads to a dysfunction of cell membrane proton pumps, accumulation of intracellular Ca^{2+} and activation of intracellular proteases, endonucleases, and phospholipases, which results in cytolysis and cell necrosis. Further, dysfunction of the OXPHOS system leads to increased ROS production and oxidative stress, which can cause damage to DNA and cellular structures. OXPHOS chain dysfunction increases the NADH/NAD ratio, which leads to decreased activity of

FAO as a secondary effect (Jaeschke *et al.* 2002, Grattagliano *et al.* 2009, Begriche *et al.* 2011).

Microvesicular steatosis is the key feature associated with impairment of the mitochondrial FAO (Fromenty & Pessayre 1995). Drugs can disrupt mitochondrial β-oxidation directly by inhibiting the enzymes of β-oxidation or indirectly by inhibiting the OXPHOS chain, which also decreases the activity of FAO, damaging mtDNA or sequestering the CoA pool (Fromenty & Pessayre 1995, Fromenty & Pessayre 1997). Inhibition of β-oxidation leads to intracellular accumulation of triglycerides or FFAs, which can then be seen as typical lipid vacuoles in liver histology. Accumulated FFAs have a toxic effect on mitochondria and further progress the mitochondrial dysfunction. Impaired FAO causes depletion of both acetyl-CoA and ATP, thus disrupting energy production of the cell. In addition, decrease in the acetyl-CoA pool leads to decreased gluconeogenesis and glucose production and decreased production of ketone bodies, which compromise the energy supply for the extrahepatic tissues (Fromenty & Pessayre 1995, Fromenty & Pessayre 1997, Begriche *et al.* 2011). All these factors lead to liver failure, encephalopathy, and severe hypoglycaemia characteristic of microvesicular hepatosteatosis (Begriche *et al.* 2011).

Certain drugs can inhibit the mitochondrial DNA pol γ, which leads to mtDNA depletion and secondary dysfunction of the OXPHOS chain and FAO as described in Chapter 2.6.5. Inhibition of the TCA cycle leads to lactic acidosis, which itself can induce mtDNA depletion. Drugs can also cause secondary DNA damage by inducing ROS production and oxidative stress. Especially, antiretroviral nucleotide/nucleoside reverse transcriptase inhibitors (NRTI) can directly inhibit pol γ and, therefore, impair the synthesis and repair of mtDNA (Grattagliano *et al.* 2009, Begriche *et al.* 2011).

Drug-induced MPTP opening causes cytolytic hepatitis. Drugs can cause MPTP opening by either directly interacting with MPTPs or indirectly inducing oxidative stress, which leads to oxidation of the thiol groups of MPTPs. Opening of MPTP disrupts the integrity of mitochondrial membranes and triggers either apoptotic or necrotic cascades, depending on the ratio of open MPTP. Membrane disruption also decreases Ψ_m, which leads to decreased activity of the ATP synthase (Jaeschke *et al.* 2002, Labbe *et al.* 2008, Grattagliano *et al.* 2009, Begriche *et al.* 2011).

2.7.3 Sodium valproate-induced hepatotoxicity

Sodium valproate (VPA) is an antiepileptic drug that can be used in various types of epileptic seizures (Perucca 2002). Structurally, VPA is a very simple, eight-carbon, branched fatty acid (Cotariu & Zaidman 1988). VPA is metabolized in the liver by various metabolic routes, including glucuronidation, oxidation by cytochrome P450, mitochondrial β-oxidation, and microvesicular ω-oxidation. Because of extensive biotransformation, 50 different metabolites of VPA have been identified (Silva et al 2008). VPA is metabolized to 4-ene-VPA by cytochrome P450 and then further metabolized to 2,4-diene-VPA by mitochondrial β-oxidation. The 2-ene-VPA is formed directly from VPA by mitochondrial β-oxidation (Silva *et al.* 2008, Surendradoss *et al.* 2012).

The therapeutic range of VPA is narrow 300-700 μmol/l (Callaghan *et al.* 1985, Silva et al 2008). VPA is a hepatotoxic drug with the potential to cause reversible or fatal liver failure (Cotariu & Zaidman 1988, Scheffner *et al.* 1988, Bryant & Dreifuss 1996, Koenig *et al.* 2006, Silva *et al.* 2008, Schmid *et al.* 2013). VPA-induced hepatic injury commonly occurs during the first 5-90 days of drug administration, but it can also cause delayed hepatic injury even after several months up to years (Bryant & Dreifuss 1996, Lee 2003, Koenig *et al.* 2006, Schmid *et al.* 2013). VPA is potentially hepatotoxic even in a therapeutic dosage (Scheffner *et al.* 1988, Bryant & Dreifuss 1996, Koenig *et al.* 2006, Schmid *et al.* 2013). Two types of hepatotoxicity are associated with VPA: a dose-dependent increase in liver enzymes and a decrease in fibrinogen, which can be reversed with full recovery by discontinuation of the drug, and idiosyncratic irreversible and fatal liver failure (Cotariu & Zaldman 1988, Koenig *et al.* 2006, Silva *et al.* 2008). Early intravenous administration of L-carnitine has been proposed to prevent the development of VPA-induced ALF and increase the survival of these patients (Ishikura *et al.* 1996, Bohan *et al.* 2001, Lheureux & Hantson 2009).

The exact pathomechanism of VPA-induced hepatotoxicity is not clear. Microvesicular steatosis and necrosis in liver histology are the characteristic features of irreversible VPA-induced liver failure (Koenig *et al.* 2006). Thus, mitochondrial dysfunction is highly likely a key factor in VPA-induced ALF as impaired mitochondrial β-oxidation leads to intracellular accumulation of triglycerides in the liver and lactic acidosis (Pessayre *et al.* 1999a, Lee 2003, Silva *et al.* 2008, Begriche *et al.* 2011). Specifically, 4-en-VPA and 2,4-en-VPA are associated with an inhibition of FAO and VPA-induced hepatotoxicity (Jaeschke *et al.* 2002, Ji *et al.* 2010, Begriche *et al.* 2011, Surendradoss *et al.*

2012). Reactive metabolites of VPA can prevent FAO by direct inhibition of β-oxidation enzymes or indirectly by depleting the intramitochondrial CoA-pool (Pessayre *et al.* 1999b, Begriche *et al.* 2011).

In addition, VPA enters mitochondria by passive diffusion, but it inhibits the function of CPT IA transporters in OMM. This action impairs carnitine-mediated transportation of long-chain fatty acids into mitochondria and provokes accumulation of intracellular triglycerides and FFAs (Fromenty & Pessayre 1997, Aires *et al.* 2010). Furthermore, VPA disrupts mitochondrial energy metabolism by inhibiting PDHG and OGDH and opening MPTPs, which disables mitochondrial respiration and ATP production, thus triggering apoptotic and necrotic cascades (Silva *et al.* 1997, Fromenty & Pessayre 1997, Pessayre *et al.* 1999b, Luis *et al.* 2007, Begriche *et al.* 2011). Dysfunctional mitochondrial respiration produces a secondary inhibitory signal to the mitochondrial FAO (Fromenty & Pessayre 1995, Begriche *et al.* 2011).

Patients with metabolic diseases, particularly those affecting mitochondrial metabolism, are at high risk of developing VPA-induced hepatotoxicity (Silva *et al.* 2008). Mutations in the *POLG1* gene can cause an impaired function of mitochondrial DNA polymerase leading to rearrangements of mitochondrial genome and mitochondrial dysfunction (Chan & Copeland 2009). Previous studies suggest that patients with mutations in the *POLG1* gene are at increased risk of valproate toxicity (Schwabe *et al.* 1997, Delarue *et al.* 2000, Simonati *et al.* 2003, Nguyen *et al.* 2006, Gordon 2006, Uusimaa *et al.* 2008, McFarland *et al.* 2009, Saneto *et al.* 2010). Stewart *et al.* (2010) showed that carrying *POLG1* gene mutations increases the risk of VPA-induced ALF by 23.6-fold. This is a problem especially in those patients suffering from Alpers-Huttenlocher syndrome; these patients typically suffer from intractable seizures and administration of VPA puts them at risk of ALF (Tzoulis *et al.* 2006, Engelsen *et al.* 2008, Uusimaa *et al.* 2013). Because of the increased risk of VPA-induced ALF associated with *POLG1* mutations, a routine *POLG1* gene analysis is proposed to all patients before the administration of VPA (Saneto *et al.* 2010).

3 Aims of the study

Depletion and deletions of mtDNA are important features in mitochondrial disorders and are related to disturbances in replication and maintenance of mtDNA. The clinical presentations and molecular aetiology of mtDNA depletion and deletion syndromes are highly heterogeneous. Patients with mitochondrial diseases, especially patients carrying mutations in *POLG1* gene, are in increased risk of sodium valproate-induced hepatotoxicity. Evidence on the prognosis of liver transplantation in patients with *POLG1* mutations is highly contradictory, and further studies are needed to identify those patients suitable for a liver transplantation. The exact pathomechanism of VPA-induced hepatotoxicity is not clear, but evidently mitochondrial dysfunction does play a central role. The specific aims of this study were:

1. To determine the molecular epidemiology and clinical phenotypes related to mtDNA depletion and deletions in paediatric patients with neuromuscular diseases (I);
2. To study the pathogenicity of homozygous *POLG1* p.R772H and compound heterozygous *POLG1* p.R722H+W748S mutation (II);
3. To investigate the prevalence of *POLG1* mutations in patients with sodium valproate-induced liver failure and study the survival of these patients after liver transplantation (III);
4. To examine the effect of sodium valproate on mitochondrial function in a HepG2 *in vitro* liver cell model (IV).

4 Subjects and methods

A detailed description of the patients and their case reports are presented in the original papers I-III, while methods used in this study are described in more detail in the original papers I-IV.

4.1 Patients (I-III)

The subjects in Studies I-II were paediatric and adult patients, who were examined for neuromuscular disorders at the Department of Paediatrics and Department of Neurology, Oulu University Hospital during the years 1992-2012. The clinical features of these patients strongly suggested mitochondrial disorder. Muscle biopsy samples from 104 paediatric (under 18 years) patients were analysed in Study I. In Study II, blood DNA samples from 751 paediatric and adult patients were screened for *POLG1* p.R722H mutation. Two families harbouring this mutation were identified and studied further. The molecular genetic data and the medical histories of the two families were also analysed.

Epilepsy patients with ALF after exposure to VPA were identified retrospectively from the register at the Department of Transplantation and Liver Surgery, Helsinki University Hospital, Finland, and included in Study III. The register included the data for all liver transplants in Finland since 1982. Five patients met the inclusion criteria of the study. Blood DNA were collected from these patients during regular follow-up visits at the transplant unit.

4.2 Clinical evaluation (I-III)

The evaluation of the clinical features of the patients included a full physical examination, radiographic imaging, electrophysiological examinations and histological studies on muscle or liver biopsies. The patients underwent brain computed tomography (CT) or magnetic resonance imaging (MRI), EEG, and ENMG. The laboratory investigations included blood lactate, pyruvate and CK, CSF lactate, and liver tests. Quantitative analyses of amino acids and quantitative analyses of organic acids in urine were done for the paediatric patients in Studies I-II. Muscle biopsies used in the molecular genetic studies (I-II) were taken as part of the diagnostic protocol. Muscle histology was studied with light and electron microscopy. OXPHOS chain enzyme activity in muscle histology specimens was also studied. All patients were screened for the common *POLG1*

mutations p.T251I, p.A467T, p.N468N, p.G517V, p.P587L, p.R722H, p.W748S and p.Y955C and the common MELAS m.3243A>G, MERRF m.8834A>G and NARP m.8993T>G mutations in mtDNA. The exons and intron-exon boundaries of the *POLG1*, *C10orf2* and *TK2* genes and the entire mtDNA coding region were sequenced, if the patient harboured mtDNA depletion or deletions. Additional molecular genetic studies were performed according to the clinical phenotype of each patient.

In Study III, clinical evaluation and follow-up were conducted at the transplant unit according to the routine procedure following liver transplantation. This procedure includes six follow-up visits during the first two years and then annually or biannually. Follow-up studies include liver function tests and control liver biopsies one, five and ten years after the transplantation. The histology of the explanted livers and control liver biopsies were studied in Department of Pathology at the University of Helsinki.

4.3 Controls (I-III)

The control muscle samples (I-II) were collected by Department of Paediatrics and Department of Surgery at Oulu University Hospital between 2008 and 2012. Altogether 14 paediatric (under 18 years) and 17 adult (18 years and older) controls were gathered. The samples were taken from patients with no signs of mitochondrial or other neurological disease during surgical treatments for orthopaedic conditions where mitochondrial function was not shown to play a major role. Muscle biopsies of 0.25-0.5 cm3 in diameter were performed during an operation that required an incision through muscle layers. The 403 control blood samples examined in Study II were obtained from anonymous blood donors recruited at Finnish Red Cross Blood Service offices in the provinces of Northern Ostrobothnia, Northern Savonia, and Kainuu. Control liver samples (III) were obtained at autopsies of subjects with normal liver histology and no signs of liver disease or liver failure. All muscle and liver controls (I-III) were matched with the patients by age.

4.4 Ethical considerations (I-IV)

The study protocol has been approved by the Ethics Committee of the Faculty of Medicine at the University of Oulu and the Ethics Committee of the Northern Ostrobothnia Hospital District (I-II, IV) and the Ethics Committee of the Helsinki

University Hospital, Helsinki, Finland (III). The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki, and written informed consent was given by each patient and control subject or their guardian prior to the study. The muscle control samples (I-II) were taken during surgical operations that required dissection of muscle layers and only, if unnecessary trauma could be avoided. The collection of muscle tissue samples did not interfere with the result of any operation or the recovery of the patient.

4.5 Molecular methods (I-III)

4.5.1 DNA extraction (I-III)

In Study I, total genomic DNA was extracted from muscle samples using standard phenol-chloroform-isoamyl alcohol extraction (PCIAA) and ethanol precipitation or a commercially available Wizard Genomic DNA Purification Kit (Promega, Madison, WI, U.S.A.) and from patient fibroblasts using QIAamp DNA Mini Kit (Qiagen, Hilden, Germany). Total genomic DNA was extracted from the muscle and buccal smear samples collected for Study II, using the standard sodium dodecyl sulphate-proteinase K method. A QIAamp Blood Kit (Qiagen) was used to extract total genomic DNA from peripheral blood leukocytes of patients and the controls according to manufacturer instructions (studies II-III). In Study III, total genomic DNA was extracted from formalin-fixed and paraffin-embedded liver samples using standard PCIAA extraction and ethanol precipitation.

4.5.2 Polymerase chain reaction (I-III)

For the analysis of *POLG1*, *POLG2*, *C10orf2*, *TK2* and *ANT1* genes, a blood DNA template was amplified by polymerase chain reaction (PCR). The polymerase enzyme used in the reaction was either Biotools DNA polymerase (Biotools B&M Labs, Madrid, Spain) or AmpliTaq Gold (Applied Biosystems, Foster City, CA, U.S.A.). Intronic primers were designed according to the reference sequences of the genes to amplify the coding exons and exon-intron boundaries. PCR reaction protocol included an initial denaturation of 1 minute and 45 seconds to 15 minutes, depending on the polymerase enzyme and 30 to 33 amplification cycles. PCR reactions were carried out using a set annealing temperature or the annealing temperature was lowered by 0.5°C per amplification

cycle from 70°C to 65°C, 66°C to 58°C or from 60°C to 52°C and then maintained at 65°C, 58°C or 52°C, respectively, for the reminder of the amplification cycles. After the PCR reaction, amplified DNA fragments were further analysed by direct DNA sequencing or restriction fragment analysis.

4.5.3 Direct DNA sequencing (I-III)

After PCR amplification, DNA fragments were purified with 6 U exonuclease I (Thermo Scientific, Waltham, MA, U.S.A.) and 0.5 U shrimp alkaline phosphatase (Thermo Scientific). The purified DNA fragments underwent cycle sequencing using a BigDye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems) and an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems). The primers used PCR amplification that was also used in cycle sequencing. Sequencing data was aligned with reference gene sequences by Sequencher 4.1.4 (Gene Codes Corporation, Ann Arbor, MI, USA).

4.5.4 Whole-exome sequencing (I)

In Study I, whole-exome sequencing (WES) was carried out in patients with MDDS (patients I:P1-P2) or mtDNA deletions (patients I:P4-P5). Total genomic DNA was extracted from skin fibroblasts of the patients. In patients I:P1-P2, WES was performed using the commercially available Agilent SureSelect in-solution target enrichment system (Agilent SureSelect Human All Exon V5, Agilent Technologies, Santa Clara, CA, U.S.A.) with mean sequencing coverage of 30x using the Illumina sequencing platform at the FIMM Technology Center, Helsinki, Finland (Sulonen *et al.* 2011). In patients I:P4-P5, WES was carried out using Agilent SureSelect Human All Exon Kit V1 (Agilent Technologies) and sequenced on the Illumina GAIIx sequencing platform at the McGill University, Montreal, Canada and Genome Quebec Innovation Center, Montreal, Canada.

4.5.5 Detection of POLG1 p.R722H mutation (II)

The *POLG1* p.R722H mutation was detected by a restriction fragment length polymorphism (RFLP) analysis. The blood DNA template was amplified by PCR with a mismatch primer, which creates a restriction site for MlsI (BalII) in the presence of the mutation. Amplified DNA fragments were digested with the restriction enzyme according to the manufacturer's instructions, and the digestion

products were electrophoresed on a 3% MetaPhor gel (Cambrex Bio Science Rockland, Inc., Rockland, ME, U.S.A.) stained with ethidium bromide. The wild-type allele yielded a 221 bp band and the mutant allele, a 183 bp band. All positive findings were verified by direct DNA sequencing.

4.5.6 Detection of POLG1 p.W748S mutation (II)

The *POLG1* p.W748S mutation was detected using allele-specific amplification. The blood DNA template was amplified by PCR reaction, using primers that contained a locked nucleic acid (LNA) nucleoside base at the 3'-end (Proligo LLC, Paris, France). The primers were designed to anneal with either the wild-type sequence or the sequence containing the mutation; a PCR product was visible only after successful annealing phase (Guo *et al.* 2009).

4.5.7 Quantification of mtDNA (I, III)

Quantitative real-time PCR (qRT-PCR) was used to determine the mtDNA content of the sample tissues. The mtDNA was amplified using PCR primers targeted at the mitochondrial NADH dehydrogenase 1 gene (*ND1*) (He *et al.* 2002). The values were normalized using the nuclear-encoded brain natriuretic peptide gene (*BNP*) as a single copy nuclear gene. Amplification products were detected by sequence specific 6FAM/TAMRA labelled fluorogenic probes (Sigma Genosys, Suffolk, U.K.). Each reaction volume of 25 µl contained 1 x iQ Supermix (Bio-Rad Laboratories Inc., Hercules, CA, U.S.A.), 0.3 µmol/l of forward primer, 0.3 µmol/l of reverse primer, 0.2 of µmol/l probe, and 13 ng of total genomic extract solution. Reaction conditions were 3 minutes at 95°C, followed by 42 cycles of 30 seconds of denaturation at 95°C, 30 seconds annealing at 63.5°C, including fluorescent signal recording, and a 1- minute extension at 72°C and a 1- minute final extension at 72°C.

Each analysis included one reaction with DNA extract replaced by H_20 (no-template control) and one reaction with DNA extracted from rho^0 cells (negative control containing no mtDNA). The control reactions were performed for the H_20 and rho^0 samples using both *ND1* and *BNP* primers separately. The 10-fold dilution series of a control DNA sample was used to plot a standard curve in every qRT-PCR assay separately. The PCR program was performed and amplification products were detected by an iCycler Thermal Cycler and an iQ5 Multicolor Real-Time PCR Detection System (Bio-Rad Laboratories Inc.,

Hercules, CA, U.S.A.). The mtDNA/nDNA ratio was calculated with the ΔCT method described by Pfaffl (2001). qRT-PCR reactions were performed as duplicates for each sample, and the analysis protocol was performed three times for each sample. For depletion analysis, the mtDNA content of the patient samples was compared to the median of age-matched controls. MtDNA depletion was defined as a mtDNA ratio <0.30 relative to the median of age-matched controls (Rahman & Poulton 2009).

4.5.8 Detection of mtDNA deletions (I-II)

Deletions of mitochondrial DNA were detected by XL-PCR of the mtDNA using Phusion DNA polymerase (New England Biolabs, Ipswich, MA, U.S.A.) in Study I or the Expand Long Template PCR System kit (Boehringer Mannheim, Mannheim, Germany) in Study II. XL-PCR was carried out with an L-strand primer, starting from the nucleotide 10 (L10), and an H-strand primer, starting from the nucleotide 16496 (H16496). The PCR products were electrophoresed with Generuler λ mix ladder and a 1 kb ladder (New England Biolabs) on a 0.7% agarose gel stained with a SYBR Safe DNA gel stain (Invitrogen, Eugene, OR, U.S.A.) or ethidium bromide.

Southern blot analysis in Study II was performed with a biotin-labelled probe. The mtDNA probe was amplified with a PCR reaction including biotin-16-dUTP (Roche, Mannheim, Germany). The biotin-labelled probe was amplified with primers targeted at between nucleotide (nt) positions 725 and 15714 to cover the D-loop area. Total genomic DNA extract was digested with FastDigest PvuII (Fermentas, Burlington, ON, Canada). The nylon membrane filter (Millipore, Billerica, ME, U.S.A.) was prehybridized, hybridized with the probe, washed, and incubated with Odyssey Blocking Buffer (LI-COR Biosciences, Lincoln, NE, U.S.A.) and Streptavidin-IRDye 800CW conjugate (LI-COR Biosciences). The blot was scanned using the Odyssey Infrared Imaging System (LI-COR Biosciences).

4.5.9 Bioinformatics (I-II)

In Study I, WES data was interpreted by Dr. Javad Nadaf, Dr. Somayyeh Fahiminiya and Prof. Jacek Majewski at the Department of Human Genetics, McGill University and Genome Quebec Innovation Center, Montreal, Canada.

To assess the pathogenicity of the *POLG1* p.R722H mutation in Study II, the secondary structure of pol γA was analysed with PredictProtein (Yachdav *et al.* 2014) and the Jpred3 server (Cole *et al.* 2008). The ClustalW software was used to create a sequence alignment of pol γA of *Homo sapiens* (human), *Pan troglodytes* (chimpanzee), *Canis lupus familiaris* (dog), *Mus musculus* (house mouse), *Rattus norvegicus* (rat), *Gallus gallus* (chicken), *Danio rerio* (zebrafish), and *Drosophila melanogaster* (fruit fly). Molecular modelling of the wild type pol γA and the protein with p.R722H mutation was performed with Modeller 8.2 software (Sali & Blundell 1993) by using the 3-D structure of the Klenow fragment of *Escherichia coli* DNA polymerase I (1kln.pdb) as a template. The results were further visualized by the MOLMOL program (Koradi *et al.* 1996).

4.6 Cell culture (I, IV)

4.6.1 Fibroblast cell culture conditions (I)

Fibroblasts were cultured for DNA extraction in Dulbecco's Modified Eagle's Medium DMEM (Sigma-Aldrich, St. Louis, MO, U.S.A.) including 5 mM D-glucose, sodium bicarbonate and pyridoxine and supplemented with 10% fetal bovine serum (FBS), 1 mM sodium pyruvate, 2 mM L-glutamine, 100 IU/ml penicillin, 100 µg/ml streptomycin. The cells were incubated in 5% CO_2 at 37°C.

4.6.2 HepG2 cell line and cell culture conditions (IV)

HepG2 cells (American Type Culture Collection, Rockville, MD, USA) were grown in 1x Eagle's minimum essential medium (EMEM) (Sigma Aldrich, St. Louis, MO, U.S.A.) supplemented with 5 mM D-glucose, 10% FBS, 1 mM sodium pyruvate, 2 mM L-glutamine, non-essential amino acids, 100 IU/ml penicillin and 100 µg/ml streptomycin. The cells were incubated in 5% CO_2 at 37°C. VPA treatments were carried out in a normal growth medium or in glucose-free medium, prepared with glucose-free 1x DMEM (Gibco, LifeTechnologies™, Thermo Fisher Scientific, Waltham, MA, U.S.A.) and supplemented with 25 mM D-galactose, 10% FBS, 2 mM L-glutamine, 1 mM sodium pyruvate, and antibiotics.

4.6.3 Course of the sodium valproate treatment (IV)

VPA was dissolved in molecular grade water and added to the treatment media. The cells were exposed to VPA at concentrations of 0.5 mM, 1.0 mM, and 2.0 mM. A 0 mM VPA control (a mock-control) was used as a reference. Cells were treated with VPA for 24, 48, and 72 hours. To study the effect of VPA on mitochondrial function under different metabolic conditions, HepG2 cells were treated with VPA in media supplemented with glucose or galactose as described in paper IV. All the treatments and measurements were carried out three times in both a glucose and galactose medium, except for Western blot assays, which were executed three times only for 72 hours in galactose medium at the VPA concentrations described above.

4.6.4 Oxygen consumption rate assays (IV)

Cellular oxygen consumption rates (OCR) were measured using a phosphorescent oxygen sensitive probe MitoXpress-Xtra-HS (Luxcel, Cork, Ireland). The cells were treated with VPA on a black 96-well plate with a clear bottom (NalceNunc, Roskilde, Denmark). After the VPA treatment, a MitoXpress probe was added on the cells according to the manufacturer's instructions in phenol-free DMEM (Gibco) supplemented with D-glucose or D-galactose, 10% FBS, 1 mM sodium pyruvate, 2 mM L-glutamine, and antibiotics according to the treatment media. The change in the probe fluorescence was detected on a FLUOstar Omega plate reader (BMG Labtech, Ortenberg, Germany) with optimal filter wavelengths of 340 nm for excitation and 615 nm for emission. The measurements were done using time-resolved fluorescence (TR-F) with a dual delay time of 30 μs and 70 μs and converted to phosphorescence lifetime values, then used to calculate the OCR. The results were expressed as relative fluorescence units (RFU)/min/100,000 cells.

4.6.5 Mitochondrial membrane potential (IV)

After the VPA treatment cells were harvested with trypsin, they were re-suspended to glucose media or galactose media, depending on the treatment media, in a concentration of 30 x 10^5/ml. The cells were incubated with MitoTracker CMXRos (Invitrogen, LifeTechnologies™, Thermo Fisher Scientific) in a final concentration of 100 nM for 30 min at +37°C in 5% CO_2

protected from light. Fluorescence was measured on the flow cytometer FACSCalibur (BD Biosciences, Franklin Lakes, NJ, USA) on channel FL2 (excitation 488 nm/emission 585 nm). A minimum of 10, 000 ungated events were detected in each sample and the data were analysed using CellQuest Pro software (BD Biosciences). The measurements were standardized by comparing the mean fluorescence of each VPA-treated sample to the mean fluorescence of the mock-control sample and calculated as a mean sample/control fluorescence intensity (FI) ratio.

4.6.6 ATP determination (IV)

ATP was extracted from VPA-treated cells by adding 150 µl of ice-cold lysis buffer containing 2 mM potassium phosphate buffer (pH 7.8), 2 mM EDTA, 0.1 mM dithiotreitol and 0.1% Triton X-100 directly to the cells. ATP levels were determined using an ATP Determination Kit (Invitrogen) according to manufacturer instructions. A standard curve was generated with solutions of known ATP concentrations. ATP levels were normalised to a total protein amount determined by using a Pierce BCA kit (Thermo Fisher Scientific). Each VPA-treated sample was compared to a 0 mM VPA sample (mock-control).

4.6.7 Mitochondrial reactive oxygen species (IV)

Mitochondrial ROS were detected using a MitoSOX Red fluorescent probe (Invitrogen). The protocol to harvest and re-suspend the cells was identical to MitoTracker Red CMXRos staining protocol. MitoSOX Red was added to the cell suspension at a final concentration of 5 µM and incubated at +37°C in 5% CO_2 protected from light. Fluorescence of 10,000 ungated events was detected by the flow cytometer FACSCalibur (BD Biosciences) on channel FL2 (Ex 488 nm/Em 585 nm). Data were analysed using CellQuest Pro software (BD Biosciences).

4.6.8 Immunoblotting (IV)

For Western blot analysis, the cells were treated with VPA only for 72 hours in galactose medium (VPA concentrations as described above). VPA-treated cells were lysed with a rubber policeman in ice-cold a radioimmunoprecipitation assay (RIPA) buffer containing 150 mM NaCl, 50 mM Tris buffer (pH 8.0), 0.5% sodium deoxycholate, 0.1% SDS and 1% Triton X-100. 20 µg of protein per

sample was used for Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) and transferred to a nitrocellulose membrane. pol γA, E3 subunit (dihydrolipoyl dehydrogenase, DLD) of pyruvate dehydrogenase (PDC) and 2-oxoglutarate dehydrogenase (OGDC) enzyme complexes, OXPHOS chain complexes I, II, and IV, ATP synthase, mitochondrial superoxide dismutase (SOD2) and glutathione peroxidase (GPX) were detected by specific antibodies. The antibodies were products of Abcam (Cambridge, UK) excep for DLD E3 (Pierce Antibodies, Thermo Fisher Scientific) and 39 kDa (Molecular Probes, LifeTechnologies™, Thermo Fisher Scientific).

4.6.9 MTT assay, cell death, and cell number (IV)

MTT, cell death and cell number assays were done in parallel with OCR assays on three clear 96-well plates (NalceNunc). One plate was used for the MTT assay (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide), which reflects the activity of NAD(P)H-dependent oxidoreductase enzymes and is frequently used to measure cell viability (Berridge & Tan 1993, Hamid *et al.* 2004). The second plate was used to determine cell death using propidium iodine (PI), and the last plate was stained with Hoechst 33342 to examine the cell number. The measurements were done on a FLUOstar Omega plate reader (BMG Labtech). MTT (Sigma Aldrich) was added to the wells at a concentration of 5mg/ml and incubated at +37°C in 5% CO2 for 1 hour. PI (Sigma Aldrich) was diluted in molecular grade water and added to the wells at a final concentration of 20µM and incubated for 30 minutes at +37°C in 5% CO_2. To determine 100% cell death, the cells were washed with PBS and exposed to ice-cold ethanol. PI fluorescence was measured with an excitation wavelength of 540 nm and an emission wavelength of 620nm. 0.1 µg/mL Hoechst 33342 (Invitrogen) was incubated on the cells for 20 minutes at +37°C in 5% CO_2 and measured at wavelengths of 355 nm (excitation) and 460 nm (emission).

4.6.10 Statistical methods (IV)

Statistical differences between the 0 mM VPA control and the VPA treatment groups were studied with a one-way analysis of variance (ANOVA) and Bonferroni correction *post hoc* test. Statistical analysis was performed using IBM SPSS Statistics 20.0 software. Differences were considered as statistically

significant with a p-value 0.01–0.05, very significant with a p-value 0.001–0.01, and extremely significant with a p-value <0.001.

5 Results

5.1 MtDNA depletion in paediatric patients (I)

Out of 104 patients with undefined encephalomyopathy or myopathy, 3 were identified to harbour mtDNA depletion (mtDNA content <0.30 compared to age-matched controls) (I: Fig. 1B). Two patients (I:P1-P2) with mtDNA depletion presented with a novel newborn-onset multisystem disorder. Patient I:P1 manifested with progressive encephalomyopathy with elevated blood lactate, pyruvate, and creatine kinase, generalized aminoaciduria and brain MRI-disclosed necrosis of the white matter and lesions in the thalamus and basal ganglia. Activity of OXPHOS chain complexes was normal. The clinical phenotype for patient I:P2 included encephalopathy, optic atrophy, pigmentary retinopathy, and sensorineural hearing impairment, together with elevated CK. Common findings in the muscle histology specimens of the patients with mtDNA depletion (I:P1-3) were atrophy, fibrosis, and fat excess (I: Fig. 2). Muscle histology of patient I:P2 showed disordered structure of myofibres, pathologic structure of ER membranes and accumulation of glycogen in addition to muscle fibrosis (I: Fig. 2B).

Direct sequencing of exons and intron-exon boundaries of the *POLG1*, *C10orf2,* and *TK2* genes and the entire coding region of mtDNA did not show any mutations in patients I:P1-P2. WES of patients I:P1-P2 did not show any mutations in the known mtDNA maintenance genes (see Chapter 2.6, Table 1). The clinical phenotype of one patient (I:P3) with mtDNA depletion was confirmed to be merosine-deficient muscular dystrophy (MDC1A) due to homozygous G1591X mutation in thelaminin subunit alpha 2 (*LAMA2*) gene.

The prevalence of mtDNA depletion was 2.9% in a cohort of patients with neuromuscular disorders. Primary mtDNA depletion syndrome was identified in two patients, suggesting a prevalence of 1.9% in this cohort. The clinical and genetic features are summarized in I: Table 1.

5.2 Large-scale mtDNA deletions and over-replication of mitochondrial DNA (I)

Patients I:P4 and I:P5 both presented with early-onset ophthalmoplegia, sensorineural hearing loss, progressive tremor, muscle hypotonia, migraine,

dysarthria, and ataxia. I:P5 presented with additional clinical features, including pigmentary retinopathy and cardiac conduction block, transient anaemia, and granulocytopaenia. The muscle histology of both patients showed COX-negative fibres and RRF (I: Fig. 3C). Further, the muscle histology of patient I:P5 revealed mitochondrial vacuoles and inclusion bodies, together with pathological mitochondrial structure and abnormal mitochondrial cristae (I: Fig. 2D). The OXPHOS chain activity measurements for patient I:P5 showed decrease activity of complex I and I+III. XL-PCR analysis of muscle DNA showed both patients to harbour large-scale 5 kb deletion and minor multiple deletions (I: Fig. 3). In addition, a quantitative analysis showed a 4.9 and 6.9-fold increase (I:P4 and I:P5, respectively) in muscle mtDNA content relative to the median of age-matched controls, suggesting over-replication of mtDNA. The clinical and genetic features of patients I:P4 and I:P5 are presented in I: Table 2.

5.3 *POLG1* p.R722H and compound heterozygous *POLG1* p.[R722H];[W748S] (II)

5.3.1 Clinical and molecular genetic findings

Homozygous *POLG1* p.R722H mutation was identified in a male patient (II:A1) presenting with external ophthalmoplegia, bilateral ptosis, sensorineural hearing impairment, dysphagia, and type 2 diabetes mellitus. The first clinical sign was hearing loss at age 72 years. A mixed-type dementia was discovered upon neuropsychological examinations at age 77 years. Blood lactate, pyruvate, and CK were normal as well as the EEG. Brain MRI showed cortical and central atrophy (II: Fig. 1). A reduction in glucose uptake in the frontal, frontotemporal, and frontoparietal regions was seen in positron emission tomography (PET) performed with ^{18}F-deoxyglucose (FDG-PET). Examination of muscle histology revealed fibrosis, COX-negative fibres, and RRFs (II: Table 1). XL-PCR and Southern blot analyses showed multiple mtDNA deletions (II: Fig. 3 and 4). Homozygous *POLG1* p.R722H mutations were also discovered in two siblings of patient II:A1 (patients II:A2 and II:A3) presenting with progressive external ophthalmoplegia, ptosis, sensorineural hearing impairment, and dementia. Muscle DNA was not available from patients II:A2 and II:A3. No mutations were found in an analysis of *POLG2*, *C10orf2,* and *ANT1*. Heterozygous *POLG1* p.R722H mutation was found in 10 out of 19 relatives of patients II:A1-3 (II: Fig. 2). The

clinical features of patients II:A1-3 and their 19 relatives are summarized in II: Table 2.

Patients II:B1 and II:B2 are daughters of non-consanguineous parents. Both were born after normal full-term pregnancy and delivery, and their early psychomotoric development was uneventful. They were diagnosed with mental retardation and learning difficulties after attending school (II:B1) or just prior to school age (II:B2). Patient II:B1 has suffered from epileptic seizures since age 11 years, and her EEG showed frontotemporal irritation with secondary generalization. Both II:B1 and II:B2 presented with juvenile-onset bilateral ptosis and neuropsychiatric symptoms, including depression, aggressiveness and suicidality. Patient II:B1 has suffered few psychotic episodes requiring hospitalization. Their mother presented with atrial valve insufficiency, atrial fibrillation in addition to a cognitive impairment, and hearing problems, but no localizing neurological symptoms. *POLG1* gene analysis of II:B1 and II:B2 disclosed heterozygous p.[R722H];[W748S] mutation (II: Fig. 5 and 6), while II:B1 also harboured heterozygous p.E1143G mutation. DNA of their father was not available for analysis, but their mother presented with heterozygous *POLG1* p.W748S mutation, but not with p.R722H (II: Fig. 5 and 6), thus confirming that p.[R722H];[W748S] in patients II:B1 and II:B2 was a compound heterozygous mutation.

5.3.2 Molecular modelling of POLG1 p.R722H and p.W748S

Sequence alignment analysis showed p.R722 and p.W748 locates in an evolutionally conserved domain (II: Fig. 7). p.R722 is not very well conserved amino residue, whereas p.W748 is highly conserved. PredictProtein and JPred3 softwares did not predict *POLG1* p.R722H mutation to cause changes in the secondary structure of pol γA. The tertiary structure was assessed based on the diffractometric data of *Escherichia coli* pol I Klenow fragment and bacteriophage T7. Alignment of human pol γA with *E. coli* Klenow fragment of pol I reveals a loosely homologous region (nt 701-724) of pol γA. The corresponding region of pol I around amino residue p.R631 is situated in a crevice, which is in close interaction with the DNA strand in the co-crystallized 1kln.pdb. This region was used as template for the homology modelling of pol γA. The p.R722H mutation was localized in a helical region, and the p.W748S in a more flexible coil region without any major effect on the secondary or tertiary structure (II: Fig. 8).

5.3.3 Carrier frequency of POLG1 p.R722H

Out of 403 anonymous blood donors, 3 harboured the heterozygous c.2447G>A (p.R722H). This finding suggests a carrier frequency of 1:135 in the population.

5.4 *POLG1* mutations in VPA-induced hepatotoxicity (III)

5.4.1 Clinical course of VPA-induced acute liver failure and survival after liver transplantation

Altogether, six patients with acute VPA-induced ALF were retrospectively identified from the register of 880 patients at the Department of Transplantation and Liver Surgery, Helsinki University Hospital, Finland (III: Table 1-2). Four patients (III:P1-P4) were treated with liver transplantation (LT). Patient III:P5 died prior to LT. One patient recovered after treatment with molecular absorbent recirculating system (MARS) and was not included in the study because of unavailability of the clinical and sample data. All patients presented with focal epilepsy with recurrent, intractable seizures. The age at onset of their first neurological symptoms ranged from 13 to 32 years, and ALF was apparent at age 14 to 36 years. The duration of VPA treatment prior to ALF ranged from 6 to 12 weeks (mean = 8.2 weeks). Histolopathological examinations of the explanted livers disclosed hepatocellular necrosis, collapse of the liver parenchyma, and ductular reaction; however, signs of chronic liver disease were not observed (III: Table 2, III: Fig 1A-C). The histopathological findings of the transplanted livers were normal (III:P1); the inflammatory cells in the portal area and mild accumulation of iron (III:P2) and diffuse steatosis, portal area fibrosis and immunoactivation had good response to anti-rejection medication (III:P4).

Based on the neurological symptoms, liver failure and elevated levels of copper in the blood or urine for three patients, they were suspected to have Wilson's disease, but Kayser-Fleischer rings were not present, and copper analyses of the explanted livers were normal. Patient III:P3 was exposed to VPA and paracetamol prior to LT. Patients III:P1, III:P2 and III:P4 have survived 4 to 19 years after LT. Two patients still suffer from occasional epileptic seizures, but one patient has been seizure-free for 11 years after the LT. Patient III:P3 developed a renal failure with cyclosporine intoxication 1.5 years after the LT and died suddenly 6 months later. However, the exact cause of death remained

unclear, as an autopsy was not performed. Patient III:P5 died at the age of 20 years due to ALF and hepatic coma before receiving a transplant.

5.4.2 Direct sequencing of POLG1

Direct sequencing of the *POLG1* gene showed that all five retrospectively identified patients with VPA-induced ALF harboured *POLG1* mutations (III: Table 2). Patients III:P1, III:P2, III:P4, and III:P5 presented with homozygous *POLG1* p.W748S and p.E1143G mutation. Patient III:P4 also carried heterozygous *POLG1* p.Q497H mutations. Patient III:P3 harboured heterozygous *POLG1* p.Q1236H, but did not present with other *POLG1* mutations.

5.4.3 Quantitative analysis of mitochondrial DNA

In quantitative analysis of the explanted livers, mtDNA depletion (as defined in Chapter 4.5.6) was found in patients III:P1, III:P2, III:P4 and III:P5 (III: Fig. 2). Patient III: P3 presented with normal liver mtDNA content (III: Fig. 2).

5.5 Effects of sodium valproate on the mitochondrial function in the HepG2 *in vitro* liver model (IV)

5.5.1 Oxygen consumption rates

OCR decreased proportionally to VPA concentration in HepG2 cells incubated in both glucose and galactose mediums. In the glucose medium, VPA caused significant decrease in OCR at 24 and 48 hours (IV: Fig 1A-B). At 72 hours, significant effect was not observed in the glucose medium (IV: Fig 1C). In the galactose medium, VPA treatment led to decreased OCR already at lower concentration (0.5 mM) and that effect was detected throughout the whole experiment all the way to 72 hours (IV: Fig. 1A-C).

5.5.2 Mitochondrial membrane potential

Decreased MitoTracker Red CMXRos fluorescence, suggesting decreased Ψ_m, was detected after 24-hour VPA treatment in HepG2 cells incubated in the glucose medium (IV: Fig. 2A). Despite the similar OCR curve in the glucose medium, no

significant differences were seen at 48 and 72 hours (IV: Fig. 2C). VPA treatment carried out in the galactose medium caused a decrease in Ψ_m after 24 and 48 hours, but no statistically significant differences were observed at 72 hours (IV: Fig. 2A-C).

5.5.3 ATP

In the galactose medium, VPA led to a dose-dependent decrease in basal ATP levels throughout the whole experiment set until 72 hours (IV: Fig. 3). VPA did not decrease ATP levels in the HepG2 cells incubated in the glucose medium (IV: Fig. 3).

5.5.4 Mitochondrial reactive oxygen species

After VPA treatment for 24 and 48 hours, MitoSOX Red staining did not show any changes in mitochondrial ROS (IV: Fig. 4A-B). After 72 hours, 2.0 mM VPA concentration caused a significant increase in ROS levels (IV: Fig. 4C).

5.5.5 Immunoblotting

Prior to immunoblotting, HepG2 cells were treated with VPA in the galactose medium for 72 hours. Immunoblotting did not show changes in protein expression of pol γA, DLD E3, OXPHOS complexes I, II and IV, nor ATP synthase. In a protein analysis of ROS scavengers, levels of GPX were not changed, whereas VPA decreased the amount of SOD2 (IV: Fig. 5A). Quantification of SOD2 showed a statistically significant decrease in the protein levels at 1.0 mM and 2.0 mM VPA concentrations (IV: Fig. 5B).

5.5.6 MTT assay, cell death, and cell number

VPA increased the MTT signal in HepG2 cells in the glucose medium after 48 and 72 hours and after 72 hours in the galactose medium (IV: Fig. 6). Increased cell death was detected with PI staining after 72-hour VPA treatment in both media (IV: Fig. 7). Hoechst 33342 staining showed a decrease in cell numbers after treating the cells with VPA for 72 hours (IV: Fig. 8).

6 Discussion

6.1 Mitochondrial DNA depletion and deletions in paediatric patients with neuromuscular diseases

6.1.1 Prevalence and onset of mtDNA depletion syndromes

Of the 104 paediatric patients with undefined encephalomyopathy or myopathy, three were identified with mtDNA depletion (I: Fig. 1B, Table 1). As one of these patients was diagnosed with merosine-deficient muscular dystrophy (MDC1A), two patients (I:P1 and I:P2) were confirmed to have MDDS. Therefore, the estimated prevalence of MDDS in this pre-selected group of patients (age under 18 years) was 1.9%. In a previous study (Macmillan & Shoubridge 1996) muscle biopsy was performed on 304 paediatric patients examined for neurological symptoms; however, only 54 patients meeting specific criteria (age under 2 years with muscle weakness, hypotonia, or developmental delay) were included for further studies. Six patients (9.0%) presented with mtDNA depletion. MtDNA depletion was found in 50% of the patients with OXPHOS chain deficiency with onset of the disease before 3 years of age (Sarzi *et al.* 2007a). Patients I:P1 and I:P2 both had onset of the disease as newborns. Using the same age criterion as Macmillan & Shoubridge (1996) and Sarzi *et al.* (2007a), in Study I, two out of 36 patients (5.6%) presented with mtDNA depletion. Thus, mtDNA depletion may be a prominent cause of neuromuscular diseases in paediatric patients, but specifically in very early-onset diseases. Still, as the genetic origin of the MDDS in our patients is yet to be confirmed, the prevalence of MDDS can be considered to be only an estimation. Although mtDNA depletion is often defined as mtDNA content <0.30 relative to age-matched controls (Rahman & Poulton 2009), patients with mtDNA ratio just above 0.30 may present with features of mitochondrial disorder. Therefore, the patients with mtDNA ratio just above 0.30 in Study I will be examined further.

Methodologically, a major drawback of Study I was the lack of healthy control subjects under 5 years. The selection process for the controls most definitely influenced the availability of the samples. As it is not ethically justifiable to collect muscle biopsies from otherwise healthy children, the control samples had to be randomly collected from patients who underwent surgical operation, and thus, it was not possible to systematically collect samples for

different age groups. Muscle mtDNA content increases in the perinatal period and after that more slowly (Poulton *et al.* 1995c, Dimmock *et al.* 2010). Therefore, the mtDNA ratio of patients under 5 years was determined relative to the median of the 5-year-old controls to make the best estimation of mitochondrial DNA depletion.

6.1.2 Clinical features of patients with mtDNA depletion syndrome, involvement of endoplasmic reticulum, and implications for further genetic studies

Patients I:P1 and I:P2 presented with clinical features that are associated with a wide variety of mutations in mtDNA maintenance genes (Alberio *et al.* 2007, Spinazzola *et al.* 2009, Suomalainen & Isohanni 2010, El-Hattabi & Scaglia 2013). Mutations in *POLG1* are related to mtDNA depletion and encephalopathies with very heterogeneous clinical presentations, including all the clinical features of I:P1 and I:P2 (Taanman *et al.* 2009, Cohen & Naviaux 2010, Sofou *et al.* 2012). In addition, mutations in *C10orf2* are relatively common in the Finnish population (Hakonen *et al.* 2007, Lönnqvist *et al.* 2009). Therefore, patients in Study I were investigated for mutations in *POLG1* and *C10orf2*; however, no mutations in these genes were found. Both patients presented with increased levels of blood CK, suggesting mutations in the *TK2* gene (Lesko *et al.* 2010), yet an analysis of *TK2* disclosed no mutations. Sequencing of mtDNA excluded pathogenic mtDNA point mutations.

Patient I:P1 presented with severe metabolic encephalopathy with increased blood lactate and pyruvate and increased cerebrospinal fluid lactate, failure to thrive, spasticity, epilepsy, and microcephaly. The brain MRI of I:P1 disclosed white matter necrosis and lesions in basal ganglia and thalamus that resembled LS (Baertling *et al.* 2014). Metabolic encephalopathy and Leigh-like lesions in brain MRI has been previously associated with MDDS caused by mutations in *SUCLA2* and *SUCLG1* (Elpeleg *et al.* 2005, Carrozzo *et al.* 2007, Ostergaard *et al.* 2007a, Ostergaard *et al.* 2007b). In addition, patient I:P1 presented with generalized aminoaciduria, which has been previously reported in association with *DGUOK* (Mandel *et al.* 2001, Dimmock *et al.* 2008) and *MPV17* mutations (Uusimaa *et al.* 2014). Interestingly, in addition to encephalopathy, *SUCLA2* and *SUCLG1* mutations present with methylmalonicaciduria and elevated level of methylmalonic acid in serum, which are diagnostic clues for mutations in these genes (Elpeleg *et al.* 2005, Carrozzo *et al.* 2007, Ostergaard *et al.* 2007a,

Ostergaard *et al.* 2007b). The combination of encephalopathy, microcephaly, elevated lactate, and generalized aminoaciduria is also suggestive for mutations in the *RRM2B* gene (Bornstein *et al.* 2008, Ascham-Roschitz *et al.* 2009, Kollberg *et al.* 2009). Considering the unique combination of clinical features in patient I:P1, previously associated with a variety of mtDNA maintenance genes, mtDNA maintenance genes were analysed using WES. No mutations were found in the known mtDNA maintenance genes and further analysis of the candidate genes are ongoing in order to establish the genetic origin of the MDDS in patient I:P1.

The clinical features of patient I:P2 included encephalopathy, myopathy, failure to thrive, optic atrophy, pigmentary retinopathy, sensorineural hearing impairment, and nystagmus. The clinical course for patient I:P2 led to death by the age of 5 years. Interestingly, muscle histology of I:P2 showed fibrosis and increased glycogen in subsarcolemmal structures in addition to structural alterations in ER (I: Fig. 2B), which has not been previously described for MDDS. Optic atrophy can be a feature of Alpers syndrome (Cohen & Naviaux 2010), but *POLG1* mutations were not found in the genetic studies. Encephalopathy and pigmentary retinopathy could also suggest KSS, but mtDNA deletions – the hallmark of KSS – were not detected in patient I:P2. Previously, one patient has reported mtDNA depletion in association with similar clinical features, including encephalopathy, failure to thrive, optic atrophy, and sensorineural hearing impairment (Renaldo *et al.* 2012). Genetic studies of this patient disclosed a missense mutation in *MFN2* gene. Interestingly, *in vitro* studies using mouse embryonic fibroblasts showed that silencing of mitofusin 2 leads to disruption in ER morphology (de Brito & Scorrano 2008).

In addition, ER is likely to take part in mitochondrial network dynamics (Friedman *et al.* 2011, Kornmann 2013). Considering the alteration of ER seen in the muscle histology and the patient as reported by Renaldo *et al.* (2012), the clinical presentation of patient I:P2 may relate to a disturbance in the dynamics of the mitochondrial network. Total genomic DNA patient of I:P2 was analysed using WES, but mutations in the known mtDNA maintenance genes, including *MFN2*, were not found. WES data of patient I:P2 is currently analysed for new candidate genes. Considering the clinical phenotype and, hitherto, unknown genetic aetiology, patient I:P2 may have offered a novel presentation of MDDS.

6.1.3 Mitochondrial DNA depletion as a secondary finding to muscle dystrophy

Patient I:P3 harboured severe mtDNA depletion with mtDNA content of only 0.10 compared to the age-matched controls (I: Fig. 1B, Table 1). Clinical examination disclosed severe myopathy with muscle fibrosis and atrophy. Based on the clinical findings and a histological examination, patient I:P3 was diagnosed with merosine-deficient muscular dystrophy (MDC1A) with heterozygous p.G1591X mutation in the *LAMA2* gene. One study has associated mtDNA depletion of a minor degree with congenital myotonic dystrophy, but no evidence of mtDNA rearrangements in the pathogenesis was found; therefore, mild mtDNA depletion was suggested to be secondary to the disease (Poulton *et al.* 1995a). Association of severe mtDNA depletion with the clinical phenotype in our patient remains uncertain, but most likely it was a secondary finding due to degeneration and atrophy of the muscle tissue.

6.1.4 Mitochondrial DNA rearrangements in Kearns-Sayre syndrome: Multiple mtDNA deletions and compensatory over-replication

Patients I:P4 and I:P5 presented with early-onset ophthalmoplegia and other neurological symptoms, including sensorineural hearing loss, muscle hypotonia, and ataxia as common clinical features. Further, patient I:P5 was found to have pigmentary retinopathy and cardiac conduction block referring to KSS (Kearns & Sayre 1958, Kearns 1965), together with transient anaemia and granulocytopaenia, suggesting PS (Pearson *et al.* 1979). Interestingly, after the pancytopaenic episode, PS may develop to KSS (Larsson *et al.* 1990, Norby *et al.* 1994), which was also the case for patient I:P5. Both of these patients harboured a large-scale mtDNA deletion approximately 5 kb in size (I: Fig. 3), which is very consistent with the finding described in KSS and PS (Zeviani *et al.* 1988, Moraes *et al.* 1989, Fischel-Ghodsian *et al.* 1992, Remes *et al.* 2005, Schapira 2006). In an XL-PCR analysis, the intact 16.6 kb mtDNA molecule was not detected in either patient. Albeit XL-PCR is not a quantitative method, this result suggests a very high heteroplasmy rate for large-scale mtDNA deletion.

In addition to single-large scale deletions, patients I:P4 and I:P5 presented with minor multiple deletions (I: Fig. 3). The single deletions observed in KSS are thought to be of sporadic origin and not genetically transmitted (Chinnery *et al.* 2004), whereas multiple deletions are typically associated with mutations in

mtDNA maintenance genes (Zeviani *et al.* 1990, Servidei *et al.* 1991, Horvath *et al.* 2006) and can be transferred as an autosomal dominant or recessive trait (Moraes *et al.* 1989, Zeviani *et al.* 1989, Van Goethem *et al.* 2001, Horvath *et al.* 2006). Although multiple mtDNA deletions have been reported in KSS (Ascaso *et al.* 2010), the presence of multiple deletions in our patients could suggest mutations in mtDNA maintenance genes. Analysis of the *POLG1* mutations and the *C10orf2* gene, which are commonly associated with multiple mtDNA deletions (Spelbrink *et al.* 2001, Longley *et al.* 2005, Wanrooij *et al.* 2007), did not show pathogenic changes. MtDNA maintenance genes were studied further using WES, but no mutations were found. These circumstances could suggest a previously unknown genetic aetiology of the disease, and further investigation on the functional characteristics of the candidate genes detected by WES is currently under way.

The qRT-PCR analysis showed a striking increase in muscle mtDNA content in patients I:P4 and I:P5 (I: Fig. 1B, Table 2). A similar finding has been reported in one female patient with KSS, who presented with a single 3078 bp mtDNA deletion with a 92% heteroplasmy rate and a 9-fold increase in muscle mtDNA content (Wong *et al.* 2003). The high mtDNA content seen in our patients and in the previously reported case could refer to over-replication of mtDNA to compensate for the mtDNA deletion with a high heteroplasmy rate (Wong *et al.* 2003). Still, it must be noted that KSS is also often associated with mtDNA duplications (Poulton *et al.* 1994, Poulton *et al.* 1995b, Odoardi *et al.* 2003), which could also suggest that the high mtDNA content detected by qRT-PCR is an error caused by a duplicated target gene. Patients I:P4 and I:P5 were not analysed for mtDNA duplications, but the mitochondrial target gene *ND1* is located in the minor arc of the mtDNA molecule and is not typically involved in mtDNA deletions (Remes *et al.* 2005, Bua *et al.* 2006, Schapira 2006, Krishnan *et al.* 2007). Thus, it is most likely that the high mtDNA content is an actual finding and refers to over-replication of mtDNA.

6.2 Pathogenicity of the *POLG1* p.R722H mutation

6.2.1 Clinical and molecular genetic features associated with homozygous POLG1 p.R722H and compound heterozygous POLG1 p.[R722H];[W748S] mutation

Homozygous *POLG1* p.R722H mutation was found in patient II:A1 (II: Fig. 5), who presented with PEO, sensorineural hearing impairment, diabetes mellitus, dysphagia, and neuropathic pain in legs as clinical features (II: Table 2). XL-PCR and a Southern blot analysis of muscle DNA disclosed multiple mtDNA deletions (II: Fig. 3-4). In addition, RRFs and COX-negative fibers were seen in the muscle histology, which together with the multiple neurological symptoms, endocrinopathy and multiple mtDNA deletions, suggested a mitochondrial disorder. *POLG1* mutations have been previously associated with PEO and multiple mtDNA deletions (Van Goethem *et al.* 2001, Di Fonzo *et al.* 2003, Winterthun *et al.* 2005, Longley *et al.* 2005, Horvath *et al.* 2006, Hudson & Chinnery 2006). In addition, the analysis of a cohort of *POLG1* patients had associated mutations in the linker region of *POLG1* with adolescence- or adult-onset ptosis and PEO with limb myopathy, ataxia, peripheral neuropathy, dysphagia, diabetes, deafness, and dementia as additional clinical features (Horvath *et al.* 2006), which correlates with the clinical features of patient II:A1. Patients II:A2 and II:A3, siblings of patient II:A1, presented with dementia and sensorineural hearing impairment as common clinical features (II: Table 2).

When assessing the clinical significance of the mtDNA deletions, it must be noted that the mtDNA deletions are also related to aging (Bender *et al.* 2006, Kraytsberg *et al.* 2006, Krishnan *et al.* 2007). Therefore, XL-PCR and Southern blot analysis was performed on muscle DNA for two age-matched controls in parallel with patient II:A1. XL-PCR showed mtDNA deletions also in the controls, but to a minor extent as in patient II:A1 (II: Fig. 3). As XL-PCR is not a quantitative method, the muscle DNA of patient II:A1 and the controls were analysed further with Southern blotting. This method did not show mtDNA deletions in the controls, but did disclose multiple mtDNA deletions in the muscle of patient II:A1, thus suggesting mtDNA deletions of a more significant extent in patient II:A1. Muscle biopsies of patients II:A2 and II:A3 were not available, and therefore, an analysis of mtDNA deletions was not performed.

Two patients (patient II:B1 and patient II:B2) were found to harbour a compound heterozygous *POLG1* p.[R722H];[W748S] mutation. Both patients

presented with mental retardation, psychiatric symptoms, and mild bilateral ptosis. In addition, patient II:B1 presented with epilepsy. The clinical presentation of patients II:B1 and II:B2 was consistent with the signs and symptoms previously associated with homozygous and compound heterozygous *POLG1* p.W748S mutations (Hakonen *et al.* 2005, Winterthun *et al.* 2005, Horvath *et al.* 2006, Tzoulis *et al.* 2006).

6.2.2 Pathogenicity of homozygous POLG1 p.R722H and compound heterozygous POLG1 p.[R722H];[W748S] mutation

Previously, heterozygous *POLG1* p.R722H mutation was reported in two patients with PD, but also in a healthy control. The mutation was considered to be a neutral polymorphism, as there was no difference in the mutation frequencies between the patients and the controls. A carrier frequency of less than 1% in healthy controls was reported (Luoma *et al.* 2007). In a study carried out on the Chinese population, *POLG1* p.R722H was found in 344 patients with PD and in 144 controls; *POLG1* p.R722H was not significantly associated with PD (Gui *et al.* 2012).

Although *POLG1* p.R722H has not been reported in neurodegenerative diseases, it has been associated with early-onset encephalopathies. Bolszak *et al.* (2009) reported a patient with severe myoclonic epilepsy in infancy (SMEI), who presented with enlarged mitochondria in muscle histology and harboured compound heterozygous *POLG1* p.[G517V];[R722H] in addition to a heterozygous p.R1645Q mutation in the *SCN1A* gene. Muscle histology is usually normal in SMEI (Ceulemans & Cras 2004), which suggests that the mitochondrial abnormalities seen in the patient reported by Bolszak *et al.* (2009) were related to the compound heterozygous *POLG1* p.[G517V];[R722H] mutation. In addition, a compound heterozygous *POLG1* p.[R722H];[V1044] has been reported in an early-onset, Alpers-like encephalopathy mitochondrial (Isohanni *et al.* 2011). Previously, more severe phenotypes with worse survival have been reported in compound heterozygous *POLG1* p.W748S mutations (Tzoulis *et al.* 2006), an instance that could refer to a dominant effect of the heterozygous *POLG1* p.W748S mutation, but may also suggest an increased pathogenic effect, when other *POLG1* mutations are present.

POLG1 p.R722H and p.W748S mutations are situated in the linker region of pol γA, which mediates the interaction between the polymerase and the exonuclease domains and also facilitates the binding of the subunits pol γA and

pol γB. Functionally, the linker domain is essential, and mutations in this domain may decrease the enzyme activity of pol γA (Luoma *et al.* 2005). *POLG1* p.R722H is not highly conserved (II: Fig. 7), but its location in the highly conserved region may denote functional importance. *POLG1* p.W748S leads to a decrease in the catalytic activity of pol γ and leads to a compromised DNA synthesis and primer extension (Chan *et al.* 2005). Although compound heterozygous linker domain mutations often present with *pol* domain mutation (Suomalainen & Isohanni 2010), it is possible that compound heterozygous *POLG1* p.[R722H];[W748S] may cause a functional defect of pol γ by altering the linker region. In Study II, the effect of *POLG1* p.R722H and p.W748S mutations on the tertiary structure of pol γ was assessed indirectly, using the known structure of the Klenow fragment of *E. coli* and bacteriophage T7 (II: Fig. 8). The p.R631 residue in this region is loosely homologous to p.R722 in the human pol γ. Site-directed mutagenesis of the Klenow fragment has shown that the mutation of p.R631 leads to a slight decrease in the enzyme activity. In addition, the conformation of this region changes during DNA binding (Minnick *et al.* 1999). Loss of the positively charged arginine in this location may alter the DNA template binding and decrease the synthesis activity or fidelity.

Considering the clinical and molecular genetic features of the patients in Study II and in the previous reports, which were consistent with mitochondrial and *POLG1*-related diseases, *POLG1* p.R722H may be a pathogenic mutation in the homozygous or compound heterozygous state. A homozygous mutation could lead to a late-onset phenotype, whereas compound heterozygous mutation may cause the onset of the disease in childhood or adolescence (Farnum *et al*. 2014). The carrier frequency of 1:135 reported in Study II and 1:95 reported by Isohanni *et al.* (2011) suggest that *POLG1* p.R722H mutation is common in Finnish population, when compared to the carrier frequencies of the common *POLG1* mutations p.A467T and p.W748S in Finland and other Nordic countries (Hakonen *et al.* 2005, Winterthun *et al.* 2005, Kollberg *et al.* 2006).

6.3 *POLG1* mutations in VPA-induced acute liver failure and prognosis after liver transplantation

6.3.1 POLG1 mutations as a risk factor for VPA-induced acute liver failure

Five patients with VPA-induced ALF were retrospectively identified from the register at the Department of Transplantation and Liver Surgery, Helsinki University Hospital, Finland. The patients received VPA treatment for focal epilepsy and intractable status epilepticus (SE). Histology of the explanted livers disclosed severe necrosis of the liver without any signs of chronic liver diseases (III: Table 1 and Fig. 1A-C). Three patients were initially suspected to have Wilson's disease, but copper staining of the explanted livers were negative.

All five patients harboured *POLG1* mutations (III: Table 1). Four patients (III: patients 1, 2, 4 and 5) presented with homozygous *POLG1* p.W748S and p.E1143G mutations and mtDNA depletion in the liver (III: Table 2 and Fig. 2). In addition, patient III:4 harboured a heterozygous *POLG1* p.Q497H mutation (III: Table 2). Clinical presentations of these patients referred to a mitochondrial disorder and resembled adolescent-onset Alpers disease or MIRAS, which have been previously associated with *POLG1* p.Q497H, p.W748S and p.E1143G mutations and mtDNA depletion (Simonati *et al.* 2003, Winterthun *et al.* 2005, Gordon 2006, Nguyen *et al.* 2006, Tzoulis *et al.* 2006, Lee & Sokol 2007, Brunetti-Pierri *et al.* 2008, Uusimaa *et al.* 2008, Wong *et al.* 2008, Horvath *et al.* 2006, Taanman *et al.* 2009, Kurt *et al.* 2010, Isohanni *et al.* 2011, El-Hattab & Scaglia 2013). *POLG1* p.E1143G mutation is often found with *POLG1* p.W748S, although its pathogenicity as a single variant is not clear (Chan & Copeland 2009). *POLG1* p.E1143G is a common variant with a frequency of 3-5% in the general population (Davidzon *et al.* 2005) and 2% in Finland (Palin *et al.* 2012). *In vitro* studies have shown that a combination of homozygous *POLG1* p.W748S and p.E1143G mutations compromises the activity of pol γ, thus leading to an inadequate mtDNA amount (Chan *et al.* 2006). The *POLG1* p.E1143G mutation has been reported in two patients with VPA-induced ALF (Stewart *et al.* 2010). This finding suggests a pathogenic nature of the mutation under special circumstances, e.g., exposure to VPA. Interestingly, Helbling *et al.* (2013) reported mtDNA depletion in a majority of cases with ALF. Furthermore, mutations in mtDNA maintenance genes were associated with an increased risk of ALF triggered by environmental factors, i.e., drugs and a viral infection (Helbling

et al. 2013). Thus, the OXPHOS chain dysfunction caused by impaired pol γ activity and mtDNA depletion was very likely the underlying cause of the clinical phenotype and VPA-induced ALF in patients III:1, III:2, III:4 and III:5.

Patient III:3 presented with a heterozygous *POLG1* p.Q1236H mutation, but no mtDNA depletion (III: Table 2 and Fig. 2). Previously, *POLG1* p.Q1236H mutation has been reported as a neutral polymorphism (Di Fonzo *et al.* 2003, Luoma *et al.* 2005, Taanman *et al.* 2009). Functionally, *POLG1* p.Q1236H increased pol γ activity in a compound heterozygous state with *POLG1* p.R627H, which suggests a compensatory effect in presence of other *POLG1* mutations (Luoma *et al.* 2005). Interestingly, in a study by Stewart *et al.* 2010 *POLG1* p.Q1236H was associated with an increased risk of VPA-induced ALF. Blood sample analysis showed no change in mtDNA content and only a slightly decreased mtDNA copy number was detected in the myotubes. Functional analysis in yeast showed increased mtDNA point mutations, but no deletions. In concert with Stewart *et al.* (2010), the results of Study III suggest that *POLG1* p.Q1236H is not a neutral polymorphism and increases the risk of VPA-induced ALF without altering the intracellular mtDNA content. Therefore, different *POLG1* mutations may alter the response to VPA exposure with various, yet unresolved, mechanisms. Considering that *POLG1* p.Q1236H mutation is common in the Caucasian population with a frequency of ≤8.6% (Stewart *et al.* 2010), this mutation may significantly contribute to VPA-induced ALF in the general population. Therefore, a *POLG1* gene analysis should be performed on all patients before administration of VPA.

6.3.2 Prognosis of liver transplantation in patients with POLG1 mutations

VPA-induced ALF can be reversible after discontinuation of VPA administration in some patients with *POLG1* mutations (McFarland *et al.* 2009, Mindikoglu *et al.* 2011), but often the liver failure is progressive and eventually requires a LT. The role of LT in treatment of these patients is very controversial, as patients with *POLG1* mutations often present with multisystem neurologic syndromes, which determine the prognosis for these patients (Thomson *et al.* 1998, Sokal *et al.* 1999, Delarue *et al.* 2000, Kayihan *et al.* 2000, Kelly 2000, Dubern *et al.* 2001, Lee & Sokol 2007). In some cases, the neurological status of the patient rapidly deteriorates after a LT, thus causing poorer survival after the operation (Thomson *et al.* 1998, Sokal *et al.* 1999, Dubern *et al.* 2001, Lee & Sokol 2007). At the

same time, successful LTs have been performed on patients with OXPHOS chain deficiency and isolated liver injury (Sokal *et al.* 1999, Dubern *et al.* 2001).

We identified four adolescents or young adults, who received LT for acute VPA-induced ALF. Patient III:5 died prior to LT. Two patients (III: patients 1 and 2) with homozygous *POLG1* p.W748S and p.E1143G mutations had survived over four years after the transplantation with occasional epileptic seizures at the time of Study III. At the time of Study III, Patient III:4 with homozygous *POLG1* p.W748S and p.E1143G mutations and a heterozygous *POLG1* p.Q497H had survived for 19 years following the LT and is currently seizure-free. Patient III:3, who harboured heterozygous *POLG1* p.Q1236H mutation, died suddenly two years after the LT, but the exact cause of death remains unknown. All three patients, who have survived following the LT, have normal cognition and functional capacity.

In a series of seven patients with *POLG1* mutations and ALF after VPA exposure, two patients were treated with LT. The patient with homozygous *POLG1* p.W748S mutation underwent a successful transplantation, whereas the patient with compound heterozygous *POLG1* p.[A467T];[W748S] died one month after the transplant (Tzoulis *et al.* 2006). Wolf *et al.* (2009) reported an unsuccessful LT in a patient with a compound heterozygous p.[W748S];p.[G848S] mutation, but two patients with a homozygous *POLG1* A467T survived following LT. On the other hand, poor survival after LT has been reported in children with VPA-induced ALF even without underlying mitochondrial disorder (Mindikoglu *et al.* 2011). Interestingly, very much like the patients in Study III, the patient with successful LT that was reported by Tzoulis *et al.* (2006) had onset of epilepsy at age 19 years. Therefore, our findings and the previous reports suggest that the mutation status and the age of the patient may influence the outcome and survival of that patient after LT. Although the number of the patient is rather small, *POLG1* mutations may not be a definite contraindication for LT after all; instead, homozygous *POLG1* gene mutations and adolescent-onset of the disease could suggest better survival. Therefore, patient's clinical phenotype, molecular genetic status and age at onset should also be taken in consideration in the treatment of VPA-induced ALF (McKiernan 2014). Considering the frequency of *POLG1* mutations in general population (Van Goethem *et al.* 2001, Hakonen *et al.* 2005, Luoma *et al.* 2005, Winterthun *et al.* 2005, Horvath *et al.* 2006, Kollberg *et al.* 2006), analysis of the *POLG1* gene should be performed on all patients prior to VPA administration.

6.4 VPA-induced mitochondrial toxicity in HepG2 *in vitro* liver model

6.4.1 Effect of VPA on mitochondrial function and cell viability

HepG2 cells were chosen as an *in vitro* model for mitochondrial toxicity, because they are easy to culture and contain a high number of mitochondria, thus offering a widely used model for drug hepatotoxicity (Pinti *et al.* 2003). The effect of VPA on the mitochondrial function in HepG2 cells was studied under two metabolic conditions: In a normal glucose-containing growth medium and a glucose-free medium supplemented with galactose and pyruvate (herein referred to as glucose medium and galactose medium). Under cell culture conditions, the galactose medium directs the cells to use oxidative phosphorylation as the main source of ATP (Marroquin *et al.* 2007, Palmfeldt *et al.* 2009). VPA concentrations were planned to cover a therapeutic dose (0.5 mM) and only a mild overdose (1.0 mM and 2.0 mM) (Callaghan *et al.* 1985, Silva *et al.* 2008, Schmid *et al.* 2013). To identify both the short-term and long-term toxic effects on mitochondrial function, the HepG2 cells were treated with VPA for 24 to 72 hours.

Previous studies on isolated rat mitochondria have shown VPA to inhibit the OXPHOS chain function (Haas *et al.* 1981, Ponchaut *et al.* 1992). We studied mitochondrial respiration by using an oxygen-sensitive MitoXpress-X-tra-HS probe. We observed a decrease in cellular respiration proportionally to VPA concentration, which refers to a VPA-induced OXPHOS chain dysfunction (IV: Fig. 1). The effect was seen both in the glucose and galactose mediums, but the effect was more potent in the galactose medium. Moreover, in the galactose medium VPA decreased the OCR rates significantly already at a therapeutic 0.5 mM VPA concentration after 24 hours. VPA has been reported to inhibit pyruvate uptake into the liver and brain mitochondria (Aires *et al.* 2010, Benavides *et al.* 1982) and directly inhibit PDC (Silva *et al.* 1997) and ODGC in the TCA cycle (Luis *et al.* 2007), which could explain the dysfunction in pyruvate-driven mitochondrial respiration. In addition, VPA also prevents cellular glucose transportation by inhibiting the GLUT1 transporter (Wong *et al.* 2005), which can interfere with glucose-driven oxidative metabolism.

In the galactose medium, a significant decrease in OCR rates was observed up to 72 hours, whereas in the glucose medium the effect was seen up to 48 hours. On the other hand, the effect of VPA on cellular respiration diminished after longer exposure. These results suggest that cells dependent on OXPHOS chain

activity are more vulnerable to the toxic effect of VPA. Over time, mitochondrial function seems to adapt in the presence of VPA. Cells supplemented with glucose were able to recover faster that the cells in galactose, which suggests a better metabolic reserve under glucose supplementation. Previous reports on VPA-treated neuroblastoma cells (Cowell *et al.* 2009) and fibroblasts (Sitarz *et al.* 2014) propose activation of mitochondrial biogenesis by induction of peroxisome proliferator-activated receptor-gamma coactivator 1α (PGC-1α), which activates the genes related to mitochondrial metabolism, glucose transportation, and FAO. Although we did not investigate the expression of these genes, immunoblotting did not show any increase in the expression of OXPHOS chain proteins (IV: Fig. 5A).

When interpreting the results of the OCR assays, it must be observed that in this setting the measurements only reflect changes in the basal cellular respiration. The measurements were not carried out with ATPase inhibitor oligomycin, complex I inhibitor rotenone, complex III inhibitor antimycin A, MPTP inhibitor cyclosporine A and mitochondrial uncouplers carbonyl cyanide m-chlorophenyl hydrazone (CCCP) or carbonyl cyanide-4-(trifluoromethoxy) phenylhydrazone (FCCP) (Lanza & Nair 2009, Jonckheere *et al*. 2010). Therefore, OCR assays did not specifically measure state 4 respiration, respiration of completely mitochondrial origin or maximal respiration rate and did not exclude the role of MPTP opening in the decreased OCR rates (Lanza & Nair 2009). Thus, the results of the OCR assays may be considered only as preliminary findings.

Previously, VPA has decreased Ψ_m in JC-1 and Rhodamine 123 assays in HepG2 cells (Wang *et al.* 2011b) and rat hepatocytes (Tong *et al.* 2005b, Pourahmad *et al.* 2012). We studied Ψ_m using MitoTracker Red CMXRos, a mitochondrion-selective and a Ψ_m-sensitive fluorescent probe, which yields fluorescence only in active mitochondria (Pendergrass *et al.* 2004, Cottet-Rousselle *et al.* 2011). With MitoTracker Red CMXRos, decreased Ψ_m was detected after VPA treatment in glucose and galactose mediums (IV: Fig. 2). In the glucose medium a significant decrease was seen only after 24-hour VPA treatment, whereas in galactose the effect was seen also, but after 48 hours. Similar to OCR assays, measurements with MitoTracker Red CMXRos suggest decreased mitochondrial activity under VPA exposure, but also an adaptation to VPA. Compared to JC-1, MitoTracker Red CMXRos may be better at detecting subtle changes in Ψ_m, and it is not affected by a quenching/non-quenching mode as opposed to rhodamine dyes (Cottet-Rousselle *et al.* 2011, Perry *et al.* 2011). On the other hand, similar to MitoTracker Orange CMTMRos (Invitrogen),

MitoTracker RedCMXRos binds covalently to thiol motifs in MM and therefore it is not distributed over IMM according to Nernst equation. In addition, the stain may inhibit complex I (Ferlini *et al*. 1998, Scorrano *et al*. 1999, Nicholls & Ward 2000). The latter two features limit the use of MitoTracker Red CMXRos as a reliable MMP probe. Furthermore, cationic dyes used in detection of MMP are often extruded from the cell by multidrug-resistance (MDR) pumps. Therefore, completely reliable MMP detection would require exclusion of MDR activity (Bernari *et al*. 1999).

As ATP production is the main function of the OXPHOS chain (Osellame *et al.* 2012), a cross-section determination of basal ATP levels was performed at each point of time. Interestingly, VPA decreased intracellular ATP proportionally to VPA concentration, but significant changes were detected only in the galactose medium (IV: Fig. 3). Therefore, the cells dependent on the OXPHOS chain cannot produce ATP, a result that very strongly suggests a dysfunction of cellular respiration. On the other hand, cells supplemented with glucose were able to maintain ATP levels via glycolysis.

MTT assay is often used in the evaluation of intracellular redox activity, cell proliferation, and cell viability (Berridge & Tan 1993, Hamid *et al.* 2004). In Study IV, MTT signal was increased after 48 and 72-hour incubations in the glucose medium and after 72-hour incubation in the galactose medium (IV: Fig. 6). As a methodological consideration, it must be noted that only 25-45% of the MTT signal is produced by mitochondria, and MTT mainly reflects the activity of oxidoreductase enzymes in the cytosol (Bernas & Dobrucki 2002). In addition, MTT may produce false positive results under oxidative stress, as $O2^{\bullet-}$ reduces tetrazolium salts to formazan (Wang *et al.* 2011a). Thus, in this setting, a MTT assay most likely does not reflect mitochondrial viability, but rather shows the activity of the cytosolic compartment as a compensation for the mitochondrial dysfunction or signifies a reaction to oxidative stress.

Curiously, PI staining showed increased cell death under 72-hour VPA exposure (IV: Fig. 7) and a decreased cell number was detected using Hoechst 33342 staining (IV: Fig. 8), which supports the findings of mitochondrial dysfunction. Although mitochondrial dysfunction was detected after 24 hours, increased cell death was apparent after longer VPA exposure, a result that is compatible to the clinical course of VPA-induced liver damage (Bryant & Dreifuss 1996, Lee 2003, Koenig *et al.* 2006, Schmid *et al.* 2013). Interestingly, increased cell death and a reduction in cell number were seen also in cells in the

glucose medium with normal ATP levels, implying that VPA has other cytotoxic mechanisms in addition to the OXPHOS chain dysfunction.

6.4.2 VPA-induced mitochondrial ROS production

MitoSOX Red, a specific probe for mitochondrial superoxide ($O2^{\bullet-}$), showed a significant increase in ROS levels after a 72-hour treatment with 2.0 mM VPA (IV: Fig. 4). Previous studies on rat hepatocytes have shown VPA to induce oxidative stress (Tong *et al.* 2005b, Kiang *et al.* 2010, Pourahmad *et al.* 2012). In these studies, VPA was used at high concentrations (6.0 mM and above), and ROS levels were increased already after 1-2 hours of exposure (Tong *et al.* 2005b, Kiang *et al.* 2010). These findings could suggest that VPA requires either high concentration or long exposure to induce detectable ROS levels. Increased ROS production could be related to a decreased OXPHOS chain function seen in OCR assays since impaired mitochondrial respiration potentially may create a backward flux of electrons in the OXPHOS chain (described in Chapter 2.1.3). VPA-induced oxidative stress has been related to the cytochrome P450-mediated activation of reactive VPA metabolites, in particular 4-ene-VPA and 2,4-diene-VPA (Begriche *et al.* 2011, Pourahmad *et al.* 2012). As HepG2 cells have only negligible cytochrome P450 activity and are not expected to produce 4-ene-VPA, or 2,4-diene-VPA (Westerink & Schoonen 2007), these metabolites are presumably not essential for VPA-induced hepatotoxicity. This finding is further supported by a previous studies on rat hepatocytes showing that 4-ene-VPA and 2,4-diene-VPA are not elevated during VPA-induced oxidative stress (Tong *et al.* 2005a) and cytochrome P450 inhibition does not affect oxidative stress or hepatotoxicity by VPA (Kiang *et al.* 2010).

SOD2 catalyses an elimination of mitochondrial $O_2^{\bullet-}$ to O_2 and H_2O_2 and the latter is eliminated by GPX (Murphy 2009). Interestingly, VPA decreased the protein levels of mitochondrial superoxide dismutase (SOD2) (IV: Fig. 5A-B). Thus, increased levels of mitochondrial $O_2^{\bullet-}$ that were detected using MitoSOX Red may be a result of a downregulation of the primary ROS scavenger (Clavijo-Cornejo *et al.* 2014) proposing oxidative stress caused by impaired elimination of mitochondrial ROS. GPX protein levels did not change (IV: Fig. 5A), therefore, suggesting that VPA does not have an effect on the H_2O_2 scavenging system.

6.4.3 Effect of VPA on expression of the pol γA, TCA cycle and OXPHOS chain proteins

As *POLG1* mutations will increase the risk of VPA-induced hepatotoxicity (Schwabe *et al.* 1997, Delarue *et al.* 2000, Simonati *et al.* 2003, Gordon 2006, Nguyen *et al.* 2006, Tzoulis *et al.* 2006, Engelsen *et al.* 2008, Uusimaa *et al.* 2008, McFarland *et al.* 2009, Saneto *et al.* 2010, Stewart *et al.* 2010, Uusimaa J *et al.* 2013), we studied the effect of VPA on the expression of pol γA, but did not detect any significant changes in the protein levels (IV: Fig. 5A). Most interestingly, VPA has been shown to increase pol γA expression in *POLG1*-deficient fibroblasts and control cells (Sitarz *et al.* 2014). Despite the induced expression of pol γA, a reduction in the mtDNA copy number was found in *POLG1*-deficient cells (Sitarz *et al.* 2014). Furthermore, mtDNA depletion has been found in a majority of patients with ALF, including drug-induced ALF (Helbling *et al.* 2013). Thus, the decreased mtDNA copy number and catalytic dysfunction of pol γA are very likely crucial in the pathogenesis of *POLG1*-related hepatotoxicity of VPA instead of in the expression of the pol γA itself.

7 Conclusions

The following conclusions can be drawn from the findings of the present research.

1. MtDNA depletion can be a fairly common cause of very early-onset neuromuscular disorders in children.
2. MtDNA depletion was identified in two patients with a novel MDDS phenotype without pathogenic mutations in any of the known mtDNA maintenance genes. The molecular genetic aetiology of the MDDS in these two patients was not yet resolved and requires further investigation on candidate genes identified by whole-exome sequencing.
3. MtDNA depletion can be found in muscle dystrophy. Most likely, it is related to degradation of the muscle tissue; the association of mtDNA depletion with the actual pathomechanism of the disease is yet unclear.
4. In addition to a single-large scale mtDNA deletion, Kearns-Sayre and Pearson syndrome can be associated with multiple deletions. This finding suggests a genetic origin of the disease instead of sporadic mtDNA deletion. High mtDNA deletion load in muscle may induce compensatory over-replication of mtDNA.
5. Instead of being a neutral polymorphism, *POLG1* p.R722H seems to be a pathogenic mutation in either the homozygous or compound heterozygous state. A homozygous mutation could lead to a late-onset phenotype, whereas compound heterozygous mutation may cause onset of the disease in childhood or adolescence.
6. *POLG1* mutations are a major risk factor for VPA-induced hepatotoxicity and acute liver failure. Homozygous *POLG1* gene mutations and adolescent-onset of the disease could suggest better survival after liver transplantation. Therefore, *POLG1* mutations may not be a definite contraindication for liver transplantation and the clinical presentation, molecular genetic status and age at onset should be taken into account in the management of VPA-induced acute liver failure. The *POLG1* gene should be studied in all patients prior to administration of VPA.
7. VPA induces mitochondrial dysfunction by inhibiting mitochondrial respiration. In addition, VPA decreases the intracellular level of mitochondrial superoxide dismutase 2. This process leads to a depletion of ATP, oxidative stress, and cell death. There was no evidence of VPA-induced

change in the expression of the catalytic subunit A of the mitochondrial DNA polymerase γ or respiratory chain subunits.

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I Komulainen T, Hautakangas MR, Hinttala R, Pakanen S, Vähäsarja V, Lehenkari P, Olsen P, Vieira P, Saarenpää-Heikkilä O, Palmio J, Tuominen H, Kinnunen P, Majamaa K, Rantala H & Uusimaa J (2014) Mitochondrial DNA depletion and deletions in paediatric patients with neuromuscular diseases – novel phenotypes. Manuscript.

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