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MITOCHONDRIAL DISEASE IN SOUTHWESTERN FINLAND

Population-based Molecular Genetic and Clinical Studies

by

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Motto:

"He who studies medicine without books sails an uncharted sea,
but he who studies medicine without patients does not go to sea at all."

-Sir William Osler

ABSTRACT

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Mitochondrial disease in Southwestern Finland. Population-based molecular genetic and clinical studies

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Mitochondria are present in all eukaryotic cells. They enable these cells utilize oxygen in the production of adenosine triphosphate in the oxidative phosphorylation system, the mitochondrial respiratory chain. The concept 'mitochondrial disease' conventionally refers to disorders of the respiratory chain that lead to oxidative phosphorylation defect. Mitochondrial disease in humans can present at any age, and practically in any organ system. Mitochondrial disease can be inherited in maternal, autosomal dominant, autosomal recessive, or X-chromosomal fashion. One of the most common molecular etiologies of mitochondrial disease in population is the m.3243A>G mutation in the *MT-TL1* gene, encoding mitochondrial $tRNA^{Leu(UUR)}$. Clinical evaluation of patients with m.3243A>G has revealed various typical clinical features, such as stroke-like episodes, diabetes mellitus and sensorineural hearing loss. The prevalence and clinical characteristics of mitochondrial disease in population are not well known. This thesis consists of a series of studies, in which the prevalence and characteristics of mitochondrial disease in the adult population of Southwestern Finland were assessed. Mitochondrial haplogroup Uk was associated with increased risk of occipital ischemic stroke among young women. Large-scale mitochondrial DNA deletions and mutations of the *POLG1* gene were the most common molecular etiologies of progressive external ophthalmoplegia. Around 1% of diabetes mellitus emerging between the ages 18 – 45 years was associated with the m.3243A>G mutation. Moreover, among these young diabetic patients, mitochondrial haplogroup U was associated with maternal family history of diabetes. These studies demonstrate the usefulness of carefully planned molecular epidemiological investigations in the study of mitochondrial disorders.

Key words: diabetes mellitus, mitochondrial disease, molecular epidemiology, progressive external ophthalmoplegia, sensorineural hearing loss, stroke.

TIIVISTELMÄ

Mika H. Martikainen

Mitokondriotautien esiintyvyys Varsinais-Suomessa. Molekyyliepidemiologinen tutkimus

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Mitokondrioita on kaikissa aitotumaisissa soluissa. Mitokondrioiden hengitysketjun avulla solut voivat hyödyntää happea soluhengityksessä ja adenosiinitrifosfaatin tuotannossa. Käsite ”mitokondriotauti” viittaa tavallisesti hengitysketjun toimintahäiriöihin, jotka johtavat puutteelliseen soluhengitykseen. Ihmisellä mitokondriotauteja esiintyy kaikenikäisillä ja kaikissa elinjärjestelmissä. Mitokondriotaudit voivat periytyä äitilinjassa, autosomaalisesti vallitsevasti, autosomaalisesti peittyvästi, tai X-kromosomaalisesti. Mitokondriaalisen leusiinin tRNA:ta koodaavan *MT-TL1* –geenin pistemutaatio m.3243A>G on tavallisimpia mitokondriotautien molekyylitason syitä. Tämän mutaation kantajien kliininen tutkimus on paljastanut useita tyypillisiä kliinisiä piirteitä, kuten aivoverenkiertohäiriön kaltaiset oireet, diabetes mellitus ja sensorineuraalinen kuulovika. Väestötasolla mitokondriotautien esiintyvyys ja ilmenemismuodot ovat kuitenkin huonosti tunnettuja. Tämä väitöskirjatyö koostuu sarjasta tutkimuksia, joissa selvitettiin mitokondriotautien esiintyvyyttä ja ilmenemismuotoja Varsinais-Suomen aikuisväestössä. Mitokondriaalisen Uk-haploryhmän todettiin nuorilla naisilla assosioituvan lisääntyneeseen takaraivolohkon aivoinfarktin riskiin. Mitokondriaalisen DNA:n deleetiot ja *POLG1* –geenin pistemutaatiot todettiin yleisimmiksi progressiivisen eksternin oftalmoplegian syiksi. Noin 1% 18 – 45 vuoden iässä ilmaantuvasta diabeteksesta liittyy m.3243A>G –mutaatioon. Lisäksi todettiin mitokondriaalisen U-haploryhmän liittyvän diabeteksen esiintymiseen äidinpuoleisessa suvussa näillä potilailla. Nämä tutkimukset osoittavat huolellisesti suunniteltujen molekyyliepidemiologisten tutkimusten käyttökelpoisuuden mitokondriotautien tutkimuksessa.

Avainsanat: aivoinfarkti, diabetes mellitus, mitokondriotaudit, molekyyliepidemiologia, progressiivinen eksterni oftalmoplegia, sensorineuraalinen kuulovika.

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ABBREVIATIONS

AChRab – acetylcholine receptor antibody
AF – atrial fibrillation
ANT1 – adenine nucleotide translocator 1
ADP – adenosine diphosphate
ATP – adenosine triphosphate
bp – base pair
CNS – central nervous system
CoQ(10) – coenzyme Q10, ubiquinone
COX – cytochrome *c* (Cyt *c*) oxidase
CSF – cerebrospinal fluid
CT – computed tomography
DGUOK – deoxyguanosine kinase
D-loop – displacement loop
DM – diabetes mellitus
DNA – deoxyribonucleic acid
dNTP – deoxyribonucleoside triphosphate
ECG – electrocardiography
ENMG - electroneuromyography
ER – endoplasmic reticulum
FADH – flavin adenine dinucleotide
Fe-S – iron-sulphur
GTPase – guanosine triphosphatase
HI – hearing impairment
h-mtRPOL – mitochondrial RNA polymerase
HSP – heavy strand promoter
H-strand – heavy strand
IBM – inclusion body myositis
ICD – international classification of diseases
IF – initiation factor
IMM – inner mitochondrial membrane
IMS – inter-membrane space

IOSCA – infantile onset spinocerebellar ataxia

ISC – iron-sulphur cluster

KSS – Kearns-Sayre syndrome

LHON – Leber's hereditary optic neuropathy

LS – Leigh syndrome

LSP – light strand promoter

L-strand – light strand

MFH – maternal family history of diabetes mellitus

MFN – mitofusin

MELAS – mitochondrial encephalomyopathy, lactic acidosis and stroke-like episodes

MERRF – myoclonic epilepsy with ragged-red fibers

MIA – mitochondrial intermembrane space assembly

MIDD – maternally inherited diabetes and deafness

MMSE – Mini-Mental State Examination

MNGIE – mitochondrial neurogastrointestinal encephalomyopathy

MR – magnetic resonance

MRC – mitochondrial respiratory chain

MRP – mitoribosomal protein

MS – multiple sclerosis

mtDNA – mitochondrial DNA

mtEF – mitochondrial elongation factor

MTERF – mitochondrial transcription termination factor

mtRRF – mitochondrial recycling factor

mtRF – mitochondrial release factor

mtSSB – mitochondrial single-stranded DNA binding protein

NADH – nicotinamide adenine dinucleotide

nDNA – nuclear DNA (i.e. 'common' DNA)

OHA – oral hypoglycaemic agent

ORL – otorhinolaryngology

OXPHOS – oxidative phosphorylation

PCR – polymerase chain reaction

PD – Parkinson's disease

(C)PEO – (chronic-) progressive external ophthalmoplegia

PET – positron emission tomography

PFO – patent foramen ovale

POLG – mitochondrial DNA polymerase γ

POLRMT – mitochondrial RNA polymerase

RBBB – right bundle branch block

RC – (mitochondrial-) respiratory chain (i.e. MRC)

RITOLS – RNA incorporation throughout the lagging strand

RNA – ribonucleic acid

ROS – reactive oxygen species

RRF – ragged-red fiber

rRNA – ribosomal RNA

SAM – sorting and assembly machinery

SIIF – Social Insurance Institute of Finland

SNHL – sensorineural hearing loss

SNP – single nucleotide polymorphism

SSB – single-stranded DNA binding protein

TFAM – mitochondrial transcription factor A

TFB1M and TFB2M – mitochondrial transcription factors B1 and B2

TOAST – Trial of ORG 10172 in Acute Stroke

TP – thymidine phosphorylase

TYMP – thymidine phosphorylase

TIM – translocase of the inner membrane

TK2 – thymidine kinase 2

TOM – translocase of the outer membrane

tRNA – transfer RNA

TUH – Turku University Hospital

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications, which are referred to in the text by Roman numerals: Studies I – IV. In addition, this thesis contains unpublished data.

I Martikainen MH, Majamaa K. Epidemiology and characteristics of occipital brain infarcts in young adults in Southwestern Finland. *J Neurol* 2010; 257: 259-263.

II Martikainen MH, Hinttala R, Majamaa K. Novel *POLG1* mutations in a patient with adult-onset progressive external ophthalmoplegia and encephalopathy. *BMJ Case Reports* 2010. doi:10.1136/bcr.01.2010.2604.

III Martikainen MH, Hinttala R, Röyttä M, Jääskeläinen S, Wendelin-Saarenhovi M, Parkkola R, Majamaa K. Progressive External Ophthalmoplegia in Southwestern Finland: A Clinical and Genetic Study. *Neuroepidemiology* 2012; 38: 114-119.

IV Martikainen MH, Rönnemaa T, Majamaa K. Prevalence of mitochondrial diabetes in southwestern Finland: a molecular epidemiological study. *Acta Diabetol* 2012 Apr 11. [Epub ahead of print]

1. INTRODUCTION

Mitochondria are intra-cytosolic, intracellular organelles present in all eukaryotic cells. They are thought to be of bacterial origin (Sagan 1967), the result of endosymbiotic colonization of eukaryotic cells by aerobic bacteria more than 10^9 years ago. As reminiscent of their separate origin, mammalian mitochondria contain their own mitochondrial DNA (mtDNA), as well as mechanisms for RNA and protein synthesis. In every cell there is a variable amount (hundreds or thousands) of mitochondria, and in every mitochondrion there are several (~2-10) mtDNA molecules. The use of molecular oxygen in the conversion of the chemical energy contained in nutrients to adenosine triphosphate (ATP), the universal energy unit of cells, is a central function of mitochondria (van der Giezen and Tovar 2005). Mitochondrial respiratory chain (MRC) consists of a series of protein complexes located in the inner mitochondrial membrane. It produces ATP by oxidative phosphorylation (OXPHOS), the reduction of equivalents produced in the Krebs cycle and in the beta-oxidation processes (Hatefi 1985, Saraste 1999).

The concept 'mitochondrial disease' conventionally refers to disorders in which the etiology is defective MRC resulting in OXPHOS defect. Mitochondrial disease in humans can present at any age, and practically in any organ system. One of the most common molecular etiologies of mitochondrial disease in population is the m.3243A>G mutation in the mitochondrial *MT-TL1* gene, encoding mitochondrial $tRNA^{Leu(UUR)}$. The m.3243A>G mutation is the most common mutation in the MELAS (mitochondrial encephalomyopathy, lactic acidosis and stroke-like episodes) syndrome (Goto et al. 1990). Clinical evaluation of patients with m.3243A>G has revealed various phenotypes (Ciafaloni et al. 1992; Goto 1995; Majamaa et al. 1997 and 1998a; Kaufmann et al. 2009), including ischemic stroke, diabetes mellitus (DM), and sensorineural hearing loss (SNHL).

The prevalence and clinical characteristics of mitochondrial disease in population are not well known. Previous studies have addressed the prevalence and the various genetic etiologies of mitochondrial disease, but only rare studies have been strictly population-based. Previous experience in Finland shows that systematic search for patients with mitochondrial disease results in new diagnoses and increased understanding of these conditions (Majamaa et al. 1998a; Lehtonen et al. 2000; Hakonen et al. 2005).

We decided to perform a series of cross-sectional prevalence studies to identify patients with mitochondrial disease in the adult population of Southwestern Finland and to assess the prevalence and characteristics of mitochondrial disease, especially that associated with the m.3243A>G mutation, in this well-defined population. Furthermore, we evaluated the overall usefulness of the approach combining clinical epidemiology and molecular genetics in the study of mitochondrial disease in population.

2. REVIEW OF THE LITERATURE

2.1 Basics of mitochondrial biology

2.1.1 Structure of mitochondria

Mitochondria are intra-cytosolic, intracellular organelles present in all eukaryotic cells. They are composed of outer membrane, intermembrane space, inner membrane, and the matrix: the region inside the inner membrane. Mitochondria have classically been depicted as bean-shaped, separate organelles, but more recent research suggests that mitochondria are interconnected, and indeed form a dynamic network, that shows an active balance of transforming fission and fusion activities which also allow exchange of genetic material between mitochondria. Moreover, mitochondria are constantly actively transported within cells according to local metabolic demand. (Hollenbeck and Saxton 2005; Chen and Chan 2006; Detmer and Chan 2007; Herzig and Martinou 2008).

2.1.2 Origins of mitochondria and mitochondrial functions

Mitochondria are thought to be of bacterial origin (Sagan 1967), the result of endosymbiotic colonization of eukaryotic cells by aerobic bacteria more than 10^9 years ago. A more recent elaboration of the endosymbiotic theory proposes that present-day mitochondria of multicellular organisms as well as anaerobic variants such as hydrogenosomes (e.g. in *Cryptosporidium*) and mitosomes (e.g. in *Entamoeba*) are derived from of a proto-mitochondrial prokaryotic organelle (Martin and Müller 1998; Hackstein et al. 2006). Moreover, it seems that an important function to all mitochondria and mitochondria-like organelles is the biogenesis of Fe-S (iron-sulphur) proteins, inorganic cofactors essential for several cellular processes such as electron transfer, catalysis, and various regulatory processes (Beinert et al. 1997, Lill 2009). Downregulation of mitochondrial iron-sulphur cluster (ISC) biogenesis leads to nuclear genome instability (Veatch et al. 2009), and defective production of the ISCs by mitochondria have a role in the pathogenesis of several human diseases such as Friedreich's ataxia (Campuzano et al. 1996; Rötig et al. 1997; Rouault and Tong 2005; Lill 2009). Mitochondria enable eukaryotic cells utilize oxygen in the production of ATP in the OXPHOS system, the respiratory chain. It produces energy with superior efficiency compared to anaerobic glycolysis. The use of molecular oxygen in the conversion of the chemical energy contained in nutrients to ATP, the universal energy unit of cells, is a central function of mitochondria (van der Giezen and Tovar 2005). Besides ATP production, mitochondria are involved in several important cellular activities, such as apoptosis, pyruvate oxidation, Krebs cycle, beta-oxidation as well as the metabolism of amino acids, fatty acids, and steroids (Newmeyer and Ferguson-Miller 2003). Mitochondria are also involved in regulation of cellular calcium levels (Pozzan et al. 2000). Moreover, mitochondria are an important source of free oxygen radicals (reactive oxygen species, ROS).

2.1.3 Mitochondrial DNA

As reminiscent of their separate origin, mammalian mitochondria contain their own mitochondrial DNA, as well as mechanisms for RNA and protein synthesis. In every cell there is a variable amount (hundreds or thousands) of mitochondria, and in every mitochondrion there are several (~2-10) mtDNA molecules. The mtDNA is a 16,569 base-pair (bp), circular, double-stranded molecule which contains 37 genes: 2 rRNA genes for mtDNA translation, 22 tRNA genes, and 13 structural genes that encode MRC subunits (Anderson et al. 1981). There are no introns in mtDNA. The only major non-coding region in the molecule is the displacement loop (D-loop), which is a 1.1kb region that contains elements of mtDNA transcription and replication (Shadel and Clayton 1997; Greaves et al. 2012). The individual strands of the mtDNA molecules are denoted heavy (H) and light (L) strand because of their different buoyant densities in a cesium chloride gradient. L-strand transcription is initiated from one single promoter (LSP), whereas H-strand transcription is initiated from two specific and differentially regulated sites, HSP1 (H1) and HSP2 (H2) (Montoya et al. 1982).

Inside mitochondria, mtDNA is organized in nucleoprotein particles called nucleoids. The nucleoid, considered a heritable unit of mtDNA, may contain several copies of the mitochondrial genome as well as several different proteins (Wang and Bogenhagen 2006; Kucej and Butow 2007). The distribution of nucleoids during mitochondrial fission and fusion events and during cytokinesis affects the segregation, transmission and complementation of mitochondrial genomes. This has particular importance in the context of primary mtDNA diseases, in which heteroplasmic cells bear a mixture of healthy and mutated mtDNA molecules (Garrido et al. 2003). Cell fusion experiments have indeed demostrated that mitochondrial nucleoids and the respiratory complexes are mobile and diffuse efficiently into mitochondria previously devoid of mtDNA (Legros et al. 2004).

Mitochondrial DNA is maternally inherited, and paternal mtDNA is eliminated during early embryogenesis (Kaneda et al. 1995). Both selection and genetic drift are thought to have effect on mitochondrial DNA evolution (Elson et al. 2004; Jenuth et al. 1996 and 1997). The precise contributions of these phenomena are not clear and are debated. Mitochondrial DNA undergoes frequent adaptive evolution (Bazin et al. 2006). Genetic draft is a result of the ‘hitchhiking’ process associated with positive selection acting on beneficial mutations (Meiklejohn et al. 2007). The mitochondria and so mtDNA molecules are distributed randomly to oocytes, but there is strong selection against defective mtDNA during embryogenesis (Cree et al. 2008). In a study of primary oocytes from a woman who harbored the m.3243A>G mtDNA mutation the frequency distribution of mutation load indicated that random drift is the principal mechanism that determines the level of mutant mtDNA within individual oocytes (Brown et al. 2001). Deleterious mtDNA mutations are selectively eliminated from the female germ line, a process which minimizes their impact on population fitness (Fan et al. 2008). A recent study presented direct experimental observations of the fate of random mtDNA mutations

in the mammalian germ line demonstrating that a purifying selection process shapes mitochondrial sequence diversity (Stewart et al. 2008a). The concept of a bottleneck mechanism governing segregation of mtDNA in the mammalian maternal germ line is at present considered well established. Most damaging mtDNA protein-coding gene mutations are removed by a process of purifying selection during oocyte development. The molecular mechanisms for rapid purifying selection and bottleneck segregation are, however, currently not fully understood (Stewart et al. 2008b).

2.1.4 Replication of mtDNA

Mitochondrial DNA is replicated in a relaxed manner, that is, independent of the cell cycle (Clayton 1982). The core mtDNA replication machinery consists of the following components. First is the mtDNA polymerase, pol γ or POLG. It is a heterotrimer molecule that consists of a catalytic subunit (encoded by *POLG1*) and two accessory β-subunits (encoded by *POLG2*). The accessory subunits bind DNA and increase the processivity of POLG. Second core component is the mitochondrial helicase Twinkle (encoded by *PEO1*) that unwinds the double-stranded DNA. A third core component is mtSSB (encoded by *SSBP1*), the mitochondrial single-stranded DNA binding protein that enhances the functions of POLG and Twinkle (Mao and Holt 2009) and maintains the integrity of single-stranded DNA in replication process. Single-stranded DNA-binding proteins (SSB) are a class of proteins that bind single-stranded DNA with high affinity. They are involved in DNA metabolism in all organisms and serve a vital role in replication, recombination and repair of DNA. Human mtSSB is a bacterial-type SSB that has an important role in DNA replication and recombination as well as its repair through binding to single-stranded DNA (Wong et al. 2009). Many molecules of mammalian mtDNA hold a short third strand, so-called 7S DNA, whose regulation is poorly understood. In addition there is a role for mtSSB in the maintenance of 7S DNA (Ruhanen et al. 2010). Mitochondrial DNA replication requires also several topoisomerases, mtDNA ligase III, RNA primers, mtDNA primase (yet unidentified), RNase mitochondrial RNA processing endonuclease (RNase MRP), endonuclease G, and RNase H1 (Smits et al. 2010).

The replication process itself is different from that of nuclear DNA, and the precise mechanisms remain to be elucidated. Three models of mtDNA replication have thus far been suggested (Holt 2009; Mao and Holt 2009). These are the strand-displacement model (Clayton 1982; Shadel and Clayton 1997; Brown et al. 2005), the coupled leading- and lagging-strand DNA synthesis model (Holt et al. 2000), and a modification of the latter, delayed lagging-strand DNA synthesis with **R**NA **i**ncorporation **t**hrough**o**ut the **l**agging **s**trand (hence RITOLS) (Yasukawa et al. 2006). In the classical strand-displacement model, the replication proceeds from two unidirectional and independent origins in an asynchronous manner. The origin of H-strand replication in the D-loop starts the mtDNA synthesis, proceeding along the L-strand to produce a daughter H-strand. When the replication reaches the second origin, the L-strand synthesis launches to the opposite

direction. In the two latter, more recent models, a coupled leading and lagging-strand synthesis exists with the original strand-asynchronous mechanism (Smits et al. 2010).

2.1.5 Nuclear genetic control of mitochondria

Mitochondria are under dual genetic control, meaning that both mtDNA genes and nuclear genes are needed in the proper function of mitochondria. Most of ~1200 gene products in mitochondria are nuclear-encoded and derived from the cytoplasm. Majority of MRC subunits are nuclear-encoded, as are assembly factors of the OXPHOS complexes, the enzymes needed in the biosynthesis of MRC cofactors and non-protein constituents such as ubiquinone and cardiolipin. All proteins needed in the replication, transcription and translation of mtDNA are nuclear-encoded. Nuclear genes are also involved in protein import to mitochondria, biogenesis of ISCs, mitochondrial protein metabolism, and mitochondrial dynamics. Moreover, it is likely that several as yet unknown nuclear proteins are needed for sound mitochondrial structure and function.

2.1.6 Regulation of mtDNA expression

The regulation of mtDNA expression is crucial for normal mitochondrial function. Despite its importance for respiratory-chain function and cell physiology, surprisingly little is known about the mechanisms of mitochondrial transcription and how the levels of transcription are regulated in response to the metabolic need of the eukaryotic cell. The replication of mtDNA is initiated by RNA primers that couple replication with transcription. The mitochondrial genome is transcribed by a specialized machinery that includes a monomeric RNA polymerase (POLRMT or h-mtRPOL) (Tiranti et al. 1997), the mitochondrial transcription factor A (TFAM) and one of the two mitochondrial transcription factor B paralogues, TFB1M or TFB2M, the termination factor MTERF (Roberti et al. 2009), and others (Spinazzola and Zeviani 2009). Human mtDNA transcription can be reconstituted in a pure *in vitro* system consisting of a promoter-containing DNA fragment and recombinant TFAM, POLRMT, and TFB1M or TFB2M (Falkenberg et al. 2002). The adequate supply of the mtDNA building blocks, deoxynucleotides and ribonucleotides, is needed as well. In addition, the components needed for the formation of mitochondrial nucleoids are essential for successful mtDNA replication and transcription (Spinazzola and Zeviani 2009).

Mitochondrial transcription factor A (TFAM) was the first mammalian protein demonstrated to regulate mtDNA copy number in vivo (Larsson et al. 1998) and it is essential for mitochondrial biogenesis and maintenance as well as embryonic development. Human TFAM is a multi-functional protein, involved in different aspects of maintaining mitochondrial genome integrity. It is also a regulator of mtDNA copy number (Ekstrand et al. 2004). TFB1M and TFB2M are dual-function proteins that not only support mitochondrial transcription *in vitro* but also act as rRNA methyltransferases *in vivo*. Both TFB1M and TFB2M can form a heterodimeric complex with POLRMT.

However, POLRMT in complex with TFB1M or TFB2M cannot initiate transcription in the absence of TFAM (Asin-Cayuela and Gustafsson 2007). There are three mitochondrial transcription units (those starting at HSP1, HSP2 and LSP). Transcription termination of the HSP1 unit is mediated by MTERF, a 39-kDa protein that binds to a 28-bp region at the 3′ end of the $tRNA^{Leu(UUR)}$ gene in a sequence-specific manner (Kruse et al. 1989; Fernandez-Silva et al. 1997). The MTERF protein can terminate transcription *in vitro* (Yakubovskaya et al. 2010), but the functional role of the protein *in vivo* remains to be established. At present, the basic components of the transcription machinery in mammalian mitochondria are known and their mechanisms of action are gradually being established (Asin-Cayuela and Gustafsson 2007). Regulatory factors govern transcription levels both at the stage of initiation and termination, but the biochemical understanding of these processes is still largely missing.

2.1.7 Protein transport into mitochondria

The vast majority of proteins in any eukaryotic cell are synthesized on cytosolic ribosomes. However, only part (~50%) of these proteins function in the cytosol; the rest function in the plasma membrane or in the various cellular organelles. This is why these proteins must be translocated across or into one of the various membranes in the cell. Specific targeting signals and protein translocases are needed in this sorting process (Wickner and Schekman 2005, Rapoport 2007). Translocases recognize the targeting signals and mediate the transport of proteins accordingly across or into the specific membranes of the organelles. The transport of proteins into mitochondria is a particularly demanding task as it not only requires targeting to the organelle but it also necessitates the proper sorting of the proteins to the correct intramitochondrial compartment. 99% of mitochondrial proteins are encoded by nuclear genes and imported into mitochondria. The mitochondrial outer membrane contains α-helical proteins and β-barrel proteins. Whereas the import pathways of α-helical proteins are only partly understood, the pathway for β-barrel proteins has been characterized and shown to require the translocase of the outer membrane (TOM) complex, small translocase of the inner membrane (TIM) chaperones of the intermembrane space and the sorting and assembly machinery (SAM) complex of the outer membrane. Several small proteins of the mitochondrial intermembrane space contain characteristic Cys motifs. Most of these proteins are imported and folded by the redox-dependent mitochondrial intermembrane space assembly (MIA) machinery. The TOM complex is the main entry for most nucleus-encoded mitochondrial precursor proteins. Presequence-carrying preproteins are then imported by the presequence TIM23 complex and the presequences are proteolytically removed by specific processing enzymes. The precursors of hydrophobic metabolite carriers of the inner membrane are imported by the TOM complex. They are bound to small TIM chaperones in the intermembrane space. Then the precursors are inserted by the carrier translocase of the inner membrane (TIM22) complex. (Chacinska et al. 2009; Schmidt et al. 2010; Marom et al. 2011).

2.1.8 Maintenance of mitochondrial dNTP pools

Deoxyribonucleoside triphosphates (dNTPs) are the precursors used by DNA polymerases for replication and repair of nuclear and mitochondrial DNA in animal cells. Accurate DNA synthesis requires adequate amounts of each of the four dNTPs (deoxyadenosine-, deoxyguanosine-, deoxycytidine-, and thymidine triphosphate) and appropriately balanced dNTP pools. Both excess and deficiency of one dNTP may be detrimental (Rampazzo et al. 2010). Complex interlinked pathways in the cytosol and inside mitochondria regulate the maintenance of mitochondrial dNTP pools which in turn depend both on de novo production of dNTPs and the action of purine and pyrimidine salvage pathways. These are operated by the two mitochondrial deoxyribonucleoside kinases, thymidine kinase 2 (encoded by *TK2*) and deoxyguanosine kinase (*DGUOK*), the former active in intramitochondrial salvage of pyrimidine nucleosides, the latter in the salvage of purine nucleosides. In non-dividing cells, the burden of dNTP pool maintenance is on these enzymes, since the cytosolic thymidine kinase 1 and dNTP synthesis are down-regulated. (Copeland 2012). Thymidine phosphorylase (*TYMP*) has a role in the cytoplasmic breakdown of thymidine nucleosides. *RRM2B* gene encodes a subunit of the cytosolic enzyme ribonucleotide reductase that provides nucleotide precursors for nDNA repair and mtDNA synhesis by reducing ribonucleoside diphosphates to deoxyribonucleoside diphosphates. The p53-inducible form of the subunit (P53R2) is needed for basal level DNA repair and mtDNA synthesis in non-proliferating cells (Copeland 2012). Other proteins essential for dNTP pool maintenance are ANT1 (*SLC25A4*), adenine nucleotide translocator of the inner mitochondrial membrane that exchanges ATP with ADP in and out of the mitochondrial matrix; and the two subunits of succinyl CoA synthetase (*SUGLG1* and *SUCLA2*), that are involved in the citric acid cycle (Krebs cycle) and nucleoside salvage. Mitochondrial phosphate carrier (*SLC25A3*) transports inorganic phosphate into mitochondrial matrix. Finally there is *MPV17*-encoded protein, the exact function of which is unknown, but which is involved in dNTP metabolism (Copeland 2008, Smits et al. 2010, Ylikallio and Suomalainen 2012).

2.1.9 Translation of mitochondrial proteins

Human mitochondria contain their own genome, encoding 13 polypeptides that are synthesized within the organelle. The molecular mechanism of human mitochondrial translation has yet to be fully described. Translation of mitochondrial proteins is first possible when several previous steps have been successfully taken. For translation to take place, the replication, transcription, and maintenance of mtDNA must be intact. In addition, essential nuclear-encoded proteins must be successfully imported from the cytoplasm (Smits et al. 2010). The mitochondrial translation system has several original features. Firstly, mitochondria use a genetic code that differs slightly from the universal one. Secondly, mitochondrial mRNAs somewhat differ from cytoplasmic ones, thirdly, mitochondria utilize a simplified decoding in translation, and fourthly, mammalian mitochondria use only a single $tRNA^{Met}$ in initiation and elongation of translation,

instead of two as in prokaryotes, cytoplasmic tRNA in eukaryotes, and mitochondria in most lower eukaryotes (Smits et al. 2010).

The mtDNA translation machinery consists of mtDNA-encoded tRNAs and rRNAs in addition to nuclear-encoded intiation, elongation, and termination translation factors, mitochondrial ribosomal proteins, mitochondrial aminoacyl-tRNA synthetases and methionyl-tRNA transformylase (Smits et al. 2010). The translation machinery consists of two initiation factors IF2 (Ma and Spremulli 1995) and IF3 (Koc and Spremulli 2002). Human mitochondria have elongation factors mtEFTu, mtEFTs, and two homologs mtEFG1 and mtEFG2 (Hammarsund et al. 2001). MtEFGs catalyze the translocation step of protein biosynthesis. Transcription termination process in mitochondria is not completely understood. Two release factors, mtRF1 and mtRF1a as well as a recycling factor mtRRF have been recognized (Zhang and Spremulli 1998; Soleimanpour-Lichaei et al. 2007; Nozaki et al. 2008; Rorbach et al. 2008). Depletion of mtRRF in human cell lines is lethal. MtRRF co-immunoprecipitates a large number of mitoribosomal proteins attached to other mitochondrial proteins, including putative members of the mitochondrial nucleoid. Elongation factor mtEFG2 is also involved in ribosome recycling (Bhargava et al. 2004). The mitoribosome is a central component of the translation system for production of proteins encoded by the mitochondrial genome. Human mitoribosomes consist of 2 rRNAs (12S and 16S) and ~81 mitoribosomal proteins (MRPs) (Smits et al. 2007). Alternative foldings are an inherent property of RNA and a ubiquitous problem in scientific investigations. Human mitochondrial tRNAs (Suzuki et al. 2011) have a secondary cloverleaf structure, and relatively weak tertiary structure possibly due to lack of multiple conserved nucleotides. The proper tertiary structure is believed to be achieved by post-transcriptional base modification (Helm 2006). So far, 19 mitochondrial aminoacyl-tRNA synthetases have been identified.

There are probably several yet unknown factors that influence mtDNA translation. Moreover, translation regulation is poorly understood. In order to be functional, proteins need to be incorporated to the OXPHOS system in the inner mitochondrial membrane after translation. This process utilizes various chaperones, proteases and assembly factors for post-translational processing (Smits et al. 2010).

2.1.10 Mitochondrial dynamics

Contrary to common depiction in cell biology textbooks, mitochondria are not isolated intracellular organelles but form an interconnected network, in which mitochondria can be transferred to different locations inside the cell along cytoskeletal tracks (Hollenbeck and Saxton 2005; Detmer and Chan 2007). This movement is thought to reflect the energy-production needs of the cell. In addition, mitochondria themselves are not static but are in a continuous process of fusion and fission as well as undergo structural transformations (Detmer and Chan 2007; Westermann 2008; Chen and Chan 2009); this means that mitochondria are joined together and then again divided in a continuous process. During this process, mtDNA molecules are also re-distributed

between mitochondria (Chen et al. 2010). Indeed, the hundreds of mitochondria in a typical cell are probably best thought as a co-operative integrated network, constantly morphed by the fusion and fission processes, and the exchange of their contents, including mtDNA molecules (Detmer and Chan 2007). Dynamins are a superfamily of large guanosine triphosphatases (GTPases) that promote the fission and fusion of membranes. These mechano-chemical proteins are thought to be of bacterial ancestry (Low and Löwe 2006). The molecular mechanisms of mitochondrial dynamics are best understood in yeast, where the core fusion machinery consists of two GTPases, Fzo1 and Mgm1. Fzo1 resides in the outer mitochondrial membrane, and its human homologues are the mitofusins MFN1 and MFN2. Mgm1 is a dynamin-related protein in the inner mitochondrial membrane, and its mammalian orthologue is OPA1. In yeast, outer membrane protein Ugo1 links Fzo1 and Mgm1 together; so far, there is no known mammalian orthologue of Ugo1 (Detmer and Chan 2007). In mitochondrial fission, dynamin-related cytosolic proteins Dnm1 in yeast and DRP1 in mammals are needed, with other essential proteins such as Fis1 in yeast and FIS1 in mammals, respectively (Detmer and Chan 2007). Because the shape, cellular distribution, and interconnectivity of mitochondria are functionally important, the balance and rates of fission and fusion are tightly controlled with elaborate mechanisms (Westermann 2008). Regulators of mitochondrial fusion include pro-apoptotic Bcl-2 family members Bax and Bak, which induce fusion by regulating MFN2. The key mammalian fission machinery component DRP1 is controlled by ubiquitin ligase MARCH-V (or MITOL) and ubiquitin-like modifier SUMO (Westermann 2008). The transport of mitochondria along cytoskeletal filaments is managed by energy-dependent molecular motors. Kinesin and dynein family member proteins have a role in anterograde and retrograde mitochondrial transport (Hollenbeck and Saxton 2005).

2.1.11 Mitochondrial respiratory chain

MRC consists of a series of protein complexes located in the inner mitochondrial membrane. It produces ATP by the reduction of equivalents produced in the Krebs cycle and in the beta-oxidation processes (Hatefi 1985, Saraste 1999). MRC is composed of five polypeptide complexes that consist of multiple subunits: NADH dehydrogenase-ubiquinone oxidoreductase (complex I, 45 subunits); succinate dehydrogenase-ubiquinone oxidoreductase (complex II, 4 subunits, the only complex that is encoded solely by the nuclear genome); ubiquinone-cytochrome *c* oxidoreductase (complex III, 11 subunits); cytochrome *c* oxidase (COX; complex IV, 13 subunits); and ATP synthase (complex V, ~16 subunits). Thirteen MRC complex subunits are mtDNA-encoded: In complex I, NADH dehydrogenases 1-4, 4L, 5 and 6; in complex III, cytochrome *b*; COX I-III (MTCO1-3) in complex IV; and subunits a (A6) and A6L in complex V. Most (>67) MRC subunits are, however, nuclear-encoded. In addition to the five complexes, MRC requires two electron carriers: ubiquinone (coenzyme Q10) and cytochrome *c*. In serial oxido-reduction reactions that have various flavins, nicotinamides, cytochromes, iron-sulphur centres and copper ions as adjuvants, electrons are transferred through the

MRC complexes I-IV. At the same time, protons are pumped across the mitochondrial inner membrane from the matrix to intermembrane space at complexes I, III and IV. This process creates an electrochemical gradient that is in turn dissipated by ATP synthase that uses the influx of these protons to condensate inorganic phosphate and ADP to form ATP.

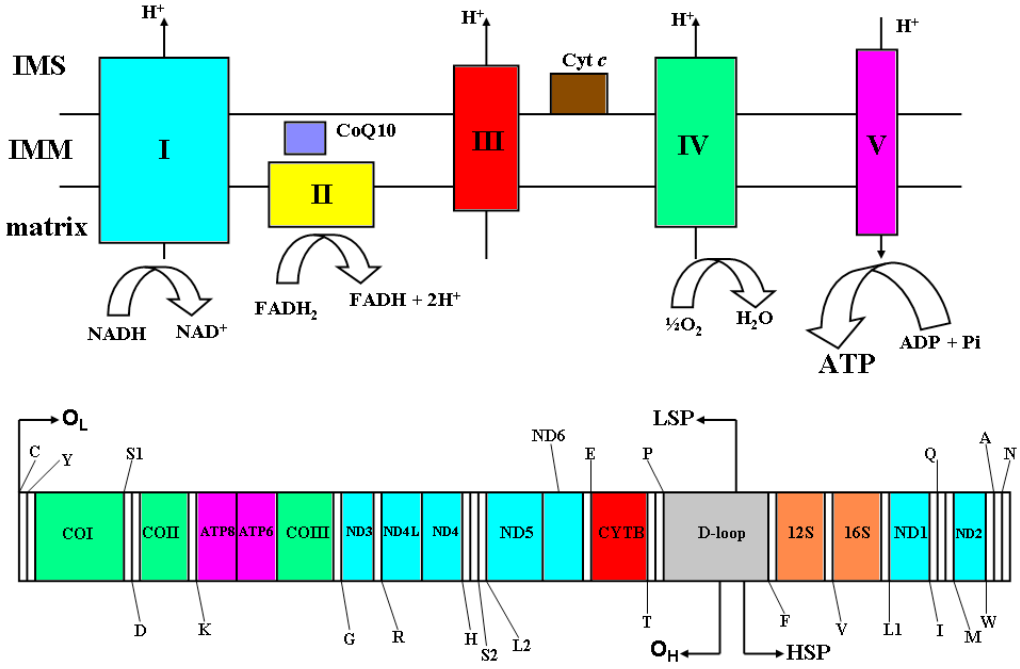

Figure 1. The OXPHOS system and mtDNA. Above: The OXPHOS system in a schematic, simplified form. Below: The circular, double-stranded human mitochondrial genome in linearized form starting at light-chain origin of replication (O_L). Turquoise, genes encoding subunits of RC complex I; red, the *MT-CYB* gene of complex III; light green, the catalytic subunits of complex IV; purple, the subunits of complex V; light brown, the 12S and 16S rRNA genes; white, the 22 tRNA genes denoted by the conventional single-letter amino acid abbreviations. The non-coding D-loop region is shown in grey. Genes in the light strand of mtDNA are marked above the genome; those in the heavy strand on or below the genome. The origins of replication O_L and O_H as well as transcription promoters of the light and heavy strands (LSP and HSP, respectively) are marked.

2.1.12 Mitochondrial DNA variation and mtDNA haplogroups

Mitochondrial DNA evolves rapidly compared to nuclear DNA (Brown et al. 1979 and 1982). The mtDNA mutation rate is ~10 – 17 times higher than that of the nuclear DNA (Vilmi et al. 2005). This is probably due to several factors, such as the lack of protective histones and non-coding mtDNA, the higher error rate of the replication

enzyme polymerase γ compared to the nuclear polymerase α, and proximity to the mutagenic ROS generated by the electron transport chain (Yarham et al. 2010; Smits et al. 2010). This results in mtDNA sequence variants that with time result in population-specific, recognizable patterns (Brown 1980; Denaro et al. 1981; Merriwether et al. 1991; Di Rienzo and Wilson 1991). MtDNA haplogroups can be formed according to similarities and differences between individuals with respect to patterns in this variation. This variation can be used in reconstruction of prehistoric origins (Cann et al. 1987) and migrations of human populations (Torroni et al. 1993 and 1998) and in estimations of genetic similarity between present human populations (Torroni et al. 1996; Ingman et al. 2000). When data on mtDNA variation is used with additional archaeological and linguistic data, human prehistoric evolution can be reconstructed with increasing precision (Cavalli-Sforza et al. 1988; Torroni et al. 2006). Additional information on Y-chromosomal variation increases further the accuracy of such population genetics analyses (Underhill et al. 2000; Lappalainen et al. 2008). In human mitochondrial DNA the sequence variation between populations is mostly considered to be neutral. However, several studies suggest that some haplogroups might interact with pathogenic mtDNA mutations and modify the severity of the resulting phenotype (Wallace et al. 1999); this has been reported e.g. in Leber's hereditary optic neuropathy (LHON) (Lamminen et al. 1997; Torroni et al. 1997). Haplogroups may also predispose individuals to disorders common in population (Gomez-Duran et al. 2010), such as type 2 DM (Achilli et al. 2011). A recent paper suggests that mtDNA variants might modulate the replication and transcription of mtDNA (Suissa et al. 2009).

A sequence variation in the major noncoding region of mtDNA was originally reported in patients with MELAS syndrome with the common m.3243A>G mutation (Morten et al. 1995; Marchington et al. 1996). In these patients, a transition at np 16 189 substituted a cytosine for a thymine residue creating a poly-cytosine tract which varied in length from 8–14 nucleotides. Later, this sequence variant has been associated with both insulin resistance (Poulton et al. 1998) and type 2 DM (Poulton et al. 2002), although a more recent study failed to support this connection (Das et al. 2007).

2.2 Mitochondrial disease – overview

2.2.1 Definition of mitochondrial disease

Traditionally, the concept 'mitochondrial disease' refers to disorder in which the etiology is defective MRC resulting in OXPHOS defect. Mitochondrial disease in humans can present at any age, and practically in any organ system. Although mtDNA is maternally inherited, the normal functioning of mitochondria is dependent on a manifold of nuclear factors; this is why mitochondrial disease – broadly defined – can be inherited in maternal, autosomal dominant, autosomal recessive, or X-chromosomal fashion (DiMauro et al. 2006). Commonly, the description of "a case of severe hypermetabolism of nonthyroid origin" with abnormal mitochondria and loosely coupled state of oxidative

phosphorylation (Luft et al. 1962) is considered as the beginning of mitochondrial medicine. It is peculiar that since then only one other patient with the same condition, in which the molecular etiology is still unknown, has been described (DiMauro and Garone 2010).

2.2.2 Particularities of mitochondrial disease

There are several distinct core features of mitochondrial disease due to mtDNA mutations. These have been described in detail in several recent reviews (DiMauro and Schon 2003; Taylor and Turnbull 2005; DiMauro and Schon 2008; McFarland and Turnbull 2009; Spinazzola and Zeviani 2009). Several basic rules of mitochondrial disease genetics differ from those commonly applied in disease due to nuclear genetic etiology. These include the concepts of *heteroplasmy* and *threshold effect*, as well as *mitotic segregation*, and *maternal inheritance* (DiMauro and Schon 2008). Heteroplasmy refers to the fact that various (i.e. not identical) types of mtDNA may be present in mitochondria of an individual, usually meaning some proportion of mutated mtDNA in addition to the normal genome. Threshold effect means that a minimum amount of mutation load (often ~80 – 90%) is needed to cause mitochondrial dysfunction; thus the threshold is "high and steep" (DiMauro and Garone 2010). On the other hand, threshold effect means that some individuals with low proportions of detectable mutated mtDNA can be clinically healthy and asymptomatic. Some mitochondrial DNA disease mutations are usually homoplasmic (i.e., 100% mutated mtDNA), but most mtDNA abnormalities are thought to be incompatible with life when homoplasmic. The three common mtDNA mutations encoding complex I subunits causing LHON are typically encountered as homoplasmic (Man et al. 2002), as is the m.1555A>G mutation in the mitochondrial 12S rRNA gene, causing aminoglycocide-induced hearing loss and non-syndromic sensorineural hearing loss (SNHL) (Prezant et al. 1993, Estivill et al. 1998). As to sporadic, large-scale mtDNA deletions, the pathogenic threshold seems to be somewhat lower (50 – 60%) (DiMauro and Garone 2010). Since the redistribution of mitochondria in the mitotic segregation of cell division is stochastic, the genetic constitution of daughter cells as to the mutated mtDNA can vary. If in this process a certain tissue reaches the pathogenic threshold, a new phenotype may arise. The exact process underlying mitotic segregation is unclear, although several plausible mechanisms have been suggested. This phenomenon also explains the tremendous age-related and tissue-related variability in mtDNA-related disease. Basically, mitochondria and so mtDNA derive from the ovum and so are maternally inherited (Giles et al. 1980). There is one reported case of paternal inheritance of mtDNA in skeletal muscle (Schwartz and Vissing 2002). This 'rule-confirming exception' was, however, encountered in association with a mitochondrial disorder.

A typical feature of mitochondrial disease is that tissues with high energy demands (e.g. brain, skeletal muscle, heart) are more sensitive to RC defects and thus these tissues are more prone to show clinical deficits. Common features present in patients with

mitochondrial disease include myopathy, including the sometimes focal presentation of PEO, axonal polyneuropathy, endocrinopathy (commonly DM), SNHL, ischaemic stroke or stroke-like episodes, cerebellar atrophy and ataxia, epilepsy, diffuse, often progressive encephalopathy, short stature, and others (Taylor and Turnbull 2005; McFarland et al. 2010). Important reason behind the perceived complexity of mitochondrial disease is the complex relationship between genotypes and phenotypes. The same genotype may result in a variety of clinical manifestations in patients, and conversely, the same clinical phenotype may be the result of various pathological genetic abnormalities (Zifa et al. 2007; Scaglia and Wong 2008). Generally speaking, the combination of such pathologies in several organ systems might lead one to consider the possibility of an underlying mitochondrial disorder.

2.2.3 Investigations of suspected mitochondrial disease

Because the genetic causes and clinical manifestations of mitochondrial disease are extremely variable, no straight-forward general guidelines for investigation of mitochondrial disease can be presented. However, there are several ways to guide the planning of investigations so as to make them as rational and resource-effective as possible (Haas et al. 2008, McFarland et al. 2010). A starting point is thus the clinical presentation of the patient. Sometimes the clinical features or constellation of core symptoms readily suggest a known mitochondrial syndrome, which in turn may guide molecular genetic investigations. In addition, the order of prevalence and likelihood of various mitochondrial disorders differ considerably depending on the patient's age; so that some conditions typically affect infants, others might be more probable in a late-onset phenotype. Obviously, eventual positive family history of similar or other suspicious symptoms is of interest. Mitochondrial disease can be sporadic (i.e. caused by a *de novo* mutation) or inherited according to materal, autosomal dominant, autosomal recessive, or X-chromosomal pattern. Thus, mitochondrial disease per se can not be excluded on grounds of any inheritance pattern suggested by the family history of an individual patient. However, the inheritance pattern may direct suspicions to certain direction. Simply put, clearly autosomal patterns favor nuclear genetic etiologies of mitochondrial disease, and maternal family history of the condition mtDNA-related etiology. If the family history should suggest X-chromosomal mode of inheritance the list of potential 'culprits' is narrowed down considerably. The precise selection of investigations depends on these pre-diagnostic considerations, but generally analysis of muscle histopathology (Filosto et al. 2007), biochemical analysis of RC function (especially in the pediatric population) and molecular genetic testing are most useful in obtaining the conclusive diagnosis of a mitochondrial disorder (Haas et al. 2008; McFarland et al. 2010). In muscle biopsy, abnormalities suggestive of mitochondrial disorder include COX-negative fibers and ragged-red fibers (RRFs) (Oldfors and Tulinius 2003; Filosto et al. 2007; Greaves et al. 2012). In brain imaging (computed tomography or MR imaging), possible findings include basal ganglia calcification, cerebellar atrophy (especially in POLG-related disease), and temporo-parietal ischemic lesions. Obviously, the findings compatible

with a stroke-like lesion should raise suspicion of mitochondrial disease (Oldfors and Tulinius 2003; Greaves et al. 2012).

Southern blotting and long-range PCR are used to detect mtDNA deletions. The possible presence of mtDNA depletion is investigated using real-time PCR (McFarland et al. 2010). If biochemical analysis of RC function points to a defect of single RC complex activity, the search for an underlying mutation is tailored accordingly. Investigating for the autosomal mutations underlying mitochondrial disease can be performed straightforward from DNA extracted from leukocytes of a conventional blood sample. For primary mtDNA defects, however, the task is more complex as the point mutations are most often in heteroplasmic state, and the level of heteroplasmy may differ considerably between different tissues (Shanske et al. 2004), being higher in postmitotic tissues such as skeletal muscle cells, urinary epithelium or hair follicles than in leukocytes (Sue et al. 1998b; Blackwood et al. 2010). Moreover, the heteroplasmy levels in leukocytes have been shown to decrease with time (Rahman et al. 2001), so that in some individuals, mutations with initially low level heteroplasmy may not be easy to detect at more advanced age. Thus, for the investigations of suspected primary mtDNA disease, a postmitotic tissue sample, such as buccal epithelium, urinary epithelium (McDonnell et al. 2004), or skeletal muscle tissue, usually results in higher detected levels of mtDNA heteroplasmy than a blood sample. Obviously, obtaining urinary epithelial cells from a urine sample is non-invasive and as such considerably more convenient for the patient than a muscle biopsy. However, the heteroplasmy levels sufficient to cause clinical manifestations are commonly high (DiMauro and Schon 2008), and leukocyte DNA is generally considered appropriate e.g. for the detection of m.3243A>G in patients with suspected MIDD (Maassen et al. 2005). Some mtDNA mutations, such as the common LHON mutations and the m.1555A>G causing non-syndromic and aminoglycoside-induced hearing loss, are usually encountered in homoplasmic (i.e. 100% mutant mtDNA) state (Taylor and Turnbull 2005).

2.3 Mechanisms of oxidative phosphorylation defects

2.3.1 Defects in mtDNA

Mitochondrial DNA abnormalities causing mitochondrial disease include several mechanisms (Di Donato 2009; Rötig 2010). 1. Rearrangements such as deletions and duplications of mtDNA (Holt et al. 1988; Poulton et al. 1989; Yamashita et al. 2008). Typical associated phenotypes are PEO and Kearns-Sayre syndrome (KSS). 2. Mutations in protein-coding mtDNA genes, such as the three common point mutations genes encoding RC complex 1 subunits that are the most common molecular aetiologies of LHON (Man et al. 2002). 3. Mutations in mitochondrial tRNA genes, which are a prevalent cause of mitochondrial disease (Zifa et al. 2007). The most common are syndromes MELAS, most commonly caused by the m.3243A>G mutation in the $tRNA^{Leu(UUR)}$ gene (Goto et al. 1990), and MERRF, most commonly due the m.8344A>G mutation in the $tRNA^{Lys}$ gene (Shoffner et al. 1990). 4. Mutations in rRNA genes, of

which the m.1555A>G mutation in the 12S rRNA gene, that is a common cause of mitochondrial sensorineural hearing loss either with or without previous exposure to aminoglycoside antibiotics (Prezant et al. 1993; Estivill et al. 1998).

2.3.2 Defects in nuclear genes

Several nuclear genetic defects may underlie clinical mitochondrial disorders (Di Donato 2009; Rötig 2010). These, in short, include: 1. Mutations in genes encoding MRC subunits or its ancillary proteins. 2. Mutations in genes involved in the MRC assembly machinery or proper MRC function. 3. Mutations in genes involved in mtDNA replication, maintenance and translation. These include genes like *POLG1* and *PEO1*; clinical phenotypes vary considerably, and often multiple mtDNA deletions or mtDNA depletion is detectable. 4. Mutations in genes involved in mitochondrial dynamics. 5. Mutations in genes that affect MRC in an indirect manner, such as defects in biosynthetic enzymes for cofactors or lipids (e.g. mutations in *TAZ* gene that encodes proteins involved in the synthesis of phospholipids of the inner mitochondrial membrane.

2.4 Important clinical syndromes mainly related to point mutations or large-scale deletions of mtDNA

2.4.1 Progressive external ophthalmoplegia and Kearns-Sayre syndrome

PEO is conventionally defined as progressive limitation of eye movements (external ophthalmoplegia) with normal pupils, and ptosis of the eyelids that is bilateral but not always symmetrical. The first clinical description of KSS dates back to 1958, when two patients with the symptoms of retinitis pigmentosa, external ophthalmoplegia, and complete heart block were described (Kearns and Sayre 1958). Later, the classical triad of KSS has been defined as symptom onset before age 20, pigmentary retinopathy, and PEO. Additionally, at least one finding of cardiac conduction block, cerebrospinal fluid (CSF) protein > 100mg/dl, or cerebellar ataxia, is required (DiMauro and Schon 2006). Many patients present with even more complex phenotypes including e.g. short stature, hearing loss, limb weakness, or encephalopathy (Yamashita et al. 2008). Symptom onset is typically in childhood with insidious ptosis and PEO, with progressive symptoms with age. Large-scale deletions of mtDNA were among the first mtDNA abnormalities found in human mitochondrial myopathies, including PEO (Holt et al. 1988 and 1989). These large-scale deletions were also found in majority of KSS patients (Zeviani et al. 1988; Moraes et al. 1989). However, the mutations are commonly not detectable in DNA isolated from blood, so a muscle biopsy is required to detect the deletion (DiMauro and Schon 2006).

2.4.2 Leber hereditary optic neuropathy

LHON has the merit of being one of the most common forms of inherited mitochondrial disease, with population prevalence of ~3.2/100000 in the North East of England population

(Man et al. 2003). LHON was also the first human condition proven to be caused by a defect of mtDNA (Wallace et al. 1988). Three point mutations encoding subunit of RC complex I m.11778G>A (Wallace et al. 1988), m.14484T>C (Howell et al. 1991; Huoponen et al. 1991), and m.3460G>A (Johns et al. 1992) are found in vast majority of patients with LHON (Mackey et al. 1996). Clinically, LHON is characterized by bilateral, subacute, painless visual failure due to degeneration of the retinal ganglion cells and the optic nerves (Man et al. 2003). Patients are typically young adults when the loss of vision occurs. In majority of patients, the causal mtDNA mutation is found in homoplasmic state. A notable feature of LHON is the incomplete penetrance and increased propensity of male mutation carriers compared to females of developing optic neuropathy (Harding et al. 1995; Riordan-Eva et al. 1995). This pattern initiated search for an X-chromosomal modifying gene, but so far results of research have been inconclusive (Vilkki et al. 1991; Pegoraro et al. 2003). Other studies suggest that mtDNA background modifies the LHON phenotype and propensity for developing optic atrophy (Carelli et al. 2006; Ghelli et al. 2009).

2.4.3 Mitochondrial encephalomyopathy, lactic acidosis and stroke-like episodes

MELAS is a multisystem mitochondrial disorder that is most commonly associated with the m.3243A>G mutation. This point mutation affects the mitochondrial *MT-TL1* gene that encodes tRNA for leucine (UUR) (Goto et al. 1990). The typical features include stroke-like episodes that begin at relatively young age, typically before age 40; encephalopathy that may present with dementia or seizures; and mitochondrial myopathy with histopathological features of cytochrome *c* oxidase (COX-) negative fibers or RRFs (Pavlakis et al. 1984). More detailed investigations of families with the m.3243A>G mutation have shown, however, that in addition to the classical MELAS phenotype, many patients present with DM, SNHL, low stature, exercise intolerance, and gastrointestinal complaints (Kaufmann et al. 2009). The clinical phenotype is related to the heteroplasmy level of the m.3243A>G, so that those with higher levels of mutated mtDNA tend to show more severe phenotypes. Classically, the stroke-like episodes have been considered the clinical hallmark of the MELAS syndrome. The occipital regions of the brain are frequently affected, and clinical signs of occipital brain dysfunction such as homonymous hemianopia or cortical blindness are not uncommon manifestations; migraine-like headaches are also relatively common in MELAS (Ciafaloni et al. 1992, Goto 1995). In brain imaging, the stroke-like lesions may change size and location with time (Iizuka and Sakai 2005). In addition to m.3243A>G, few other *MT-TL1* point mutations have been shown to result in MELAS phenotype, and comprise less than 15% of the MELAS phenotype. Other causal mutations of MELAS are, according to present knowledge, very rare (GeneReviews: MELAS).

2.4.4 Myoclonus epilepsy with ragged-red fibers

MERRF is one of the classical mitochondrial syndromes. Its defining characteristics include: myoclonus, generalized epilepsy, ataxia, and ragged-red fibers in muscle biopsy

(DiMauro and Schon 2006). Associated relatively common features include hearing impairment, axonal peripheral neuropathy, exercise intolerance, and dementia. The most common etiology of MERRF is the m.8344A>G point mutation in the mitochondrial $tRNA^{Lys}$ gene (Shoffner et al. 1990). This gene seems to be a hotspot for MERRF mutations, since other pathogenic point mutations in this gene resulting in MERRF phenotype have subsequently been described (Silvestri et al. 1992; Ozawa et al. 1997; Rossmanith et al. 2003). One of these, the point mutation m.8356T>C in $tRNA^{Lys}$, has in addition to MERRF been described in a MERRF-MELAS overlap phenotype (Zeviani et al. 1993). One of these four $tRNA^{Lys}$ point mutations is found in ~90% of patients with MERRF phenotype. In addition, point mutation m.611G>A (Mancuso et al. 2004b) in the *MT-TF* $tRNA^{Phe}$ and m.15967G>A mutation in *MT-TP* (Blakely et al. 2009) have been reported to result in MERRF phenotype; in the latter with pigmentary retinopathy. The m.3291T>C mutation in the $tRNA^{Leu(UUR)}$ gene has been reported in a complex phenotype with MERRF and KSS features (Emmanuele et al. 2011); in addition, a recent study reported the common MELAS mutation m.3243A>G in a 13-year-old girl with the classic MERRF phenotype (Brackmann et al. 2012).

2.4.5 Neuropathy, ataxia and retinitis pigmentosa

The syndrome of neuropathy, ataxia, and retinitis pigmentosa (NARP) is defined by the combination of sensory neuropathy, ataxia, seizures, dementia, and retinitis pigmentosa (DiMauro and Schon 2006). The most common molecular etiology of NARP is m.8993T>G point mutation in the *MTATP6* gene encoding the ATP synthase subunit 6 (Holt et al. 1990). The same mutation has been found quite common also in pediatric Leigh syndrome (Santorelli et al. 1993).

2.5 Nuclear gene defects mainly leading to secondary multiple mtDNA deletions

2.5.1 POLG-associated mitochondrial disease

Mitochondrial polymerase pol γ, or POLG, is the only human mtDNA polymerase (Clayton 1982). POLG is a 195kDa heterotrimer that consists of a 140kDa catalytic subunit and two accessory subunits of 55kDa size (Longley et al. 1998a and 1998b). The catalytic subunit is encoded by *POLG1* (Ropp and Copeland 1996); the accessory subunits by *POLG2* (Lim et al. 1999). The accessory subunits act as DNA binding factors, increasing the holoenzyme processivity. POLG mediates mtDNA replication and base-excision repair (Pinz and Bogenhagen 2000). POLG exonuclease and polymerase functions are essential for mtDNA maintenance (Spelbrink et al. 2000). Since the first report of a POLG-associated human disorder (Van Goethem et al. 2001), rapidly increasing evidence has proven that mutations in the *POLG1* gene are an important cause of human mitochondrial disease. Several recent reviews cover the wide spectrum of clinical phenotypes in POLG-associated disease (Horvath

et al. 2006; de Vries et al. 2007; Blok et al. 2009). Clinical phenotypes related to POLG mutations comprise a continuum of phenotypes that are partly overlapping. Broadly defined, these syndromes can be divided into the following groups (GeneReviews: POLG-related disorders): Alpers-Huttenlocher syndrome, spectrum of myocerebrohepatopathy syndromes with childhood onset, spectrum of ataxia-neuropathy phenotypes, including the distinct mitochondrial syndromes of sensory ataxic neuropathy with dysarthria and ophthalmoparesis (SANDO) and spinocerebellar ataxia with epilepsy (SCAE); and PEO with either autosomal dominant (adPEO) or autosomal recessive form of inheritance (arPEO). Mutations in *POLG1* are at present regarded as the most common autosomal dominant defect to cause PEO. In addition to ophthalmoplegia, several other clinical features such as parkinsonism, cerebellar dysfunction, dysphagia and dysphonia, can be present depending on the mutation. Also recessive mutations of *POLG1* are prevalent etiologies of PEO, with other possible clinical features being parkinsonism, peripheral neuropathy, depression and endocrine abnormalities. Mutations in *POLG2* have been reported in adPEO with multiple mtDNA deletions (Longley et al. 2006).

2.5.2 ANT1

ANT1 or *SLC25A4* (solute carrier family 25, member 4) encodes the isoform of mitochondrial adenine nucleotide translocator that is specific to skeletal muscle and heart (ANT1). ANT1 regulates the mitochondrial and cytosolic adenine nucleotide pools by exchanging ATP with ADP in and out of mitochondrial matrix. ANT1 protein forms a homodimer that is imbedded in the inner mitochondrial membrane (NCBI Gene website). Mutations of *ANT1* are a rare cause of slowly progressive, autosomal dominant PEO (Kaukonen et al. 2000). A recessive mutation in *ANT1* has been reported to cause hypertrophic cardiomyopathy, myopathy with exercise intolerance, RRF and lactic acidosis but without PEO (Palmieri et al. 2005). A patient with mutations both in *ANT1* and *POLG1* had initially PEO but later developed a more complex phenotype with cerebellar ataxia, peripheral neuropathy, parkinsonism and depression (Galassi et al. 2008).

2.5.3 PEO1

PEO1 or *C10orf2* (chromosome 10, open reading frame 2), encodes Twinkle, the sole mitochondrial DNA and RNA helicase, and so is centrally involved in the mtDNA replication alongside POLG, the mtDNA polymerase, and the mtSSB, encoded by *SSBP1*. The Twinkle helicase opens short stretches of double-stranded DNA in the 5' to 3' direction (NCBI Gene website). Clinically, as the gene name suggests, mutations of *PEO1* are mainly associated with PEO (Spelbrink et al. 2001), but also L-dopa responsive parkinsonism, ataxia, epilepsy, hearing loss, and optic atrophy have been described. In muscle biopsy, multiple mtDNA deletions are not always detectable. Mouse models have shown that PEO1 mutations result in multiple mtDNA deletions and a progressive

MRC dysfunction with COX deficiency. A specific point mutation in *PEO1*, Y508C, causes infantile-onset spinocerebellar ataxia (IOSCA) (Koskinen et al. 1994; Nikali et al. 2005).

2.5.4 OPA1

OPA1 gene encodes a dynamin-related GTPase-like OPA1 protein, that is involved in mitochondrial dynamics. OPA1 has been described in optic atrophy type 1, a dominantly inherited condition that results in progressive loss of visual aquity (Alexander et al. 2000; Delettre et al. 2000). Further studies have shown that OPA1 results also in more complex phenotypes ('optic atrophy plus') with multiple mtDNA deletions (Amati-Bonneau et al. 2008; Yu-Wai-Man et al. 2010).

2.5.5 MFN2

MFN2, mitofusin 2, is the human homolog for the transmembrane GTPase encoded by the *fzo* gene in yeast and *Drosophila* (Bach et al. 2003). It is another dynamin-like GTPase protein that is involved in mitochondrial dynamics. Expression of MFN2 is crucial in proper mitochondrial metabolism (Bach et al. 2003). MFN2 tethers endoplasmic reticulum (ER) to mitochondria; this contact is necessary for uptake of Ca^{2+} ions to mitochondria (de Brito and Scorrano 2008). The encoding gene *MFN2* has previously been shown to be mutated in Charcot-Marie-Tooth 2A type hereditary neuropathy (Züchner et al. 2004). The clinical picture of MFN2-associated disease was later shown to be more complex with reports of axonal neuropathy with optic atrophy (Züchner et al. 2006) and other complex phenotypes such as axonal neuropathy with associated cognitive impairment, corticospinal tract involvement, and sensorineural hearing loss (Del Bo et al. 2008). Quite recently mutation of *MFN2* has been shown to cause optic atrophy 'plus' phenotype with multiple mtDNA deletions (Rouzier et al. 2012). At present, the exact mechanism that causes mtDNA deletions and their clonal expansion in the context of abnormalities of mitochondrial dynamics is a target of intensive investigation (Yu-Wai-Man and Chinnery 2012).

2.6 Mitochondrial disease due to mtDNA depletion

2.6.1 Overview of mtDNA depletion syndromes

Mitochondrial DNA depletion syndromes are autosomal recessive conditions in which mtDNA copy numbers are profoundly decreased in affected tissues (Spinazzola and Zeviani 2008). Maintenance of adequate mtDNA copy numbers is dependent of supply and balance of mitochondrial dNTP pools as well as on functional mtDNA replication system. Three main presentations include myopathic (associated with *TK2* and *RRM2B* mutations), encephalomyopathic (*SUCLA2*, *SUCLG1* mutations) and hepatocerebral (*PEO1*, *POLG1*, *DGUOK*, *MPV17* mutations) (Spinazzola and Zeviani 2008).

Mitochondrial DNA depletion syndromes typically affect infants and children, although in *POLG1* mutations, the age of onset may be more variable.

2.6.2 Mitochondrial neurogastrointestinal encephalomyopathy

With the discovery that thymidine phosphorylase deficiency causes destabilization of mitochondrial DNA and a severe multisystemic syndrome (Nishino et al. 1999) the importance of dNTP pool balance was extended to mitochondria. Following that first discovery, mutations in other genes coding for mitochondrial or cytosolic enzymes of dNTP metabolism have been associated with mitochondrial DNA depletion syndromes. Mitochondrial neurogastrointestinal encephalomyopathy (MNGIE) is an autosomal recessive multisystem disease, characterized by features of PEO (ptosis and external ophthalmoplegia), peripheral neuropathy, severe gastrointestinal dysmotility, and leukoencephalopathy (Hirano et al. 1994). Age of onset varies considerably (from infancy to 43 years, average age of onset 19 years). This disorder results from loss-of-function mutations in *TYMP*, a gene encoding thymidine phosphorylase (TP). This enzyme catalyzes the reversible phosphorolysis of thymidine. The produced molecules are then utilized as carbon and energy sources or in the rescue of pyrimidine bases for nucleotide synthesis. In MNGIE, the defect of TP function leads to accumulation of thymidine and deoxyuridine, which, in turn, lead to depletion and multiple deletions of mtDNA, resulting in RC defect (Nishigaki et al. 2003). Thus, MNGIE can be classified to the group of mitochondrial disorders due to nucleotide pool imbalances (DiMauro and Schon 2006).

2.6.3 RRM2B

RRM2B gene encodes small subunit of the cytosolic p53-inducible ribonucleotide reductase. Its mutations were first shown to cause severe mtDNA depletion (Bourdon et al. 2007). Later, mutations in *RRM2B* were reported in adPEO with multiple mtDNA deletions (Tyynismaa et al. 2009). At present, it is not known how large a proportion of PEO is due to mutations in *RRM2B*, but a recent study suggests that these mutations might be frequent in familial PEO (Fratter et al. 2011). In addition, mutations of *RRM2B* have been associated with the MNGIE syndrome (Shaibani et al. 2009).

2.7 Leigh syndrome

Leigh syndrome (LS) is an extremely variable and multi-etiological entity in mitochondrial disease (Rahman et al. 1996; Dahl 1998). Typically, LS is a devastating encephalopathy of infants and children, and results from a severe defect of the mitochondrial OXPHOS. Clinically, characteristic features of LS include progressive psychomotor regression, hypotonia, ataxia, seizures, respiratory difficulties, dystonia, and problems with swallowing (Dahl 1998). In brain imaging, bilateral necrotizing lesions within the basal ganglia, thalami and in the brainstem are encountered (Lee et al.

2009). Lactate concentration is commonly elevated in blood and CSF. Genetic etiology is highly variable, and includes mutations in the nuclear *SURF1* gene (Tiranti et al. 1998), and mutations in various nuclear genes encoding for the subunits of complex I (Dahl 1998). The most common mitochondrial mutations causing Leigh syndrome are the point mutations m.8993T>G and m.8993T>C in the *MTATP6* gene encoding the F_O subunit 6 of complex V (Makino et al. 2000).

2.8 Accumulation and clonal expansion of mtDNA mutations

2.8.1 Overview

Mitochondrial DNA mutations (most commonly, deletions) accumulate with age. These mutations are known to expand clonally (Moslemi et al. 1996) so that identical mutant molecules become prevalent in mitochondria at close proximity to each other (Nekhaeva et al. 2002; Bua et al. 2006, Nicholas et al. 2009). Clonal expansion of mtDNA mutations is a widespread process in various human tissues (Coller et al. 2002). The mechanisms underlying the clonal expansion of mtDNA mutations are not yet fully elucidated. This phenomenon is likely to be of major importance in the context of both mtDNA dysfunction in both neurodegenerative conditions and healthy aging (Nekhaeva et al. 2002; Khrapko and Vijg 2009). It has been suggested that mtDNA deletions are most likely to occur during repair of damaged mtDNA rather than during replication (Krishnan et al. 2008). The sizes of clonal expansions appear to span a wide range and thus, may affect samples of various sizes, from individual cells to individuals (Khrapko et al. 2003). The clonal expansion of a single type of mutated mtDNA might arise as a purely stochastic process (Elson et al. 2001) due to random drift. In addition, the mtDNA molecules with deletions may have replicative advantage because of their smaller size compared to wild-type molecules (Hayashi et al. 1991; Diaz et al. 2002).

2.8.2 Mitochondrial dysfunction in inclusion body myositis and inflammatory muscular disease

The presence of COX-negative fibres and mtDNA deletions in muscle of patients with inclusion body myositis (IBM) was demonstrated already twenty years ago (Oldfors et al. 1993; Oldfors et al. 2006). PCR analysis of isolated, single muscle fibers showed presence of mtDNA with only one type of deletion (clonal expansion) and deficiency of wild-type mtDNA in each COX-deficient muscle fiber (Oldfors et al. 1995). Further studies indicated that common factors were involved in the development of multiple mtDNA deletions in IBM, autosomal dominant PEO, and aging (Moslemi et al. 1996 and 1997). Multiple mtDNA deletions and COX-negative fibers over the amount expected due to normal ageing have been found as well as in patients with polymyositis and dermatomyositis (Chariot et al. 1996; Blume et al. 1997). Commonly these changes have been interpreted as secondary, but the precise mechanism that causes these changes is unclear.

2.8.3 Mitochondrial dysfunction in ageing and neurodegeneration

Mitochondrial dysfunction has been implicated in the context of normal ageing (Brierley et al. 1998; Kujoth et al. 2005) and in various neurodegenerative conditions (Filosto et al. 2011). The most robust evidence of mitochondrial dysfunction in a common neurodegenerative disease comes from studies in Parkinson's disease (PD) (Schapira 2008). Findings of multiple mtDNA deletions in the substantia nigra of patients with PD (Bender et al. 2006; Kraytsberg et al. 2006; Reeve et al. 2008) suggest that mitochondrial dysfunction might have a role in the pathogenesis of this neurodegenerative disease. Further, the fact that mutations in mitochondrial genes such as *parkin* (Kitada et al. 1998) and *PINK1* (Valente et al. 2004) result in parkinsonian phenotypes similar to that in idiopathic PD support the central role of mitochondrial dysfunction in both the hereditary parkinsonian syndromes and the idiopathic PD (Belin et al. 2008; Klein et al. 2009; Martin et al. 2011). Clinical parkinsonism is also encountered in association of other genetic defects resulting in mitochondrial dysfunction, such as mutations in *POLG1* (Luoma et al. 2004; Orsucci et al. 2011) and mutations in mtDNA (Horvath et al. 2007; Orsucci et al. 2011). Multiple deletions of mtDNA have been found in the cortical neurons of patients with secondary progressive multiple sclerosis (MS) (Campbell et al. 2011). These mtDNA deletions result in respiratory deficiency in the affected cells and probably contribute to the neurodegeneration observed in MS. In addition, signs of profound oxidative damage have been found in the brain cells of MS patients (Haider et al. 2011). Moreover, a phenotype identical to common MS has been reported in two patients with *POLG1* mutations (Echaniz-Laguna et al. 2010). Multiple mtDNA deletions with impaired RC function have been reported also in Alzheimer's disease (Krishnan et al. 2011). In a study of clinically typical amyotrophic lateral sclerosis patients, 46% had COX-negative fibers in muscle biopsy, and one had multiple mtDNA deletions (Crugnola et al. 2010).

The contribution of mitochondrial dysfunction (via the production of free oxygen radicals) as a fundamental cause of aging has been hypothesized decades ago (Harman 1956). Mitochondrial DNA point mutations, as well as large-scale deletions, have been shown to associate with cytochrome *c* oxidase deficient muscle fibre segments in ageing. Their focal accumulation causes significant impairment of mitochondrial function in individual cells in spite of low overall levels of mitochondrial DNA mutations in muscle (Fayet et al. 2002). The incidence of mutations in various aged tissues may be on the order of one mutant per mitochondrial genome copy, and most of the cells are likely to be affected by intracellular clonal expansions of mitochondrial genomes. Thus aged tissue may be considered a mosaic of cells with different mutant mitochondrial genotypes (Kraytsberg et al. 2003). Studies on the interconnections of mitochondrial dysfunction, accumulation of somatic mtDNA mutations and aging phenotype in the 'mutator mouse' (Trifunovic et al. 2004) further increased the interest in mitochondrial contributions to aging in health and disease. Intriguingly though, the aging phenotype of the mutator mouse does not seem to be caused by oxidative stress (Trifunovic et al. 2005). Recent deep-sequncing work on mutator mice suggests that most somatic

mtDNA mutations occur as replication errors during development and do not result from damage accumulation in adult life (Ameur et al. 2011). The premature ageing -like phenotype of the mutator mouse with defective mitochondrial polymerase γ and enhanced production of multiple mtDNA mutations (Trifunovic et al. 2004) has been interpreted to support the role of mitochondria also in normal ageing, but this view has also been contested (Khrapko and Vijg 2007; Vermulst et al. 2007; Kraytsberg et al. 2009). To make things more complicated, the so-called 'deletor mice' with defective mtDNA helicase Twinkle (Tyynismaa et al. 2005) accumulate multiple mtDNA deletions that result in progressive respiratory dysfunction and chronic late-onset mitochondrial disease phenotype. However, the deletor mice do not show premature aging, indicating that the accumulation of mtDNA deletions and progressive respiratory chain dysfunction are not sufficient to create a phenotype of accelerated or premature aging. Indeed, other mouse studies have even found that COX-deficient mice did not show elevated ROS production and actually showed less signs of neuronal oxidative damage than healthy littermates (Fukui et al. 2007). The role of somatic mtDNA mutations especially in the context of healthy human ageing is at present not quite clear and further investigations are needed (Larsson 2010).

2.9 Features of m.3243A>G –associated disease

2.9.1 Overview

The clinical features of m.3243A>G –associated disease are diverse. The classical phenotype of MELAS (Pavlakis et al. 1984), which was first associated with this mutation (Goto et al. 1990), is now considered a rather uncommon presentation among people with this mutation. DM, SNHL, short stature, migraine, myopathy, and PEO are among the reported phenotypical features. In individual cases, these features can present as various combinations. Generally it seems to be so that those patients with higher mutation heteroplasmy levels show more clinical associated features, and the full-blown MELAS syndrome is associated with the most severe disease. MIDD is a subtype of diabetes mellitus caused in most cases by the m.3243>G mutation (van den Ouweland et al. 1994).

2.9.2 Ischemic stroke, stroke-like episodes and m.3243A>G

Ischemic stroke or stroke-like episodes occur as complications of mitochondrial disease (Michelson and Ashwal 2004). Ischemic stroke may be the initial or sole manifestation of a mitochondrial disorder (Martínez-Fernandez et al. 2001). Especially the posterior part of the brain is vulnerable in mitochondrial disorders that manifest themselves in various ways. One possible manifestation is an episodic brain dysfunction that resembles ischaemic stroke (stroke-like episode). This is the case especially with the mtDNA m.3243A>G mutation (Goto et al. 1990). Clinical evaluation of patients with m.3243A>G has revealed various phenotypes, including stroke because of either

metabolic or ischaemic vascular processes (Ciafaloni et al. 1992; Goto 1995; Majamaa et al. 1997 and 1998a). The m.3243A>G mutation leads to stroke-like episodes that have a predilection in the occipital and parietal regions of the brain (Michelson and Ashwal 2004; Iizuka and Sakai 2005). Headache and seizure are common presenting symptoms (Iizuka et al. 2002). Other features commonly seen in MELAS patients include migrainous headache, seizures and hemianopia or cortical blindness (Pavlakis et al. 1984, Montagna et al. 1988), the latter two suggesting occipital cortical involvement in the disease process. In acute phase of a stroke-like episode, hypodensities in brain computed tomography (CT) imaging and hyperintensities in both T2-weighted and diffusion-weighted brain MR imaging are seen (Ohshita et al. 2000; Ito et al. 2008); these lesions are most common in the temporo-parieto-occipital regions of the brain (Michelson and Ashwal 2004). The stroke-like lesions seen in brain imaging evolve and spread with time (Iizuka et al. 2003). Later imaging commonly reveals brain atrophy and gliosis (Michelson and Ashwal 2004). The exact pathophysiology of stroke-like episodes and the resulting stroke-like lesions has been investigated with no conclusive results, one major obstacle being the differences in brain imaging findings between hyperacute, acute, and chronic stages of stroke-like episodes. Both primary cytopathy and angiopathy have been suggested (Ito et al. 2011). Some recent studies suggest that the stroke-like lesions could be caused by vasogenic rather than cytotoxic edema with hyperperfusion and neuronal damage (Ohshita et al. 2000, Ito et al. 2008). Underlying acute defect of oxidative phosphorylation in brain vessels is suggested by the findings of high mutated mtDNA content in brain vessels (Tokunaga et al. 1993; Betts et al. 2006). Cortical spreading depression is possibly involved in the topographic progression of the stroke-like episode (Betts et al. 2006).

There are few studies on the brain metabolism in patients with m.3243A>G mutation. In a positron emission tomography (PET) study on patients with the m.3243A>G mutation, the cerebral metabolic rate of oxygen was decreased in the grey as well as the white matter of the brain (Lindroos et al. 2009a). In the same study a decrease in the metabolic rate of glucose was found with predilection to the posterior part of the brain. These results suggest that the m.3243A>G mutation leads to a global decrease in oxygen consumption in the grey matter including areas where no other signs of disease were present.

In differential diagnostic perspective it should be noted that stroke-like episodes are not pathognomonic for the m.3243A>G mutation. Stroke-like episodes and histopathological findings of cortical brain infarcts have also been described in rare patients with m.8344A>G mutation, the most common mutation in the MERRF syndrome (Chinnery et al. 1997, Tanji et al. 2003). Recently, mutations in the mitochondrial polymerase γ (*POLG1*) have been associated with occipital epilepsy. In 11 out of 17 patients with occipital epilepsy and with *POLG1* mutations, brain MR imaging showed occipital cortical lesions similar to those found in MELAS patients (Engelsen et al. 2008).

2.9.3 Mitochondrial diabetes mellitus and m.3243A>G

The importance of maternal contribution to the inheritance of non-insulin dependent DM has been observed already 20 years ago (Alcolado and Alcolado 1991), and subsequent molecular genetic studies have identified maternally transmitted diabetes and deafness due to the m.3243A>G mutation in mtDNA (van den Ouweland et al. 1992; Reardon et al. 1992; Remes et al. 1993; Kadowaki et al. 1994). Mitochondrial diabetes (MIDD) is now recognized as a distinct subtype of DM that most commonly results from the m.3243A>G mutation in the *MT-TL1* gene (van den Ouweland et al. 1994; Maassen 2002). Previous studies have suggested a prevalence of ~1% for MIDD in diabetes patients (Murphy R et al. 2008). In a cohort of Finnish and Swedish patients with familial early-onset DM (Lehto et al. 1999) the m.3243A>G mutation was detected in three out of 115 families. In MIDD, the mean age at diagnosis of DM is around 37 years, with range from 11 to 68 years (Guillausseau et al. 2001; Murphy R et al. 2008).

The persistent hyperglycaemia characteristic of DM can be caused by impaired insulin production by the beta-cells of the pancreas or it may arise as consequence of the increased insulin resistance of tissues, importantly the skeletal muscle. The contributions of pancreatic beta-cell dysfunction and insulin resistance in skeletal muscle were investigated in a recent PET study. The results showed that in subjects with m.3243A>G skeletal muscle is insulin resistant even when pancreatic beta-cell function was not markedly impaired or glucose control compromised. Moreover, the results of the study suggested that both the skeletal muscle insulin sensitivity and the beta-cell function are affected before the onset of the mitochondrial diabetes caused by the m.3243A>G mutation (Lindroos et al. 2009b).

The association of mtDNA haplogroups with DM has been studied in different populations, although without conclusive results. In principle, mtDNAs of different haplogroups might be functionally different (Wallace et al. 1999). Previous studies in the UK (Chinnery et al. 2007) and Finnish populations (Mohlke et al. 2005) have reported no definite association of mtDNA haplogroups with type 2 DM. However, the possible associations of mtDNA haplogroups and maternal family history of DM in young adult patients with DM of any type are at present not well known.

2.10 Sensorineural hearing loss (SNHL) due to m.1555A>G and m.3243A>G mutations

SNHL is a common clinical feature in mitochondrial disease. It is encountered in association with several pathologic mutations (Taylor and Turnbull 2005, McFarland and Turnbull 2009) and both syndromic and non-syndromic forms occur (DiDonato 2009). Research suggests that in the population level, the most common molecular etiologies of mitochondrial hearing loss include the m.3243A>G and m.1555A>G mutations (Fischel-Ghodsian et al. 2004). A study in Japanese population found the m.3243A>G mutation in 3% of patients with bilateral SNHL of unknown origin (Oshima

et al. 1999), and a previous study in Finnish patients with matrilineal SNHL found this mutation in 4.3% of patients (Lehtonen et al. 2000). Prevalence of the m.1555A>G mutation in patients with hearing loss has been studied in different populations with prevalence figures around 3% (2.4 – 3.6%) (Usami et al. 2000; Østergaard et al. 2002; Wu et al. 2007; Nahili et al. 2010). Notably, a study of familial SNHL in Spain found m.1555A>G in 27.1% of the investigated families (Estivill et al. 1998). The age at onset of hearing loss is reported to be around 20 years in patients with m.1555A>G. The age range is wide being from 1 to 65 years, but ~80% of cases have age at onset before 40 years (Estivill et al. 1998).

As to m.3243A>G, the hearing loss is of cochlear origin, typically symmetrical, and steadily progressive (Sue et al. 1998a) with median age of onset at 34 years with considerable variation (10-50 years) (Uimonen et al. 2001). Specifically in MIDD, the diagnosis for SNHL has been reported around age 35 years, with range from 2 to 61 years (Guillausseau et al. 2001).

High population prevalence figure of 1 in 500 (Vandebona et al. 2009) for the m.1555A>G was found in an Australian adult population cohort study of 2856 subjects of European descent and over the age of 49 years. These subjects were not pre-selected for hearing loss. Similarly, a British birth cohort study of 9371 subjects resulted in a prevalence estimate of 1 in 520 for the m.1555A>G mutation (Bitner-Glindzicz et al. 2009).

2.11 PEO and mitochondria

PEO is a common manifestation of mitochondrial disease. Conventionally, PEO is defined as progressive limitation of eye movements (external ophthalmoplegia) with normal pupils, and ptosis of the eyelids that is bilateral but not always symmetrical. Some patients present with only ptosis without any apparent restriction of eye movements. Diplopia may occur. 'PEO-plus' patients have involvement of other organs as well (Van Goethem et al. 2003). Molecular etiologies of PEO include mitochondrial DNA (mtDNA) rearrangements such as large-scale deletions (Holt et al. 1988; Zeviani et al. 1988; Holt et al. 1989) and mutations in mitochondrial tRNA genes (Lauber et al. 1991; Moraes et al. 1993; Raffelsberger et al. 2001). Several nuclear gene defects may result in multiple mtDNA deletions and PEO with autosomal dominant (Zeviani et al. 1989) or autosomal recessive (Bohlega et al. 1996) mode of inheritance. These genes include *PEO1*, *ANT1*, *POLG1* and *POLG2* (Suomalainen et al. 1995; Spelbrink et al. 2001; Kaukonen et al. 2000; Van Goethem et al. 2001; Lamantea et al. 2002; Longley et al. 2006). Mutations in nuclear genes *OPA1* and *RRM2B2* have recently been identified as causes of multiple mtDNA deletions in patients with PEO (Amati-Bonneau et al. 2008; Hudson et al. 2008; Stewart JD et al. 2008; Tyynismaa et al. 2009; Fratter et al. 2011).

2.12 Treatment of mitochondrial disorders

At present, there are few treatments that can directly affect the pathophysiologic processes in mitochondrial disease (Chinnery et al. 2006). The mainstay of current therapeutic interventions is in amelioration of symptoms as well as in providing suitable physical therapy, psychological support and encouragement as well as treatment of various more manageable conditions secondary to mitochondrial disease (e.g. DM) (Horvath et al. 2008). Diabetes mellitus is a common feature of mitochondrial disease. The most common phenotype of mitochondrial DM is MIDD (van den Ouweland et al. 1994). Mitochondrial DM patients are commonly not overweight, and usually become insulin-dependent with time (Guillausseau et al. 2001; Maassen 2002; Murphy R et al. 2008). Use of metformin should be avoided in patients with mitochondrial DM, since it may predispose these patients of lactic acidosis (Mancuso et al. 2012). A notable point of concern is the risk of potentially fatal liver failure induced by sodium valproate antiepileptic medication in patients with POLG-associated disease (Tzoulis et al. 2006). Cardiomyopathy and cardiac conduction abnormalities are not uncommon in mitochondrial disease (Santorelli et al. 2001; Sproule et al. 2007). Patients with cardiomyopathy benefit from beta blockers, angiotensin converting enzyme (ACE) inhibitors or angiotensin 2 receptor antagonists; patients with Wolff-Parkinson-White syndrome and conduction block may require ablation therapy or eventually an implanted pacing device (McFarland et al. 2010). ECG recording should be obtained from all patients. Patients with PEO may benefit from operative treatment of ptosis or strabismus (McFarland et al. 2010), although the progressive underlying muscle disease may compromise results.

Aerobic exercise can improve the exercise intolerance common in mitochondrial myopathies (Taivassalo et al. 2006), and moderate strength training is thought to stimulate satellite cell activation (Murphy JL et al. 2008). A recent study showed that in people with mitochondrial disease, level of habitual physical activity is low (Apabhai et al. 2011). Overall, some evidence supports beneficial effect of moderate aerobic exercise and strength training in patients with mitochondrial myopathy (Voet et al. 2010).

In MNGIE, loss of TP activity results in toxic accumulations of the nucleosides thymidine and deoxyuridine that cause deoxynucleoside triphosphate pool imbalances. These imbalances cause mtDNA instability that in turn results in mtDNA deletions and depletion. Allogeneic hematopoetic stem cell transplantation is a promising therapy for MNGIE since it restores TP activity and toxic metabolites are eliminated (Hirano et al. 2006 and 2012). Based on preliminary clinical experience, a standardized treatment approach has been suggested (Halter et al. 2011). In addition, early results from a murine model suggest that hematopoietic gene therapy may be an alternative treatment option in MNGIE (Torres-Torronteras et al. 2011).

Coenzyme Q10 (CoQ(10), ubiquinone) is an antioxidant and essential electron carrier in the mitochondrial respiratory chain. Deficiency of CoQ(10) is a clinically and molecularly heterogeneous, autosomal recessive syndrome. Primary CoQ(10)

deficiencies, due to mutations in genes required for ubiquinone biosynthesis, and secondary deficiencies, caused by genetic defects not directly related to CoQ(10) biosynthesis, often improve with CoQ(10) supplementation (Quinzii and Hirano 2010; Hirano et al. 2012). An analog of ubiquinone called idebenone has been investigated in the treatment of conditions due to mitochondrial dysfunction such as LHON and Friedreich's ataxia. Two recent studies suggest that patients with LHON might benefit from idebenone treatment (Carelli et al. 2011; Klopstock et al. 2011). In patients with Friedreich's ataxia, however, idebenone did not improve overall neurological function (Lynch et al. 2010) or cardiac dysfunction in patients with Friedreich-associated cardiomyopathy (Lagedrost et al. 2011).

2.13 Genetic counseling

The genetic counseling of individuals and families with mitochondrial disease depends critically on the identification of causal genetic defect and thus the mode of inheritance. Since a clinical phenotype of mitochondrial disease can arise because of autosomal dominant, autosomal recessive, X-chromosomal, or, as for mtDNA, maternally inherited, genetic defect, no meaningful counseling is possible without the identified cause of the condition. For autosomal or X-chromosomal conditions, the form of inheritance is straightforward and the risk of transmitting the disease mutation to progeny can be calculated according to conventional methods. As to mtDNA mutations, however, things get decisively more complicated because of the heteroplasmy and mitotic segregation phenomena. It means that the proportion of mutated mtDNA in the ovum and then in the offspring cannot be reliably predicted by the heteroplasmy levels in the mother. Recent work on mitochondrial gene replacement and pronuclear transfer methods gives promise that possibly the risk of transmitting mutated mtDNA could be dealt with (Tachibana et al. 2009; Craven et al. 2010; Craven et al. 2011).

2.14 Clinical epidemiology

2.14.1 Overview

Epidemiology means the study of disease occurrence in the population level (Fletcher and Fletcher 2005; Rothman et al. 2008). Clinical epidemiology may be defined as the study of determinants and consequences of clinical decisions (Spitzer 1986). As a 'bridge science', it combines clinical medicine and epidemiology in that epidemiological data is used to answer clinical questions. The purpose of clinical epidemiology is to provide clinical decision making with information that makes decisions of patient care more valid and less prone to systematic error and chance (Fletcher and Fletcher 2005). Since the improvement of clinical decisions is a proximal objective of clinical epidemiological work (Spitzer 1986), it stands closer to the dilemmas of the clinical physician than more basic biomedical research or traditional epidemiology.

2.14.2 Fallibility of human heuristics and intuition. The need for clinical epidemiological studies

In clinical practice the ability of the doctor to suspect a given condition as a cause of the patient's symptoms and to rationally decide what investigations to pursue is crucial. Casual estimations and formulations of prevalence are highly ambiguous (Bryant and Norman 1980; Toogood 1980) and they do not lead to uniform practical conclusions. Research has shown that human thinking under uncertainty is prone to several biases that can lead to severe and systematic errors (Tversky and Kahneman 1974). Human decision making and the evaluation of probabilities and outcomes has as well been shown to depend strongly upon the formulation (framing) of the problem (Tversky and Kahneman 1981). This is why the common clinical work that often relies on heuristic rules such as familiarity of the condition, personal experience on a limited number of similar cases, and the like, may well lead to suboptimal decision making. Rational and cost-effective, evidence-based diagnostic algorithms instead of such *ad hoc* diagnostic decision-making are needed. Such guidelines, in turn, are based on knowledge on the true population prevalence and characteristics of the medical conditions, that enables the estimation of pre-test probabilities and planning of most prudent diagnostic testing. Such knowledge can be obtained via clinical epidemiological studies of disease in population.

2.14.3 Epidemiology of neurological disease

An early example of descriptive epidemiological study of neurological disease was conducted in the population of Rochester, Minnesota in the 1950s (Kurland 1958). Epidemiological studies in the field of neurology are particularly challenging, since reaching the correct diagnosis in an individual patient is not always straightforward (Kurtzke 1984). Due to diagnostic challenges and ascertainment bias, there is always some proportion of the true affected persons that are lost from the count of individuals recognized in an epidemiological study. Nevertheless, epidemiological studies may provide important information on the prevalence, incidence and clinical course of neurological disorders. This information is valuable when socioeconomic plans for adequate financial resources, facilities, and personnel are made to meet the burdens of illness (Kurtzke 1984). Increasing knowledge of genetic bases of neurological disease provides possibilities for more definite diagnoses and improved insight into the pathophysiological processes in various neurological conditions. An early epidemiological study of single-gene neurological disorders was conducted in the South Wales region of Great Britain (MacMillan and Harper 1991). In Scandinavian countries, reliable census data and communal, state-funded health-care systems provide excellent opportunities for conducting population-based studies. A Swedish study of the epidemiology of childhood neuromuscular disorders (Darin and Tulinius 2000) showed that these conditions were more common than was previously thought, thus representing the strength of conducting population-based studies instead of case series in these relatively rare conditions.

2.14.4 Prevalence studies in epidemiology

Why is there need for prevalence studies of medical conditions? The answer to this question is two-fold. Firstly, disease prevalence represents the total burden of illness in the population and so knowledge on prevalence helps appreciating the scope of health care needs of the population with respect to the condition in question. Secondly, cross-sectional prevalence studies form the basis of rational diagnostic testing, as knowledge on pre-test probabilities (determined by *a priori* prevalence of the disease and its typical characteristics, enabling probability assessment on individual basis) is crucial for choosing what tests to perform on which patients in a reasonable and cost-efficient way (Fletcher and Fletcher 2005).

2.14.5 The use of medical record data in prevalence studies

In order to make use of hospital patient records in epidemiological study, one must make sure that the population in question meets several requirements (Anderson et al. 1988). Population should be motivated to obtain medical care; relevant medical expertise should be available to the community; patient data should be recorded in a sufficiently detailed and standardized manner; the medical data should be available to investigators; and practically all patients of the community should be treated in the local medical care from which the patient data are obtained. Major drawback of using patient record data is that those persons with undiagnosed conditions are not included in the survey. This is why asymptomatic or very mildly symptomatic patients are less likely to be identified.

2.14.6 Prevalence studies of rare conditions

Rare disease is defined as one that affects fewer than 5 people per 10,000 (Health-EU). A central problem in the epidemiological study of rare populations is efficient sampling. In a world of scarce resources, economical data collection is of importance because screening costs increase rapidly as the rarity of the condition increases. A resource-effective way to estimate population prevalence of a rare condition is to use two-phase sampling (Kish 1965; Kalton and Anderson 1986). In this method, a relatively cheap but imperfect screening is performed first to select a subsample for more expensive (and more accurate) further investigations. The probability of a positive screening result in the presence of the disease (sensitivity) should be high (ideally, 1.0) and the probability of negative screening result in the absence of the disease (specificity) should as well be reasonably high (but not too high, to avoid false negatives) in the first phase of the study. This saves expenses (in terms of time, money, and labor) in the confirmatory second phase. These factors crucially affect the usefulness of the sampling method. Quite obviously, when a rare condition is investigated, the screening in the first phase must be carefully planned to be sensitive enough so that the number of false negatives in the first phase is negligible, otherwise the prevalence of the condition will be underestimated (Morvan et al. 2008). In addition to the two-phase method, multiple frames may be applied (Kish 1965; Anderson and Kalton 1990). Sometimes the total number of identified members

of the rare population can be increased by using multiple selection frames that are complementary; i.e. they select persons from the total population based on different characteristics. It should be noted that overlaps (i.e., multiple identification of the same individual from different frames) must be taken into account when this method is used.

2.14.7 Epidemiology of rare diseases, special features

The epidemiological study of rare diseases has distinctive features due to the very definition of these conditions. In particular, it is difficult to establish reliable prevalence data with conventional epidemiological methods. The prevalence figures based on patients already ascertained on clinical basis may represent too conservative minimum estimates. Moreover, these identified patients may be those with most severe or otherwise noticeable ('typical') clinical features. So it is possible that a number of patients with less severe or untypical phenotypes go unnoticed. In such case, the general understanding of the rare condition may be hampered by the biased data, leading to false impressions on the prevalence and characteristics of the condition. This, in turn, hinders identification of those patients who do not fit the stereotype of the 'typical' patient. For many rare conditions, prevalence data do not exist or are available only for few populations. It is well known that in many human diseases, the population prevalence figures vary considerably between different populations. There is no reason to suppose that this should be otherwise regarding many rare disorders.

2.15 Molecular epidemiology and Genetic epidemiology

2.15.1 Overview

Molecular epidemiology means the use of various molecular biological markers, such as gene mutations or polymorphisms, in the study of exposures, susceptibility, and outcomes in epidemiological research (Schulte and Perera 1998). Conventionally, genetic epidemiology refers to the study of genetic factors in the occurrence of disease in population level (Rothman et al. 2008). These definitions are in some ways overlapping, but generally genetic epidemiology refers to more large-scale association studies of various genetic markers with disease in population, whereas the methods of molecular epidemiology with respect to the use of molecular genetic data in the study of human disease can be applied also in studies of smaller scale.

In clinical medicine, the molecular genetic diagnosis represents a 'golden standard' for a definitive disease etiology. This type of diagnosis has obvious and important implications for the patient and the family. This alone is a good reason to pursue a genetic diagnosis when it is reasonable. The difficulties concerning the epidemiologic study of mitochondrial disease (there are similar difficulties in the study of any rare genetic disease) mentioned above are, however, practical and one may expect them to be amenable with increasing knowledge on mitochondrial disease. In addition, the developments in molecular genetic

methods allow one to carry out more extensive investigations faster and with less expense than before. With expanding knowledge on the molecular genetic background of several diseases, there are at present several thousands known genetic rare disorders (Orphanet). There is increasing awareness in the medical community of the group of rare diseases (Schieppati et al. 2008): conditions that as separate units are not significant in population level, but which are of self-evident importance to the affected persons and their families, and which as a group are not that rare.

2.15.2 Resource-effective use of molecular genetic testing in rare disorders

Individually inherited neurological (neurogenetic) disorders are rare; the typical prevalence being <1/10000 (Edlefsen et al. 2007). The knowledge of molecular genetic backgrounds of several conditions and the availability of potential molecular genetic testing has increased rapidly in recent years. The practical use of these tests, however, depends on the limited resources of both money and effort. Prudent use of these investigations requires primarily knowledge on the prevalence and characteristics of the condition in question, since these crucially influence the pre-test probabilities of testing (Reyna 2001). Clinical epidemiological studies of rare conditions are valuable in this perspective. Expert clinicians who ordered neurogenetic investigations in a tertiary center obtained a positive result overall in 21.5% of patients; in this material, the diagnostic yield for mitochondrial testing was 12.5% (Edlefsen et al. 2007). It should be noted that the patient material in such a tertiary center is already pre-selected in a way that should increase the pre-test probabilities considerably. This means that there is still room for improvement in the efficacy of the diagnostic processes. Importantly, data obtained from the patient files of such tertiary center should be used very cautiously when estimating the morbidity due to neurogenetic conditions in the whole population, since the number and characteristics of those patients who for any reason have not made it to the diagnostic process cannot be estimated based on such data.

2.16 Molecular Epidemiology of mitochondrial disease

Molecular epidemiological studies of mitochondrial diseases were not possible before knowledge on the genetic mechanisms underlying these conditions started to accumulate. In the late 1980s and early 1990s, the first reports on the molecular genetic etiologies of mitochondrial disorders were published. Single, large-scale mtDNA deletions in patients with mitochondrial myopathies (Holt et al. 1988), and, in more detail, in patients with KSS (Zeviani et al. 1988) and PEO (Moraes et al. 1989) were accompanied by the first published mtDNA mutation causing LHON (Wallace et al. 1988), and the common mutations of the mitochondrial MELAS and MERRF syndromes (Goto et al. 1990; Shoffner et al. 1990, respectively). In the 1990s, 2000s and onward, the knowledge on the molecular genetic backgrounds of mitochondrial disease has increased in proverbially exponential manner.

Mitochondrial disease as a group is today thought to represent one of the most common forms of inherited neuromuscular disorders. The population prevalence of mtDNA point mutations m.3243A>G and m.1555A>G has been estimated as high as 1:400 (Manwaring et al. 2007; Vandebona et al. 2009; Bitner-Glindzicz et al. 2009). It should be noted, however, that these figures do not represent clinically manifest mitochondrial disease, and it is unclear how these results should affect clinical practice. The prevalence of mitochondrial disease due to mtDNA mutations has been estimated to be ~9.2/100 000 (Chinnery et al. 2000; Schaefer et al. 2008). The overall prevalence of mitochondrial disease (of all genetic backgrounds) may be considerably higher, given that prevalence ~1:200 for pathogenic mtDNA mutations has been reported in population (Elliott et al. 2008).

The first population-based study on mitochondrial disease was conducted in the province of Osthrobothnia in Northern Finland. This study examined the prevalence of m.3243A>G in adult population (Majamaa et al. 1998a). In this population, several further studies have examined in more detail the characteristics of people with this mutation with respect to e.g. hearing loss (Uimonen et al. 2001) or myopathy (Kärppä et al. 2005). In addition, similar epidemiological approach has been used in investigating pediatric population with mitochondrial disease (Uusimaa et al. 2007) and patients with other mitochondrial genetic defects such as the m.8344A>G mutation associated with the MERRF syndrome (Remes et al. 2003) and mtDNA deletions (Remes et al. 2005). In addition, the epidemiology and characteristics of LHON have been investigated in the entire Finnish population, as part of the long tradition of Finnish LHON research (Puomila et al. 2007). Studies assessing the prevalence of mitochondrial disease have been carried out also in Sweden (Darin and Tulinius 2000; Darin et al. 2001), Northeast of England (Chinnery et al. 2000; Man et al. 2003; Schaefer et al. 2008) and Australia (Skladal et al. 2003).

There seems to be variation between populations in the prevalence of overall mitochondrial disease and also between prevalences of various molecular etiologies. For this reason, the results of studies performed in one population may not be directly applicable elsewhere. Moreover, there is lack of epidemiologically sound, population-based studies on the prevalence of mitochondrial disease. Studies in clinical epidemiology are not performed for solely academic purposes. Previous experience in Finland shows that systematic search for patients with mitochondrial disease results in new diagnoses for individual patients and families as well as increased understanding of these often complex conditions (Majamaa et al. 1998a; Hakonen et al. 2005).

Previous studies have addressed the prevalence and the various genetic etiologies of PEO, but these studies have not been strictly population-based (Agostino et al. 2003; Hudson et al. 2006; Virgilio et al. 2008). A study in a northern Finnish adult population determined the prevalence of large-scale mtDNA deletions, but the prevalence of any nuclear gene mutations was not investigated (Remes et al. 2005).

2.17 Genetic composition of the Finns. Implications for molecular epidemiology in Finland

Finland is considered a classic example of a genetic isolate (Lao et al. 2008; Nelis et al. 2009), and Finnish population has been thought to be genetically quite homogenous. However, the reality may be somewhat more complex. Several studies on mtDNA variation as well as Y-chromosomal variation and single nucleotide polymorphisms (SNPs) have revealed, somewhat contrary to previous presuppositions, substantial genetic differences between Finns living in the southern and western regions and Finns living in the northern and north-eastern parts of the country (Lappalainen et al. 2006; Salmela et al. 2008; Palo et al. 2009). These differences are probably due to the population history of Finland, i.e. the late settlement of the northern and north-eastern parts of the country by a relatively small founding population (Peltonen et al. 2000; Kere 2001; Norio 2003a; Norio 2003b). These findings highlight the fact that supposed genetic homogeneity on the basis of linguistic or cultural homogeneity of a population might turn out not to be supported by empirical evidence. In addition, prevalences of various genetic conditions might vary between different populations, so that, in case of Finland, previous results on the prevalence of mitochondrial disease in northern and north-eastern Finland might not be directly applicable to southern and western parts of the country. Furthermore, genetic studies show that the Finns, especially those from the southern and western parts of the country, have strong genetic similarities with other populations surrounding the Baltic Sea (Lappalainen et al. 2008) and as a group these populations have strongest genetic roots in Central Europe.

2.18 Why study *clinical molecular epidemiology* of mitochondrial disease?

The benefits of accurate data on the characteristics and prevalence of any medical condition apply to mitochondrial disease. However, there is also a need to develop algorithms for the rational pursuit of a diagnosis of mitochondrial disease. Despite the comparative rarity of these conditions individually, as a whole mitochondrial disease is one of the most common causes of inherited neurological disease in population. Moreover, the special characteristics of mitochondrial disease complicate the molecular genetic diagnostics: there are large overlaps between the clinical manifestations of various genetic defects, there is large variety in the phenotypes caused by the same genetic defect, and many individual features that are typical in mitochondrial disease are not so rare in the general population either (e.g. diabetes mellitus). All this adds up to a situation where the possibility of a mitochondrial disorder might be entertained among a great many of patients, but the scarcity of resources (including expert consultations and referrals, extensive clinical and molecular genetic investigations, and the related expenses) in practice require that the diagnostic efforts be directed according to best possible analysis of pre-test probabilities of reaching a correct diagnosis of a mitochondrial disorder. Thus studies that combine the clinical epidemiological and molecular genetic approaches in

the field of mitochondrial disease may help the clinician wondering whether her patient might have a mitochondrial disorder, and how she should proceed in trying to find out.

3. AIMS OF THE STUDY

Few population-based, epidemiologically sound studies have investigated the prevalence of mitochondrial disease among patients selected by clinical features that are commonly encountered in mitochondrial disease. For resource-effective clinical practice it is of interest to know what proportion of patients with such phenotypes have a clinically probable mitochondrial disease and in what proportion of these patients a relevant molecular diagnosis of a mitochondrial disorder can be achieved. In this thesis, the prevalence and clinical characteristics of mitochondrial disease, particularly that related to the m.3243A>G mutation, were investigated in the adult population of southwestern Finland among patients with occipital ischemic stroke, PEO, DM, and SNHL. These clinical cohorts were chosen based on previous experience in Finland (Majamaa et al. 1998a) and other previous reports on prevalent clinical features of mitochondrial disease (e.g. DiMauro and Schon 2003; Taylor and Turnbull 2005). In particular, the objectives of the study were as follows.

1. To determine the prevalence of the mtDNA mutations m.3243A>G and m.8344A>G and common *POLG1* mutations among young adults with occipital ischemic stroke and to study the possible associations of mtDNA haplogroups with occipital brain infarcts among these stroke patients.

2. To determine the prevalence of large-scale mtDNA deletions, mtDNA point mutations m.3243A>G, m.8344A>G, and mutations in the nuclear genes *ANT1*, *PEO1*, *POLG1*, and *POLG2* among patients with PEO.

3. To determine the prevalence of the m.3243A>G mutation among patients with DM and disease onset as young adults, and to study the possible associations of mtDNA haplogroups with DM among these DM patients.

4. To determine the prevalence of the m.1555A>G and m.3243A>G mutations among patients with early-onset severe SNHL.

5. To assess the overall usefulness of combined clinical epidemiological and molecular genetic methodology in the study of mitochondrial disorders.

4. PATIENTS AND METHODS

4.1 Setting

In Finland, the registers of the public health care system represent well the total morbidity of the population. Specialized medical care is provided at provincial level, and in the area of Southwestern Finland, it is provided by Turku University Hospital (TUH). For these reasons the medical charts of TUH provide a good representation of the prevalence of diseases in this population. Practically all young patients with an ischemic stroke in this region are referred to TUH, as are patients with conditions relevant in the differential diagnostics of PEO, such as suspected myasthenia gravis or neuromuscular disease, or patients in need of neuro-ophthalmologic or eye surgery evaluation. Patients with hearing loss requiring a hearing aid receive this equipment from the otorhinolaryngology (ORL) department hearing loss unit of TUH, in which there has since 1988 been a computerized record of all delivered hearing aids. Since these patients are also examined by the otorhinolaryngologists of the same unit, the medical charts of TUH contain these patients' clinical data relevant to the hearing loss.

In Finland, patients with DM receive special reimbursement for their expenses for both insulin and other types of medications for this condition. This reimbursement is provided by the Social Insurance Institution of Finland (SIIF). The reimbursement records of SIIF provide a good representation of the prevalence of DM in this population, since practically all patients with medically treated DM have this reimbursement benefit.

The population of Southwestern Finland was 455 584 on 31 December 2005 (the prevalence date for stroke and PEO cohorts), and 457,789 on 31 December 2006 (the prevalence date for the DM cohort).

4.2 Patient identification and clinical investigations

In all investigated cohorts, the patient had to be of Finnish origin. For the occipital ischemic stroke and PEO studies, the medical charts of TUH covering the years 1987 – 2005 were reviewed. We performed a computerized medical files search for relevant ICD-9 and ICD-10 diagnoses. In the occipital stroke cohort, the patients had to be aged 18 – 45 years at the time of diagnosis; in the PEO cohort, the patients needed to be at least 18 years of age in order to be included in the study. The medical charts of these identified patients were then reviewed. For the DM cohort, the reimbursement registers of SIIF were searched for patients that had been registered for special reimbursement for DM medication (insulin and all oral hypoglycaemic agents) during 1.1.1987 – 31.12.2006. The patients had to be 18 – 45 years old at the time of the reimbursement decision. For the SNHL cohort, the hearing aid records of TUH ORL hearing aid unit were searched

for patients that had been delivered a hearing aid during 1.1.1998 – 31.5.2009. The patients had to be 18 – 45 years old at the time of the hearing aid delivery.

In the stroke cohort, the patients were ascertained if they had an occipital brain infarct in CT or MR imaging or a homonymous hemianopia or quadrantanopia, suggesting an occipital infarct and with no signs of other etiology in brain imaging. We then reviewed all available medical charts of the ascertained patients, and data were collected concerning the previous medical history and risk factors for ischaemic stroke (detailed in Study I). The medical chart information did not allow the differentiation between migraine with or without aura. We used the TOAST criteria (Adams et al. 1993, Goldstein et al. 2001) in order to determine the etiology of ischemic stroke. We then mailed a request to all living identified patients, asking them to take part in the study by giving a blood sample for the analysis of possible mtDNA mutations. As to the PEO cohort, the patients were excluded, if they had diplopia because of strabismus, if they had myasthenia gravis and acetylcholine receptor antibodies, and if they had a single ocular muscle paresis or strictly unilateral ptosis. We included all patients with external ophthalmoplegia, multiple ocular muscle pareses, or bilateral ptosis. Also those patients in whom the medical record information was insufficient to confirm the exclusion criteria were included in the study.

All living patients in the DM cohort that were identified from the registers of SIIF were requested to take part in the study and to fill in a family and medical history questionnaire. Those patients who returned the questionnaire were then asked to give a blood sample for the analysis of possible mtDNA mutations. The received samples were analyzed for the mtDNA 3243A>G mutation and for mtDNA haplogroups. As to the SNHL cohort, those with conductive-type or mixed-type hearing loss were excluded by scrutinizing the medical records of the patients after the initial identification of patients with hearing aids. The remaining identified patients were asked to take part in the study and to fill in a family and medical history questionnaire. All patients were also asked to give a blood sample for the analysis of possible mtDNA mutations. The received samples were analyzed for the m.1555A>G and m.3243A>G mutations.

In the PEO cohort, the medical charts of the deceased subjects and those who declined to participate in the clinical study were reviewed to obtain the relevant clinical data. The living consenting patients were interviewed and examined clinically by a neurologist in order to determine whether the clinical features were compatible with mitochondrial disease. Head CT, echocardiography, 24-hour ECG recording, electroneuromyography (ENMG), and a set of laboratory examinations (detailed in Study III) were performed. Furthermore, a deltoid muscle biopsy was obtained for histopathological investigations and for molecular genetic diagnostics. The two-phased screening of the patient cohorts and the numbers of patients and obtained tissue samples (muscle biopsy in the PEO cohort, blood sample in others) for molecular investigations are depicted in Table 1.

Table 1. Original patient cohorts, number of remaining patients after first phase screening exclusions, and number of tissue samples obtained for molecular genetic investigations.

	Original cohort	Screened to second phase † N (%)	Samples for molecular investigations‡ N (%)
Occipital ischemic stroke	619	49 (7.9)	27 (64*)
PEO	620	10 (1.6)	6 (100*)
DM	1532	561 (37)	299 (53)
SNHL	379	231 (61)	52 (23)

DM = diabetes mellitus. DNA = deoxyribonucleic acid. PEO = progressive external ophthalmoplegia. SNHL = sensorineural hearing loss. † = percentage of original cohort. ‡ = percentage of those screened to second phase. Of the 49 stroke patients screened to second phase, 7 were deceased. Of the 10 PEO patients screened to second phase, 4 were deceased. * = percentage counted from the living patients screened to second phase.

The patient described in Study II was a member of the PEO cohort (one of the deceased patients). The clinical and molecular investigations in this patient had been performed previously on clinical grounds; the case report was published *post mortem*. Muscle biopsy from the right vastus lateralis muscle had been obtained for histopatological investigations during life. Other clinical investigations of this patient are described in Study II.

4.3 Molecular methods

Total DNA was extracted from blood by using QIAgen Blood Kit (QIAgen, Hilden, Germany). From muscle specimen, total DNA was extracted by using the standard sodium dodecyl sulfate–proteinase K method.

The molecular methods for detecting the m. 1555A>G, m.3243A>G, and m.8344A>G point mutations using restriction-fragment analysis and for the determination of mtDNA haplogroups were as described elsewhere (Zeviani et al. 1991; Prezant et al. 1993; Torroni et al. 1996; Majamaa et al. 1998a). Deletions of mtDNA were analyzed by long range PCR (Expand Long Template PCR System kit; Roche, Mannheim, Germany).

In the stroke cohort, the *POLG1* gene (NM_002693) was analyzed for seven common mutations. Restriction fragment analysis was used to detect the p.T251I (BseNI), p.A467T (MscI), p.G517V (BstXI), and p.P587L (XmaI) mutations. The p.R722H mutation was detected by restriction fragment analysis using a mismatch primer that creates a restriction site for MlsI in the presence of the mutation. The p.W748S mutation and the p.Y955C mutation were detected by allele-specific amplification using primers with a locked nucleic acid 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. In the PEO study, muscle DNA was used as a template to amplify and sequence the 23 coding exons of the *POLG1* gene (NM_002693) by automated

sequencing (ABI PRISM 3100 Genetic Analyzer, Applied Biosystems, Foster City, CA, U.S.A.) using the BigDye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems) after treatment with exonuclease I and shrimp alkaline phosphatase. The novel sequence variants found in *POLG1* (Study II) were confirmed using restriction fragment length polymorphism analysis, and the the variants were additionally assessed using segregation analysis and PolyPhen (HumVar) and SIFT predictions. *ANT1* and *PEO1* genes were sequenced and analyzed for mutations as described elsewhere (Kaukonen et al. 2000; Spelbrink et al. 2001). *POLG2* gene was analysed as described (Ferraris et al. 2008) with minor improvement to cover the whole coding region of the gene.

4.4 Statistical methods

In the PEO cohort, Poisson confidence intervals (CI's) were used to estimate the population prevalences. In the DM and SNHL cohorts, binomial confidence intervals (CI's) were used for the number of identified patients with mtDNA mutations. For the DM cohort, modified Wald method and Fisher's exact test with two-tailed p values were applied in the analysis of haplogroup frequencies.

4.5 Ethical considerations

The study protocols were approved by the Ethics Committee of TUH. Permission for the use of the TUH patient medical chart data for this study was obtained from Finland's Ministry of Social Affairs and Health. A written informed consent was obtained from all patients who took part in these studies. As to the case report, written informed consent for the publication was obtained from the late patient's daughter.

5. RESULTS

5.1 Clinical characteristics and investigations – Occipital stroke and PEO cohorts

In the occipital stroke cohort, we identified 49 patients (31 women) with an occipital brain infarct. The clinical chararcteristics and etiologic risk factors of stroke are detailed in Study I. The living patients in the PEO cohort fulfilling the inclusion criteria were examined clinically (P1 – P5) or the available medical history (P6) was reviewed. The clinical diagnoses of the four deceased patients (D1 – D4) were evaluated on the basis of available medical history information (Study III, Table 1.). Patient D2 (Study II) was identified in the medical record search for PEO patients, but had been investigated already previously on clinical grounds.

5.2 Clinical history of the PEO patient with *POLG1* mutations

The patient D2 was a woman with an uneventful medical history until the age of 50 years when she had breast cancer operated. Axillary lymph node evacuation and postoperative radiation therapy was performed at age 54 years. She had a history of psychiatric symptoms of unknown quality, and she had been on anti-depressive medication. Her parents had had no known medical conditions. Her father had died at age 75 years and her mother at age 85 years. The patient was her parents' only child. She had two healthy adult daughters.

At age 64 years she was referred to ophthalmologist for surgical treatment consideration because of bilateral ptosis. She had no history of diplopia, headache, or difficulties with swallowing. At that time her medication consisted of bisoprolol, losartan, quetiapine, and escitalopram. The ophthalmologist confirmed bilateral ptosis, but in addition, external ophthalmoplegia was diagnosed. Neurological examination revealed symptoms of diffuse encephalopathy: The patient presented with general cognitive slowness, problems in understanding and following commands in clinical examination, confusion and disorientation. She also showed symptoms of echolalia, automatic laughter, and general clumsiness. There were no signs of hemiparesis. Tendon reflexes were weak but symmetric, and she had flexor plantar responses. The disease history and the clinical assessment were not suggestive of dementia of Alzheimer type. The patient had no history of seizures or other symptoms suggestive of epilepsy. Her Mini-Mental State Examination (MMSE) score at age 64 years was 27 points out of 30, which is decreased but not indicative of dementia. MMSE was not repeated, but later clinical notes indicated definite progression of the cognitive problems leading to dementia at age 67 years. At that time, the patient had slowly progressive symmetric limb muscle weakness. She was not able to move unaided and was not able to live independently. She had severe bilateral

ptosis and complete external ophthalmoplegia. She died from pneumonia at age 67 years in a nursing home.

In this patient, histological examination of a sample from the right vastus lateralis muscle showed abnormally frequent COX-negative fibres at age of 64 years. Electrophysiological examination revealed myopathic changes that were most prominent in facial muscles. Furthermore, a sensory more than motor, axonal neuropathy was observed. Results of other clinical investigations are detailed in Study II.

5.3 Molecular investigations – Occipital stroke and PEO cohorts

We requested blood samples from the 42 living patients with occipital stroke, and received samples from 27 patients (21 women). None of the 27 samples harbored the m.3243A>G or the m.8344A>G mutation or any of the investigated common mutations in the *POLG1* gene (0 – 13%; 95% confidence interval). As to the molecular findings of the investigated patients in the PEO cohort, patient P1 had been found to harbor the common 5-kb deletion in mtDNA and she was the only subject with a previous molecular genetic diagnosis. None of the patients harbored mutations in *ANT1*, *PEO1*, or *POLG2* genes. Patients P1 and P5 were heterozygous for the c.3708G>T (p.1236Q>H) transversion in the *POLG1* gene. This is considered a neutral polymorphism. Patient P2 had c.2492A>G (p.831Y>C) in *POLG1* in heterozygous state. This change is also considered a neutral polymorphism, although it has been previously reported in patients with autosomal dominant PEO and parkinsonism (Mancuso et al. 2004a). None of the patients harbored mtDNA point mutations m.3243A>G or m.8344A>G. The long-PCR analysis confirmed the common 5-kb deletion in mtDNA of patient P1, but no large-scale or multiple mtDNA deletions were detected in the remaining patients. The deceased patients D1 and D3 in the PEO cohort had previously been diagnosed with KSS and a large-scale mtDNA deletion had been detected in Southern blotting (Study III, Table 1.).

Patient D2 of the PEO cohort had been investigated previously on clinical grounds because of suspected mitochondrial disease, and DNA had been extracted from muscle biopsy for molecular investigations. Initial analysis for the common mtDNA point mutations m.3243A>G, m.8344A>G, and m.8993T>C, as well as southern blot analysis to demonstrate large mtDNA deletions had been negative. Further molecular analysis with long range PCR revealed multiple mtDNA deletions. Sequencing of the entire *POLG1* gene revealed two heterozygous nucleotide substitutions, c.2993C>T (p.998S>L) and c.3550G>C (p.1184D>H). Both were previously unreported. PolyPhen (HumVar) and SIFT predictions of these changes suggested that these compound heterozygous changes were pathogenic and caused the patient's phenotype. The two daughters of the patient as well as one child of the elder daughter harboured heterozygous p.1184D>H, but p.998S>L was found only in the proband. These findings confirmed that the two novel base exchanges in the proband were heterozygous *in trans*.

In total, six of the ten patients identified in the PEO cohort (P1, P4, P5, D1, D2, and D3) had clinically definite PEO. Four of these six patients had a definite mitochondrial molecular etiology for their condition. Three patients (P1, D1, and D3), two of them with KSS, had large-scale mtDNA deletions. The fourth patient (D2) had multiple mtDNA deletions in muscle and she was a compound heterozygote with respect to two mutations in *POLG1*. Two other patients with PEO (P4 and P5) were considered to have a probable mitochondrial disorder on the basis of suggestive clinical features, myopathic findings in electrophysiological examination, and COX-negative fibers in muscle histology.

5.4 Molecular investigations – DM and SNHL cohorts

We received blood samples from total 299 patients in the DM cohort. The analysis of blood samples revealed three patients (1.0%, 95%CI 0.2; 2.9) with the m.3243A>G mutation. One of these (Patient 1 in Study IV, Table 1) had been tested positive of m.3243A>G already previously on clinical grounds, the other two diagnoses were novel. The clinical characteristics of these identified patients in the DM cohort are presented in table 1 of study IV. Notably, all three patients had SNHL in addition to DM. Among the 231 patients of the SNHL cohort, we identified one patient with m.1555A>G and m.3243A>G mutations each (SNHL cohort; unpublished data).

5.5 Mitochondrial DNA haplogroup analyses – Occipital stroke and DM cohorts

We analyzed mtDNA haplogroups in patients with occipital stroke (Study I) and in patients with DM,(Study IV) and compared them to those haplogroup frequencies previously reported in the Finnish population (Torroni et al. 1996). Among patients in the stroke cohort, haplogroup Uk was more frequent among women with stroke giving an odds ratio 3.06 (0.95 – 9.9, 95% confidence interval), while the frequency observed among men did not differ from the general population (odds ratio 1.2; 0.13 – 11.9, 95% confidence interval). Among the 299 patients in the DM cohort, the frequencies of mtDNA haplogroups (Study IV, Table 2) did not differ from the general Finnish population. However, among the 124 patients (71 women, 57%) who reported a maternal family history of DM (MFH), 50 patients (40%; 95% CI 32; 49) belonged to haplogroup U, whereas only 39 patients out of the 175 patients with no MFH (22%; 95% CI 17; 29) belonged to this haplogroup ($p = 0.0013$ for difference).

6. DISCUSSION

6.1 Overview

In the studies of this thesis, we investigated the population prevalence of certain molecular genetic etiologies of OXPHOS defects among patients with several phenotypes that are encountered in mitochondrial disease (occipital ischemic stroke, PEO, DM, SNHL). The overall purpose of these studies was to systematically detect mitochondrial disease patients presenting with these phenotypes and to improve the estimation of pre-test probabilities of correctly diagnosing mitochondrial disease in a given patient with such clinical features. In addition, the purpose was to investigate the usefulness of carefully planned two-phase, population-based molecular epidemiological studies in a relatively rare condition such as mitochondrial disease.

We used the two-phase sampling method to investigate the prevalence of mitochondrial disease among the investigated clinical phenotypes. In the occipital stroke and PEO cohorts, the patients were initially ascertained from the computerized medical records of TUH during a period of 18 years using relevant ICD-9 and ICD-10 diagnoses (619 and 620 patients respectively; population 455,584 on prevalence date). For the DM cohort, the SIIF reimbursement records for DM medications in Southwestern Finland were screened through a period of 19 years (1532 patients; population 457,789 on prevalence date). SNHL patients were first identified of the hearing aid registry of TUH ORL unit during more than 11 years (total 379 patients). After the initial screening, those patients deemed (using pre-set criteria) at risk of having mitochondrial disease were investigated further.

Ischemic stroke or stroke-like episodes are encountered in patients with mitochondrial disease, especially with the m.3243A>G mutation. In these events, the occipital region of the brain is most commonly affected. In the occipital stroke cohort, we investigated the clinical characteristics and the prevalence of m.3243A>G and m.8344A>G mutations as well as seven common *POLG1* mutations in young adults with occipital ischemic stroke. In addition, we investigated the possible associations of mtDNA haplogroups with occipital stroke in these patients. PEO is a classical phenotype of mitochondrial disorder, and its molecular etiologies are diverse. From the original cohort of 620 patients, we determined a total of six patients with clinically definite PEO. In the DM cohort, we investigated the prevalence of MIDD among diabetic patients with DM onset as young adults. We concentrated the molecular studies on the m.3243A>G mutation, since previous research suggests that it is by far the most common etiology for mitochondrial DM (Tsukuda et al. 1997) and other mtDNA mutations reported to result in DM have been mostly in single pedigrees (Maassen et al. 2005). The identified cohort of young patients with severe SNHL was investigated for the m.1555A>G and m.3243A>G mutations, known to comprise the majority of

molecular etiologies of mitochondrial hearing loss (Fischel-Ghodsian et al. 2004) (unpublished data).

6.2 Occipital stroke cohort

A considerable part of ischaemic strokes of young people remain etiologically unexplained despite thorough investigations (Kristensen et al. 1997; Putaala et al. 2009). The same applies to infarcts in the posterior circulation, in general and also among young patients (Pessin et al. 1987; Naess et al. 2004). Indeed, among patients in the occipital stroke cohort, the etiology of stroke according to TOAST criteria remained undetermined in most cases. Various prothrombotic states and cardioembolism due to PFO were found to be common etiologic factors, whereas those etiologic factors common in more elderly stroke patients such as large artery atherosclerosis and atrial fibrillation were absent. A fairly large proportion of the identified female stroke patients (39%) had a history of migraine. This finding adds to previous evidence suggesting migraine as a risk factor for stroke among young women (Tzourio et al. 1993 and 1995). On the basis of our results, it seems that young women with migraine are at increased risk of posterior circulation ischaemic stroke. Thus special attention should be payed to the various manageable risk factors of ischaemic stroke (i.e., smoking, oral contraceptives, hypertension, etc.) in this patient group. The large variety of etiologic factors and relatively high frequency of rare causes stresses the need for thorough etiologic work-up in young patients with occipital ischaemic stroke in order to determine the etiology as reliably as possible so that secondary prevention can be optimized.

Among young patients with occipital ischemic stroke we found no patients with the m.3243A>G or m.8344A>G mutations, nor with one of the seven common mutations of the *POLG1* gene. This result seems to differ from the estimated frequency of 10% for mitochondrial disorder in young patients with occipital brain infarcts ascertained in the population of northern Finland (Majamaa et al. 1997). Although these results suggest that a difference in the prevalence of mitochondrial disease associated stroke between these two regions in Finland is possible, the interpretations should be cautious: the confidence intervals for prevalence of m.3243A>G in Study I overlap with the ones of the previous study (Majamaa et al. 1997).

6.3 PEO cohort

Large-scale mtDNA deletions were found in three of the six identified patients with definite PEO, and multiple mtDNA deletions with two novel variations *in trans* in the *POLG1* gene in one of the six patients. In addition, the remaining two patients with PEO were considered to have a probable mitochondrial disorder based on suggestive clinical features and investigations, although no definite molecular diagnosis could be established (Study III, Table 1). The estimated prevalence of PEO with definite or

probable mitochondrial etiology in southwestern Finnish population, based on these results, is 1.3/100,000 (95 % CI 0.5; 2.9). Further, the estimate for prevalence of PEO due to large-scale mtDNA deletions is 0.66/100,000 (95 % CI 0.14; 1.9). Moreover, it should be noted that these figures represent a minimum estimation regarding the whole population as they are derived from an investigation in an adult population with previous neurological or ophthalmological diagnoses. In a previous study in the adult population of North East of England, an estimate of 1.17/100,000 (95% CI 0.7; 1.9) for overall prevalence of large-scale mtDNA deletions was reported (Schaefer et al. 2008).

Patient D2 (reported in Study II) had an unusual 'PEO plus' phenotype with late-onset PEO and progressive encephalopathy. PEO and encephalopathy are both frequent clinical features in mitochondrial diseases, but in patients with *POLG1* mutations, encephalopathic phenotypes present most often in childhood. The combination of adult-onset PEO and encephalopathy is, however, uncommon (Horvath et al. 2006; Wong et al. 2008). Our patient had bilateral ptosis, external ophthalmoplegia and progressive encephalopathy as dominant features. Molecular genetic analysis revealed multiple mtDNA deletions in muscle and two novel, heterozygous *in trans* changes p.998S>L and p.1184D>H in the *POLG1* gene. Another pathogenic mutation has previously been described in the position 1184. The p.1184D>N mutation has been described *in trans* with the exonuclease domain mutation p.227R>W in children with failure to thrive, mental retardation and hypotonia (de Vries et al. 2007), and in adults with a linker region mutation p.468N>D with PEO and tetraparesis (Gónzalez-Vioque et al. 2006). Both p.998S>L and p.1184D>H changes are located in the polymerase domain of pol-γ in positions that are evolutionarily conserved. Also SIFT and PolyPhen predictions suggested that these changes were pathogenic.

The results of the PEO cohort suggest that large-scale mtDNA deletions could be the most common etiology of PEO in the population. A previous report on a case series suggested that the proportion of PEO patients with major mtDNA deletions is indeed high (Holt et al. 1989). Previously, the prevalence of large-scale mtDNA deletions has been estimated to be 1.6/100,000 in the province of Northern Ostrobothnia in northern Finland (Remes et al. 2005). However, cases of the previous study were ascertained from a multitude of phenotypes, which at least partly explains the difference in the prevalence. Since 1989, nuclear defects have been implicated as possible etiologies of mitochondrial disease (Zeviani et al. 1989). Mutations of *POLG1* gene are an important etiology of human mitochondrial disease, including both autosomal dominant and autosomal recessive PEO (Van Goethem et al. 2001; Lamantea et al. 2002; Hudson et al. 2006; Horvath et al. 2006). We found one patient with multiple mtDNA deletions and two novel compound heterozygous *POLG1* mutations. Recent investigations have found that mutations in *POLG1* are common among such patients with sporadic (i.e., non-familial) PEO with multiple mtDNA deletions, with varying reported mutation frequencies (between 8 and 34.6%) (Agostino et al. 2003; Hudson et al. 2006; Virgilio et al. 2008). Mutations in *ANT1*, *PEO1*, or *POLG2* genes were not detected in our patients with PEO.

The reported frequencies of mutations in *PEO1* are variable, but these mutations may be more common in PEO with autosomal dominant inheritance than in sporadic PEO cases (Virgilio et al. 2008). Based on current knowledge, mutations in *ANT1* and *POLG2* seem to be rare causes of PEO. We found no patients with m.3243A>G or m.8344A>G mutations among PEO patients.

Even though we sequenced the *ANT1*, *PEO1*, *POLG1*, and *POLG2* genes, we failed to confirm a molecular genetic diagnosis to one third of patients with clinically definite PEO. There are several other possible molecular etiologies for PEO, but these are, according to present knowledge, either individually very rare (e.g. other mtDNA point mutations) or their prevalence among PEO patients is not yet well established (e.g. mutations in *OPA1* and *RRM2B*). A recent report suggests that *RRM2B* mutations are frequent among patients with familial PEO and multiple mtDNA deletions (Fratter et al. 2011), and the roles of *RRM2B* and *OPA1* in PEO obviously warrant further study.

As to the patient ascertainment, we argue that with the selection criteria used in the PEO cohort most patients in whom the medical records information would allow an experienced clinician to consider the diagnosis of PEO were detected. Importantly, the patients with pre-existing diagnoses of PEO or KSS were not included to the study *ad hoc* but were detected from the medical records search strictly according to the pre-set criteria. With the criteria used in the present study, six (P1, P4, P5; D1, D2, D3) of the initially identified ten patients were found to have a clinically definite PEO.

6.4 Mitochondrial diabetes cohort

Among the investigated 299 patients of the DM cohort, we detected three patients with MIDD and the m.3243A>G mutation. This result suggests a 1.0% prevalence of MIDD among patients who have started anti-diabetic medication between the ages of 18 and 45 years. This figure is in line with the mean prevalence (0.8%) calculated from previous studies in European populations (Murphy R et al. 2008). Our result represents a minimum estimate of the prevalence of MIDD, since patients were not considered for the study if they had started DM medication before the age of 18 years or after the age of 45 years. Moreover, it is plausible that extending investigations to the maternal relatives of the identified patients would have resulted in the identification of further cases with m.3243A>G.

Previous studies suggest that the age of diabetes onset varies widely in MIDD, but is in young adulthood in most patients (Guillausseau et al. 2001; Murphy R et al. 2008). The prevalence of the ‘common’ type 2 DM is considerably higher among the older age groups in the Finnish and many other European populations (Shaw et al. 2010). Thus, data on the MIDD age of onset and on the epidemiology of type 2 DM suggest that the *a priori* probability of MIDD diagnosis decreases among older DM patients. This is why screening older age groups means increased expenses of the study in terms of time,

money and labour. We decided to limit the search for MIDD to patients aged 18 – 45 years at DM onset, since the principal aim of Study IV was to estimate the population prevalence of MIDD among these patients.

The clinical manifestations of the m.3243A>G mutation are diverse (Murphy R et al. 2008) and classic phenotypes such as MELAS are probably more an exception than the rule. All the three patients with DM and m.3243A>G in Study IV had severe hearing impairment requiring use of hearing aid. Patient 1 had had migraine since her teens, and had suffered a cerebrovascular incident at age 51 years that was considered an ischaemic stroke rather than a stroke-like episode. She did not have clinically manifest myopathy or encephalopathy, and blood lactate levels were normal. Thus, her symptom constellation does not fulfill the diagnostic criteria of MELAS (Hirano et al. 1991), but seems to be on a continuum between MIDD and MELAS (Suzuki et al. 2003; Takeshima and Nakashima 2005). Patient 2 had albuminuria, probably reflecting an early renal complication of DM, which have been reported frequently in mitochondrial DM. Proteinuria in adult age is a common manifestation and often there is progression to end-stage renal failure (Guillausseau et al. 2001; Guéry et al. 2003). Cases of m.3243A>G MIDD in patients with renal failure and hearing loss, but with no hematuria have been misdiagnosed as Alport syndrome (Jansen et al. 1997; Nakamura et al. 1999). Patient 3 had a history of gastrointestinal problems including diagnosed non-alcohol-induced pancreatitis and constipation. Gastrointestinal symptoms have been estimated to be quite common in mitochondrial DM (Narbonne et al. 2004).

Among the identified MIDD patients, Patient 3 did not report any relatives with either DM or hearing impairment, and Patient 2 did not report a definite maternal history of these symptoms. These examples stress the fact that mitochondrial DM is a possibility even in the absence of suggestive maternal history. Although both patients 2 and 3 in retrospect had quite typical clinical features suggestive of mitochondrial DM, they had not been previously genetically tested and they had not been referred to clinical genetics or mitochondrial specialist consultation. Such patients with ‘classical’ mitochondrial DM but without suggestive family history may easily go unnoticed in regular clinical practice.

We used blood DNA for the detection of the m.1555A>G and m.3243A>G mutations. This method has been used in many previous prevalence studies of mitochondrial DM (Katagiri et al. 1994; Newkirk et al. 1997; Tsukuda et al. 1997; Guillausseau et al. 2001). Leukocyte DNA has generally been considered appropriate for the detection of m.3243A>G (Maassen et al. 2005), although it is known that mutation heteroplasmy is lower in leukocytes than in e.g. urinary sediment or cheek mucosa (Shanske et al. 2004), and that the heteroplasmy detected in leukocytes tends to decrease over time (Rahman et al. 2001). In MIDD patients, the leukocyte heteroplasmy levels of the m.3243A>G mutation vary in the range of 1 to 40% (Maassen et al. 2005).With the restriction-fragment analysis method used in these studies, heteroplasmy levels as low as 2% are

reliably detected (Smith et al. 1997; Wong and Lam 1997). Thus it is possible that a patient with 1 – 2% heteroplasmic m.3243A>G could have gone unnoticed.

6.5 Mitochondrial DNA haplogroups and disease

The analysis of mtDNA haplogroups in patients with occipital brain infarct showed that 17% of men and 33% of women belonged to haplogroup Uk whereas the frequency of this haplogroup is 17% in a population sample best conforming to Southwestern Finnish population (Torroni et al. 1996). Interestingly, previous research has linked this haplogroup with occipital stroke in migraine (Majamaa et al. 1998b). No sex-related association of haplogroup Uk and occipital brain infarcts has been previously reported, but a recent study in the Japanese population (Nishigaki et al. 2007) found mtDNA haplogroup A to be a risk factor for atherothrombotic cerebral infarctions in women. Our findings in Study I suggest that haplogroup Uk may be a risk factor for occipital brain infacts in young women.

Previous studies have reported no clear associations between mtDNA haplogroups and DM. In the DM cohort, we found mtDNA haplogroup U to be more prevalent among patients who reported maternal family history of DM. Previous studies on the mtDNA haplogroups in DM have focused only on type 2 DM, and maternal family history has either not been investigated or only affected mothers have been registered. In the present work, all first- and second-degree maternal relatives with DM were reported, and DM was defined by the need for medication for DM, i.e., not differentiating between type 1 or 2 or otherwise. Furthermore, a recent study in Italian population has found that mtDNA haplogroups are not associated with the risk of developing type 2 DM, but are associated with risk of DM complications (Achilli et al. 2011), suggesting that the associations between mtDNA haplogroups and DM may be even more complex.

6.6 Results of the present studies in the population perspective

Recent studies have suggested genetic differences between Finns living in the southern and western regions and Finns living in the northern and northeastern parts of the country (Lappalainen et al. 2006; Salmela et al. 2008). These differences are probably due to the population history of Finland, i.e. the late settlement of the northern and northeastern parts of the country by a relatively small founding population (Peltonen et al. 2000; Kere 2001; Norio 2003a; Norio 2003b). These genetic differences between Finnish populations of different geographical areas might be one explanation to the fact that the results of these studies conducted in southwestern Finland differ in some ways from previous results from Ostrobothnia in northern part of Finland (Majamaa et al. 1997; Remes et al. 2005). On the other hand, genetic studies show that Finns from the southern and western parts of the country have strong genetic similarities with other populations surrounding the Baltic Sea (Lappalainen et al. 2008) and as a group these populations

have strongest genetic roots in Central Europe. Thus, the results of our studies should be applicable, albeit with caution, at least to other European populations around the Baltic Sea.

In every epidemiological study, a crucial aspect in assessing the results is how well they represent the true morbidity in population. Two major points in this respect are addressed here. Firstly, since the studies of this thesis were initially based on medical records information, we would have missed those patients who had not been seeking medical attention for their symptoms. Formal assessment of this type of selection bias in prevalence studies of rare conditions such as forms of mitochondrial disease is difficult, since identification of possible false negatives would require thorough analyses of a large number of individuals who were not screened in these studies. Secondly, in the setting of a two-phase study, the first phase selection criteria are of critical importance in order to minimize losing the searched patients. These criteria may always be criticized *post hoc* of not being optimal for the purposes of the study (that is, resulting in a biased sample not representative of the true prevalence of the investigated condition), but this criticism remains speculative until other studies on mitochondrial disease prevalence with similar overall approach but different selection criteria should prove otherwise.

6.7 Practical implications of the present studies

Altogether, based on results in the occipital stroke cohort it seems that mitochondrial disease is a rare cause of occipital brain infarct in young adults, and genetic testing for mitochondrial disease in clinical practice should probably remain reserved for those stroke patients whose other clinical characteristics or family history raise the suspicion of mitochondrial disease. These features could be e.g. bilateral sensorineural hearing impairment, diabetes mellitus, hypertrophic cardiomyopathy, or epilepsy, and any combination of these features.

Regarding PEO, it seems reasonable to pursue a mitochondrial diagnosis in patients presenting with symptoms similar to those used in the ascertainment of the PEO cohort, since six out of ten patients in the second phase of the study met the criteria of clinically definite PEO. Sporadic, large-scale mtDNA deletions are, based on this study and others' results, likely to be the most frequent molecular etiology of PEO. The clinical features of the PEO patient with novel *POLG1* changes, a relatively late adult-onset symptom combination of PEO and progressive encephalopathy, demonstrate the wide phenotypic variety associated with the *POLG1* mutations. For this reason, the possibility of a *POLG*-associated disease should be considered in any patient with unexplained or unusual neurological features (Chinnery et al. 2008). Altogether, the results of the PEO cohort study suggest that molecular investigation of patients with PEO, sporadic or familial, should commence with an analysis for mtDNA deletions, followed by analysis of the *POLG1* gene. We advise to refer patients with negative results in these initial

investigations to a center specialized in mitochondrial diseases for evaluation of most appropriate further genetic analyses.

Based on the results in the DM cohort, some 1% of diabetes emerging between the ages 18 – 45 years is associated with the m.3243A>G mutation. In population, these young patients with mitochondrial disorder are probably under-diagnosed, and we suggest that patients in this age group presenting with both DM and hearing impairment should undergo investigation for the m.3243A>G mutation. Moreover, among the patients with DM onset in young adulthood, mtDNA haplogroup U seems to be associated with maternal family history of DM. Among non-selected young patients with SNHL requiring hearing aid, the mutations m.1555A>G and m.3243A>G seem to present with ~0.5% prevalence each (SNHL cohort, unpublished data).

Overall, the results of these studies suggest that a carefully planned two-phase sampling approach with a clinical data analysis of a large population followed by comprehensive investigations in selected individuals is a useful and resource-efficient method in the difficult task of obtaining prevalence data of rare genetic conditions, such as various forms of mitochondrial disease.

6.8 Future directions

The studies of this thesis provide with material and interesting hypotheses for future investigations. Since in both Studies I and IV we found an association between mtDNA haplogroup U and occipital ischemic stroke and DM, respectively, that have been reported as a manifestation of a mitochondrial disorder, it would be intriguing to investigate whether there are some other clinical features that associate with this haplogroup, in population level. If haplogroup U predisposes people to mitochondrial dysfunction, there might be associations with e.g. general aerobic condition and healthy ageing. As to Study IV, it would be interesting to investigate mtDNA sequence also for other possible mutations apart from m.3243A>G, especially in those with both DM and SNHL, as this combination is quite suggestive of a mitochondrial disorder. In addition, the possible other associations of mtDNA haplogroups apart from the maternal family history of DM are probably worth investigating.

7. CONCLUSIONS

These studies resulted in detection of novel pathogenic mutations, new definite molecular genetic diagnoses for several individual patients, increased understanding of the clinical phenotypes associated with nuclear and mtDNA mutations causing mitochondrial disease as well as mtDNA haplogroups as risk factors for ischemic stroke and DM. In addition, we used population-based data to assess the prevalence of mitochondrial disease among several clinical phenotypes with more reliability than previous studies. This type of studies forms an important bridge between the everyday clinical work with suspected mitochondrial disease and the fundamental molecular genetic understanding of these conditions.

The specific conclusions of the studies of this thesis are as follows.

1. Mitochondrial DNA point mutations m.3243A>G and m.8344A>G or common *POLG1* mutations seem to be rare among young adults with occipital ischemic stroke. Mitochondrial haplogroup Uk seems to be associated with increased risk of occipital ischemic stroke among young women.

2. Large-scale mtDNA deletions and mutations of the *POLG1* gene are probably the most common molecular etiologies of PEO. MtDNA point mutations m.3243A>G, m.8344A>G and mutations in nuclear genes *ANT1*, *PEO1*, or *POLG2* seem to be rare among PEO patients.

3. Around 1% of DM emerging between the ages 18 – 45 years is associated with the m.3243A>G mutation. In population, these patients are probably under-recognized. Moreover, among these young patients with DM, mtDNA haplogroup U seems to be associated with maternal family history of DM.

4. In population, the mtDNA mutations m.1555A>G and m.3243A>G are found with ~0.5% prevalence among early-onset severe SNHL requiring use of hearing aid (unpublished data).

5. Altogether, the studies of this thesis show that carefully planned studies with combined clinical epidemiological and molecular genetic methodology are useful even in the study of rare conditions, such as mitochondrial disorders.

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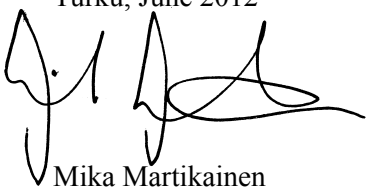

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