OULU 2006
D 904

ACTA UNIVERSITATIS OULUENSIS

Reetta Hinttala

GENETIC CAUSES OF MITOCHONDRIAL COMPLEX I DEFICIENCY IN CHILDREN

FACULTY OF MEDICINE, DEPARTMENT OF NEUROLOGY, DEPARTMENT OF PAEDIATRICS,
DEPARTMENT OF MEDICAL BIOCHEMISTRY AND MOLECULAR BIOLOGY,
UNIVERSITY OF OULU;
CLINICAL RESEARCH CENTER,
OULU UNIVERSITY HOSPITAL;
DEPARTMENT OF PEDIATRICS, NIJMEGEN CENTRE FOR MITOCHONDRIAL DISORDERS,
RADBOUD UNIVERSITY NIJMEGEN MEDICAL CENTRE

ACTA UNIVERSITATIS OULUENSIS
D Medica 904

REETTA HINTTALA

GENETIC CAUSES OF MITOCHONDRIAL COMPLEX I DEFICIENCY IN CHILDREN

Academic dissertation to be presented, with the assent of the Faculty of Medicine of the University of Oulu, for public defence in Auditorium 101 A of the Faculty of Medicine (Aapistie 5 A), on January 5th, 2007, at 12 noon

OULUN YLIOPISTO, OULU 2006

Acta Univ. Oul. D 904, 2006

Supervised by
Professor Kari Majamaa
Doctor Johanna Uusimaa

Reviewed by
Professor Brian H. Robinson
Doctor Robert W. Taylor

ISBN 951-42-8287-6 (Paperback)
ISBN 951-42-8288-4 (PDF) http://herkules.oulu.fi/isbn9514282884/
ISSN 0355-3221 (Printed)
ISSN 1796-2234 (Online) http://herkules.oulu.fi/issn03553221/

Cover design
Raimo Ahonen

OULU UNIVERSITY PRESS
OULU 2006

Hinttala, Reetta, Genetic causes of mitochondrial complex I deficiency in children
Faculty of Medicine, Department of Neurology, Department of Paediatrics, Department of Medical Biochemistry and Molecular Biology, University of Oulu, P.O.Box 5000, FI-90014 University of Oulu, Finland; Clinical Research Center, Oulu University Hospital, P.O.Box 5000, FI-90014 University of Oulu, Finland; Department of Pediatrics, Nijmegen Centre for Mitochondrial Disorders, Radboud University Nijmegen Medical Centre, P.O. Box 9101, 6500 HB Nijmegen, the Netherlands
Acta Univ. Oul. D 904, 2006
Oulu, Finland

Abstract

The mitochondrial oxidative phosphorylation system is composed of five multisubunit enzyme complexes. Complex I is the first and largest of these, containing 46 subunits, seven encoded by mitochondrial DNA (mtDNA) and the rest by nuclear DNA. Isolated complex I deficiency is a major cause of metabolic errors in infancy and childhood, presenting as encephalomyopathies or multisystem disorders. Due to the bigenomic origin of complex I, the genetic causes of these defects can be either mitochondrial or nuclear.

The object of the present work was to identify the underlying genetic cause in cases of children with complex I deficiency and to obtain more information on the structurally and functionally important sites of complex I subunits. The complete coding region of mtDNA was analysed by conformation-sensitive gel electrophoresis and subsequent sequencing. In addition, nine nuclear genes encoding conserved subunits of complex I were sequenced. The structural and functional consequences of the new sequence variants were further elucidated using mutagenesis of homologous residue in bacterial NDH-1 or by studying complex I assembly and expression in patient cell lines.

Analysis of the mtDNA coding region in 50 children revealed four definitely pathogenic mutations, 3460G>A, 10191T>C, 11778G>A and 14487T>C, in seven patients. In addition, two novel mtDNA base pair substitutions were identified, 3866T>C in a patient with muscle weakness and short stature and 4681T>C in a patient with Leigh syndrome. The latter mutation causes a Leu71Pro amino acid exchange in the ND2 subunit. Cybrid clones harbouring this mutation retained the complex I defect, and reduced amounts of fully assembled complex I were detected in patient cell lines. The 3866T>C mutation leads to a Ile187Thr amino acid substitution in the ND1 subunit, and functional studies of the homologous amino acid substitution in *E. coli* showed that this had an effect on the assembly or stability of the NDH-1 holoenzyme. Sequencing of the nine nuclear-encoded complex I genes revealed only one novel base pair substitution with pathogenic potential. Further studies are needed, however, to establish the role of the Arg18Cys substitution in the mitochondrial leading peptide of the TYKY subunit.

The above findings emphasize the contribution of mtDNA mutations to the aetiology of pediatric patients with complex I deficiency. Furthermore, two LHON primary mutations were identified in the present cohort of patients, although the clinical signs differed considerably from the classical symptoms of LHON. This suggests that the phenotype caused by primary LHON mutations is more variable than has so far been thought.

Keywords: DNA mutational analysis, inborn errors of metabolism, mitochondria, mitochondrial DNA, mitochondrial encephalomyopathies, NADH dehydrogenase, oxidative phosphorylation, site-directed mutagenesis

To my family

Acknowledgements

This work was carried out at the research laboratories of the Departments of Neurology and Medical Biochemistry and Molecular Biology, University of Oulu and the Department of Pediatrics, Nijmegen Centre for Mitochondrial Disorders, Radboud University Nijmegen Medical Centre during the years 2001-2006.

I devote my deepest gratitude to my principal supervisor, Professor Kari Majamaa, whose expert knowledge and kind support have been essential for this work. I really appreciate his understanding and down to earth attitude towards young students while guiding us to the fascinating world of science. I am equally grateful to my second supervisor Dr. Johanna Uusimaa for her precious support. Our discussions, whether about scientific mysteries or gardening, have inspired me in so many ways. It has been a priviledge to have two such dedicated supervisors providing an excellent research atmosphere, and their enthusiasm towards mitochondrial research has been of great motivation for me.

I want to acknowledge Professor Vilho Myllylä, chairman of the Department of Neurology, University of Oulu and Professor Matti Hillbom, head of the Department of Neurology, Oulu University Hospital for the opportunity to carry out my research in their department. I also want to express my gratitude to Professor Taina Pihlajaniemi, the head of the Department of Medical Biochemistry and Molecular Biology and Professor Tatu Juvonen, head of the Clinical Research Center for the research facilities.

I am indebted to all my collaborators. I wish to express my respect to Professor Emeritus Ilmo Hassinen, Oulu Bioenergetics Group, for providing me guidance and facilities to work in the difficult field of enzyme activities. I also want to thank Professor Jan Smeitink for the opportunity to work in his laboratory at the University Medical Centre Nijmegen. Professor Jan Smeitink, Professor Nils-Göran Larsson, Professor Heikki Rantala, Assistant Professor Leo Nijtmans, Docent Anne Remes, Dr. Cristina Ugalde, Dr. Marko Kervinen, Dr. Jukka Moilanen and Roel Smeets deserve likewise my special acknowledgements for their invaluable contribution to this research. It has been an honour to work with such talented and highly motivated people.

I wish to express my gratitute to the official referees Professor Brian Robinson and Dr. Robert Taylor for their careful review of the manuscript of this thesis. I also want to thank the expertise of Malcolm Hicks, MA, in revisioning the English language of this thesis.

I would like to warmly thank all my colleagues and co-authors. I wish to thank every member of the Northern Mitochondria Team for creating such a cheerful and supportive atmosphere in which this work was carried out. My special thanks go to colleagues Heidi Soini, Johanna Annunen-Rasila, Anna-Kaisa Niemi, Johanna Krüger and Anna-Leena Heula for their invaluable friendship. Their support has made the hard times easier to handle and the good times to feel even better; together we have had many happy moments both at work and on free time. Pirjo Keränen, Anja Heikkinen, Irma Vuoti and Aila Holappa deserve my warm thanks for their accurate, skilful and highly motivated technical assistance. The work they have carried out in the laboratory has been essential for this research. Risto Helminen and Antti Kiviniemi are acknowledged for the valuable assistance with computer problems. I am also greatly indebted to the patients and their families who participated in this research.

All my friends and relatives outside the research world deserve my special thanks for their support. Especially I need to thank Kaisa Hahto and Eija Sippola for many happy weekends that really have provided me an escape from everyday life. I wish to thank warmly my mother Maria Mehtälä for her loving care, encouragement and confidence in me. I also send my deepest gratitude to my father Jouko Mehtälä up in the stars for all the support he provided me during his life. Above all, I owe my deepest gratitude to my husband Jukka Hinttala, whose genuine love and positive attitude towards life have carried me through these years. I also thank Sulo the cat for his comforting presence at home.

This work was supported financially by the Academy of Finland, the Sigrid Juselius Foundation, the Alma and K. A. Snellman Foundation, Oulu, Finland, Apteekin rahasto, the Arvo and Lea Ylppö Foundation, the Foundation for Pediatric Research, the Neurology Foundation, Oulu University Hospital, and the Päivikki and Sakari Sohlberg Foundation.

Oulu, October 2006 Reetta Hinttala

Abbreviations

ATP	adenosine triphosphate
BN-PAGE	blue native polyacrylamide gel electrophoresis
CSGE	conformation-sensitive gel electrophoresis
DB	decylubiquinone
DNA	deoxyribonucleic acid
d-NADH	deamino nicotinamide adenine dinucleotide
Fe-S	iron sulphur cluster
FMN	flavin mononucleotide
HAR	hexammine ruthenium
LHON	Leber hereditary optic neuropathy
LNA	locked nucleic acid
MELAS	mitochondrial encephalomyopathy, lactic acidosis and stroke-like episodes
mRNA	messenger ribonucleic acid
mtDNA	mitochondrial deoxyribonucleic acid
NADH	reduced nicotinamide adenine dinucleotide
NAD+	nicotinamide adenine dinucleotide
NDH-1	proton pumping NADH-quinone oxidoreductase
OMIM	Online Mendelian Inheritance in Man
OXPHOS	oxidative phosphorylation
PCR	polymerase chain reaction
UQ	ubiquinone
QH2	ubiquinol
rCRS	revised Cambridge reference sequence
RFLP	restriction fragment length polymorphism
ROS	reactive oxygen species
RRF	ragged red fiber
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
SNP	single nucleotide polymorphism
tRNA	transfer ribonucleic acid
VNA	N-vanillylnonanamide

List of original papers

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

I Hinttala R, Uusimaa J, Remes AM, Rantala H, Hassinen IE & Majamaa K (2005) Sequence analysis of nuclear genes encoding functionally important complex I subunits in children with encephalomyopathy. J Mol Med 83: 786-794.

II Esteitie N, Hinttala R, Wibom R, Nilsson H, Hance N, Naess K, Teär-Fahnehjelm K, von Döbeln U, Majamaa K & Larsson NG (2005) Secondary metabolic effects in complex I deficiency. Ann Neurol 58: 544-552.

III Hinttala R, Smeets R, Moilanen JS, Ugalde C, Uusimaa J, Smeitink JAM & Majamaa K (2006) Analysis of mitochondrial DNA sequences in patients with isolated or combined oxidative phosphorylation system deficiency. J Med Genet 43: 881-886.

IV Ugalde C, Hinttala R, Timal S, Smeets R, Rodenburg RJT, Uusimaa J, van den Heuvel LP, Nijtmans LGJ, Majamaa K & Smeitink JAM (2006) Mutated ND2 impairs mitochondrial complex I assembly and leads to Leigh Syndrome. Mol Genet Metab. In press.

V Hinttala R, Kervinen M, Uusimaa J, Finnilä S, Rantala H, Remes AM, Hassinen IE & Majamaa K (2006) A rare 3866T>C mutation in the mitochondrial ND1 gene and mutagenesis of a homologous position in *Escherichia coli*. Manuscript.

Contents

1 Introduction

The mitochondria are the power plants of the eukaryotic cell, storing energy from nutrients into chemical bonds of adenosine triphosphate (ATP) in a process referred to as respiration. The reaction is carried out by the oxidative phosphorylation (OXPHOS) system, which is situated in a highly folded mitochondrial inner membrane. The OXPHOS system is composed of five multisubunit enzyme complexes, the first and largest of which, NADH:ubiquinone oxidoreductase (complex I), is also structurally by far the most complicated. Complex I is formed of 46 subunits encoded by two separate genomes, 14 being encoded by the mitochondrial DNA (mtDNA) and the rest by nuclear DNA. As a result of intensive investigations during recent years, significant progress has been made in the field of complex I assembly, structure, function and pathology.

Complex I deficiency is the most common OXPHOS system disorder in children, and because of the dual genomic origin of the complex, it may follow either a maternal or Mendelian inheritance pattern. The clinical spectrum of complex I deficiency is wide, varying from severe lactic acidosis in infants to muscular weakness in adults. The many devastating neurodegenerative disorders of childhood associated with complex I deficiency emphasize the need for identifying the underlying genetic defect and further elucidating the molecular mechanisms involved in the functioning of complex I. There is no simple way at present to prioritise candidate genes for mutation analysis. Mutations have been reported in both nuclear and mitochondrial genes encoding subunits for complex I, but these still cover only approximately 40% of the patients analysed. Thus the origin of the defect in the majority of cases seems to lie in functional rather than structural genes. Only two genes encoding factors involved in the assembly of human complex I have been identified so far, however.

The aim of this work was to determine the underlying genetic defect in 50 children with isolated or combined complex I deficiency or other OXPHOS enzyme deficiency. The mutation analysis covered the complete coding region of mtDNA and nine nuclear genes encoding the best-conserved and functionally most important subunits of complex I. The pathogenic role of the novel mutations identified was further established by means of bioinformatics and by *Escherichia coli* mutagenesis, or by studying the assembly and expression of complex I at the protein level.

2 Review of the literature

2.1 Energy metabolism in cells

Organisms need energy for mechanical work, for the active transport of molecules and ions and for the synthesis of macromolecules and other biomolecules. The free energy used in these processes comes from the environment, but before the energy from nutrients can be utilized by the cell, it has to be converted to chemical energy and stored in a useful form, that known as ATP. This energy conversion takes place in mitochondria and is termed oxidative phosphorylation (Fig. 1). The glucose metabolism under aerobic conditions begins with glycolysis in the cytosol, and the overall reaction results in conversion of the glucose into two pyruvate molecules, giving a net energy yield of two ATP molecules. In addition, two nicotinamide adenine dinucleotide molecules (NADH) are reduced in the reaction. Under aerobic conditions pyruvate and NADH are transferred to the mitochondria. When pyruvate enters the matrix, it reacts with coenzyme A to generate acetyl-CoA, CO_2 and reduced NADH, the acetyl-CoA being further oxidized in the Krebs cycle. The net energy yield of the overall oxidation of two pyruvate molecules from one glucose molecule is conserved in ten NADH, two flavin adenine dinucleotide ($FADH_2$) and two ATP molecules. Under anaerobic conditions pyruvate is converted into lactate by lactate dehydrogenase in a reversible reaction which enables glycolysis to proceed transiently in active tissues such as contracting muscle. The lactate formed in these active tissues is secreted into the bloodstream and then oxidized back to pyruvate in the liver. (Berg *et al.* 2002.)

Another way to produce acetyl-CoA for the Krebs cycle is through the oxidation of fatty acids in the mitochondrial matrix. Free fatty acids are coupled to coenzyme A to form acyl-CoA in the outer mitochondrial membrane. Long-chain acyl-CoA esters, being unable to traverse the mitochondrial membrane by themselves, are carried across as carnitine esters. On the matrix side, the regenerated acyl-CoA molecules are then shortened by two carbon atoms in a cyclic process called β-oxidation. This continues until all the carbon atoms in the acyl-CoA have been converted into two carbon acetyl-CoA molecules, which enter the Krebs cycle. One molecule of NADH, and one of $FADH_2$ and two of acetyl-CoA are produced in every cycle. (Berg *et al.* 2002.)

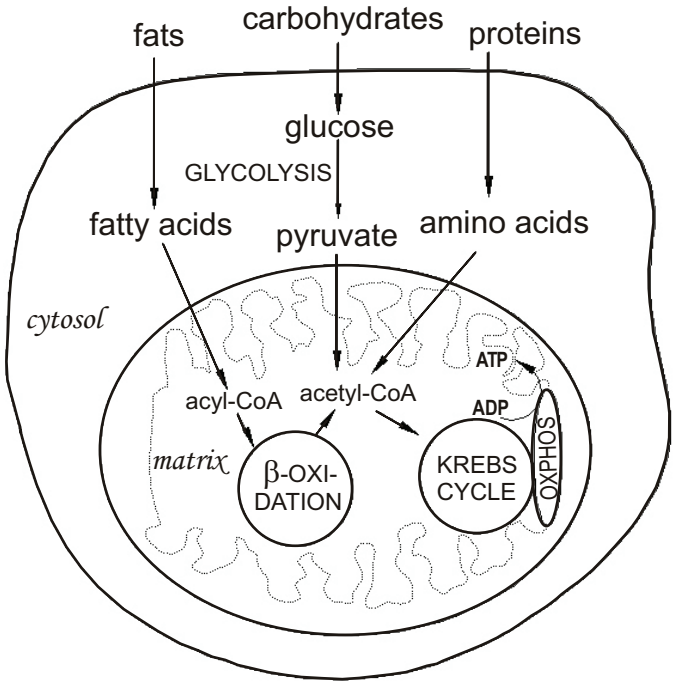

Fig. 1. Energy metabolism in cells. Oxidation of nutrients in the cytosol and in the mitochondrial matrix leads to ATP generation by the oxidative phorphorylation system. The ATP is then used to drive biosynthetic reactions and other processes in the cell that require energy. OXPHOS = oxidative phosphorylation, ADP = adenosine diphosphate, ATP = adenosine triphosphate.

2.2 Mitochondria

Mitochondria are membrane-enclosed organelles present in the cytosol of every eukaryotic cell. Depending on the energy need of the cell it may contain hundreds or even thousands of mitochondria. The main function of a mitochondrion is to produce energy by utilizing ogygen and the byproducts of the oxidation of nutrients. (Mathews *et al.* 2000.)

The origins of mitochondria are thought to date back to the time around 1.5 billion years ago when the Earth's atmosphere first became rich in oxygen. Since the ancestral eukaryotic cells were not able to use oxygen in their energy production, a primitive eukaryotic cell united with free-living oxygen-metabolizing α-proteobacteria in order to obtain that ability. The symbiosis between these two types of cell evolved over time, the host cell received the ability for power generation in return for sheltering the bacterial cell (Gray *et al.* 1993). This serial endosymbiosis theory has recently been revised (Gray *et al.* 1999, Vellai & Vida 1999) to incorporate the view that there were no amitochondriate eukaryotes, but rather the fusion of anaerobic archaeobacteria with

respiration-competent proteobacteria represented the origin of the eukaryotes. (Scheffler 2001, Alberts *et al.* 2002.)

2.2.1 Structure of mitochondria

Mitochondria are traditionally described as distinct cell organelles surrounded by a membrane, although recent developments in the imaging of living cells have shown that mitochondria are mobile and plastic, as they are constantly changing their shape and location. Also, rather than being distinct organelles, they are seen as forming continuous tubular networks that undergo fusions and fission. (Bereiter-Hahn & Voth 1994.)

Mitochondria are surrounded by two highly specialized membranes, the outer and inner membranes (Fig. 2A). The area enclosed by the inner membrane is called the matrix. Another compartment, left between the outer and inner membranes, is called the intermembrane space. Each of these membranes and spaces contains a unique set of proteins, according to which they can be distinguished from one another. The outer membrane is permeable to molecules of size ≤5000 daltons, enabling their entry into the intermembrane space from the cytosol. Thus the cytosol and intermembrane space are highly similar with regard to their small molecule content. The inner mitochondrial membrane, on the other hand, is highly selective, containing cardiolipin and several selective transport proteins for molecules needed on the matrix side. The inner mitochondrial membrane is also highly folded, forming structures called cristae that project into the matrix. These structures increase the area of the inner mitochondrial membrane, which is where ATP production occurs via respiratory chain and complex V. The electron transfer of the respiratory chain is coupled to proton pumping across the inner mitochondrial membrane, leading to a pH gradient with a higher pH on the matrix side than in the cytosol. In addition, proton transfer generates a voltage gradient across the membrane, with the negative pole on the matrix side and the positive pole on the outside. Together, the pH gradient and the membrane potential form an electrochemical proton gradient across the inner mitochondrial membrane which is essential for the synthesis of ATP and for the import of molecules into the mitochondria. (Alberts *et al.* 2002.)

2.2.2 Oxidative phosphorylation in mitochondria

Oxidative phosphorylation is carried out by five multisubunit enzyme complexes (Fig. 2B). Electrons from NADH and $FADH_2$ are transported to oxygen by a respiratory chain composed of NADH:ubiquinone oxidoreductase (complex I), succinate:ubiquinone oxidoreductase (complex II), ubiquinone:cytochrome *c* oxidoreductase (complex III) and cytochrome *c* oxidase (complex IV). The final step, the production of ATP from ADP and inorganic phosphate is carried out by ATP synthase (complex V). Rather than being isolated complexes, these enzymes seem to occur together, forming supercomplexes (Schägger and Pfeiffer 2000, Schägger and Pfeiffer 2001), with varying stoichiometries depending on the use of different solubilization detergents (Schägger 2002, Schafer *et al.*

2006). The functional significance of this supercomplex formation is thought to involve catalytic enhancement by channelling of the substrates and prevention of competition from other enzymes, prevention of the generation of reactive oxygen species (ROS) and stabilization of the individual OXPHOS enzyme complexes (Schägger 2002, Acin-Perez *et al.* 2004).

The operation of the respiratory chain is characterized by two processes, electron flow within the enzyme complexes and the transport of protons across the inner mitochondrial membrane. NADH is oxidized by the first and largest enzyme of the respiratory chain, complex I. Electrons are passed through the enzyme via prosthetic groups flavin mononucleotide (FMN) and seven iron-sulphur clusters, to ubiquinone (UQ). $FADH_2$ donates the electrons to complex II. The enzyme operates both in the respiratory chain and in the Krebs cycle, where it oxidizes succinate to fumarate, yielding $FADH_2$. Electrons are carried through complex II via flavin adenine dinucleotide (FAD) and three iron-sulphur clusters to ubiquinone. Complex II also contains one *b* heme, the functional role of which is currently unclear. The reaction of complex II is reversible and the direction of electron flow through the enzyme is dictated by the relative concentrations of the reactants and products. Thus, in addition to being an entry point for electrons into the respiratory chain, complex II also participates in regulation of the Krebs cycle. Electrons from complexes I and II reduce ubiquinone (UQ) to ubiquinol (QH_2), which is hydrophobic and shuttles within the inner mitochondrial membrane, transferring the electrons further to complex III. The electrons pass through the complex III dimer via cytochrome *b*, a binuclear iron-sulphur cluster and cytochrome c_1, which donates them to ferricytochrome *c*. Complex IV catalyzes the final step of the respiratory chain, the transfer of the electrons from ferrocytochrome *c* to dioxygen, to produce water. The electron transportation occurs via four redox centres, a binuclear copper centre (Cu_A), a mononuclear copper centre (Cu_B) and two hemes (*a* and a_3). In addition to electron transport, complexes I, III and IV serve as proton pumps. The enzymes use energy from the electron transfer to translocate protons from the matrix side into the intermembrane space, producing an electrochemical gradient across the inner mitochondrial membrane. The free energy released from the flow of protons back across the membrane is then used by complex V for chemical work to produce ATP from ADP and inorganic phosphate. (Schultz & Chan 2001, Berg *et al.* 2002.)

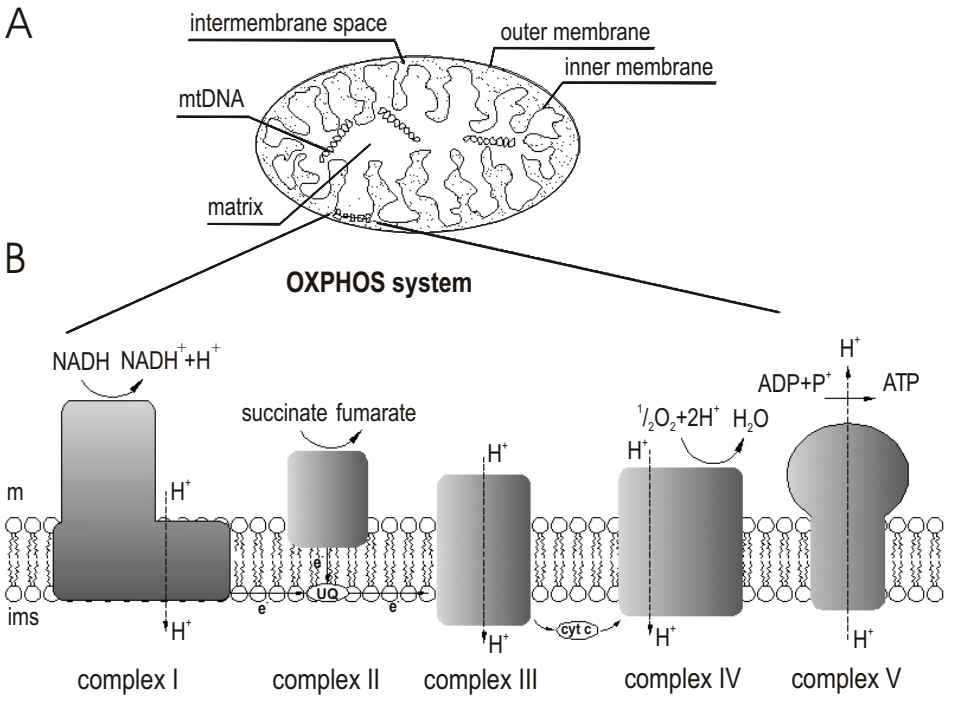

Fig. 2. Structure of the mitochondrion (A). Schematic representation of the respiratory chain complexes I-IV and complex V in the mitochondrial inner membrane (B). The electron (e^-) and proton (H^+) flows are indicated by arrows. OXPHOS = oxidative phosphorylation, m = matrix, ims = intermembrane space, UQ = ubiquinone, cyt c = cytochrome *c*.

2.3 Mitochondrial DNA

Mitochondria contain their own genome, mitochondrial DNA (mtDNA) (Nass & Nass 1963). Each cell can harbour hundreds or even thousands of mtDNA molecules, with 2-10 copies per mitochondrion. The mtDNA is maternally inherited, as although a few mitochondria from the sperm cell may enter the oozyte during fertilization, they are eliminated by a ubiquitin-dependent mechanism (Sutovsky *et al.* 2000). Interestingly, recent data related to human disease has shown that the paternal mtDNA can escape this elimination and be transmitted to the muscle tissue of the offspring (Schwartz & Vissing 2002).

MtDNA has a circular double-stranded structure, and its complete 16 568 bp nucleotide sequence has been resolved (Anderson *et al.* 1981, Andrews *et al.* 1999) (Fig. 3). The two strands of mtDNA have significantly different base compositions, the heavy (H) strand being rich in guanines and the other, light (L) strand rich in thymines. MtDNA encodes 37 genes, of which 28 are encoded by the H-strand and 9 by the L-strand. The majority of the genes encode an RNA product, so that there are 22 mitochondrial tRNAs

and two rRNAs (23S and 16S). The remaining 13 genes encode proteins of the OXPHOS system. The mitochondrial genome is compact, as the genes lack introns and are tightly packed, overlapping each other, or else, in most cases, the coding sequences of adjacent genes are contiguous or separated by only one or two non-coding bases. There are only two non-coding regions in mtDNA, a displacement (D) loop of about 1 kb between tRNA (Phe) and tRNA (Pro) and a region of ~30 nucleotides located inside a tRNA cluster about two-thirds of the way from the D-loop. (Strachan & Read 1999.)

2.3.1 Replication and transcription

The mitochondrial genome is replicated and transcribed within the organelle. The *cis*-elements responsible for the regulation of these processes are mainly located within the D-loop region of the mtDNA. By contrast, all the *trans*-acting factors, such as mtRNA polymerase, mtDNA polymerase γ and the regulatory factors, are nuclear-encoded. (Garesse & Vallejo 2001.)

The mechanism of mtDNA replication is currently under debate. The first model, suggested three decades ago (Kasamatsu and Vinograd, 1973) describes it as an asynchronous displacement reaction involving two independent, unidirectional origins O_H and O_L (Clayton, 1982) (Fig. 3). This model has recently been challenged by a new bidirectional strand-coupled mechanism of mtDNA replication (Holt *et al.* 2000) in which the origin of replication of both the leading and lagging strands is located downstream from the original O_H. The replication fork is thought to move bidirectionally from the origin of replication along the parental mtDNA strand until the O_H is reached (Bowmaker *et al.* 2003). Differences have been detected between mtDNA replication in cultured cells and in solid tissues (Yasukawa *et al.* 2005), and it remains to be resolved whether or not both of these replication mechanisms exist, perhaps regulated by different physiological conditions in the cell.

The replication and transcription are linked together, as the same RNA primer is used in both transcription of the L-strand and replication of the mtDNA. Transcription of the L-strand begins at the initiation point (L), located in the LSP promoter area of the D-loop (Fig. 3). The L-strand is transcribed as a single polycistronic precursor RNA containing eight tRNAs and the ND6 mRNA (Attardi & Schatz 1988). Two models exist for the transcription of the H-strand. Montoya *et al.* (1983) suggested that the RNA synthesis starts at two transcription initiation points, H_1 and H_2, located in the HSP promoter area of the D-loop, while another model proposes the existence of only one major transcription initiation point, H_1 (Clayton 1992). The transcription of both strands of mtDNA leads to a polycistronic primary RNA molecule in which both of the rRNA genes and almost all the protein genes are flanked by tRNA genes. This unique genetic organization has led to a proposal that the cloverleaf secondary structures of the tRNA sequences may act as a signal for the processing enzymes. Precise endonucleolytic excision of the tRNAs from the polycistronic transcripts would then yield correctly processed rRNAs and mRNAs. (Ojala *et al.* 1981.) In a few cases where there are no tRNAs flanking the mRNA, there may be secondary structures resembling tRNA cloverleafs that are recognized by the processing enzymes.

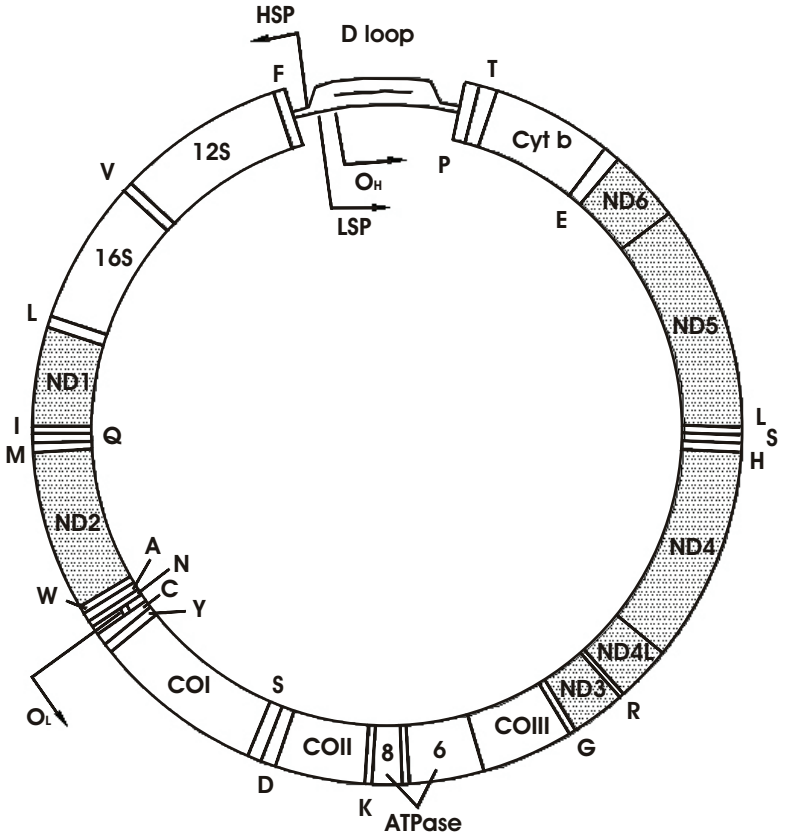

Fig. 3. Mitochondrial DNA encodes two rRNAs (12S and 16S), 22 tRNAs (marked with one-letter amino acid codes) and 13 subunits of OXPHOS enzyme complexes: ND1-ND6 and ND4L for complex I (highlighted in figure), cyt b for complex III, CO I-III for complex IV and ATPase 6 and 8 for complex V. D loop = displacement loop, HSP and LSP = heavy and light strand promoters for transcription, O_H and O_L = heavy and light strand replication origins.

2.4 Complex I

Complex I is the largest and most complicated enzyme of the mitochondrial respiratory chain. It can be separated into distinct subunit fractions upon exposure to certain reagents. This feature has been exploited extensively to resolve the subunit composition of both bacterial and mammalian complex I. Although purified already almost 50 years ago (Hatefi *et al.* 1962), it is only very recently that any substantial progress has been made towards resolving the high-resolution structure of complex I (Hinchliffe & Sazanov 2005, Sazanov & Hinchliffe 2006).

2.4.1 Structure of complex I

Electron microscopic studies on species such as *Neurospora crassa* (Guénebaut *et al.* 1997), *Escherichia coli* (Guénebaut *et al.* 1998) and *Bos taurus* (Grigorieff 1998) have revealed an overall L-shaped structure for complex I. The enzyme is formed of a hydrophobic membrane arm lying within the inner mitochondrial membrane or the bacterial cytoplasmic membrane and a hydrophilic arm protruding into the mitochondrial matrix space or the bacterial cytoplasmic phase (Hofhaus *et al.* 1991, Guénebaut *et al.* 1998). The core of the complex I is formed by 14 subunits which are common in species ranging from bacteria to humans. Seven of these are predominantly hydrophilic (the 75, 51, 49, 30 and 24 kDa, TYKY and PSST subunits) and seven are highly hydrophobic subunits dominated by transmembrane helices (ND1-ND6 and ND4L subunits). These 14 core subunits contain all the known redox cofactors and the substrate binding site of complex I. (Walker 1992.)

2.4.1.1 Complex I in Escherichia coli

The proton-pumping NADH-ubiquinone oxidoreductase NDH-1 is a bacterial counterpart of mitochondrial complex I (Yagi 1993). The NDH-1 contains 13-14 subunits, NuoA-N, and it is about 530 kDa in size (Weidner *et al.* 1993). The genes encoding the subunits are organized in what is known as the *nuo*-operon (NADH-ubiquinone oxidoreductase) with the particularity that the genes *nuoC* and *nuoD* are fused in *E.coli* (Weidner *et al.* 1993, Braun *et al.* 1998, Friedrich, 1998) (Table 1).

NDH-1 can be fragmented into three modules by means of biochemical procedures: an NADH dehydrogenase part, a connecting part and a membrane part (Leif *et al.* 1995) (Fig. 4). The NADH dehydrogenase fragment contains three hydrophilic subunits, NuoE, F and G, with FMN and up to six iron-sulphur clusters as cofactors, and it is considered to represent the electron input part of the enzyme (Leif *et al.* 1995, Braun *et al.* 1998, Friedrich 2001, Hinchliffe & Sazanov 2005). The connecting amphipathic fragment is composed of subunits NuoB, CD and I and contains the rest of the iron-sulphur clusters, including N2 (Leif *et al.* 1995, Hinchliffe & Sazanov 2005) which presumably interacts with ubiquinone (Ohnishi 1998). The membrane fragment contains seven hydrophobic subunits NuoA, H and J-N, which participate in proton transportation and possibly in ubiquinone binding (Leif *et al.* 1995, Friedrich 1998, Hinchliffe & Sazanov 2005). The structural and functional similarities between the bacterial and human forms of complex I allow NDH-1 to be used as a minimal model for studying this enzyme. In addition, *E. coli* is an ideal organism for studying the mitochondrially encoded subunits, since the genetic manipulation of mtDNA is extremely laborious.

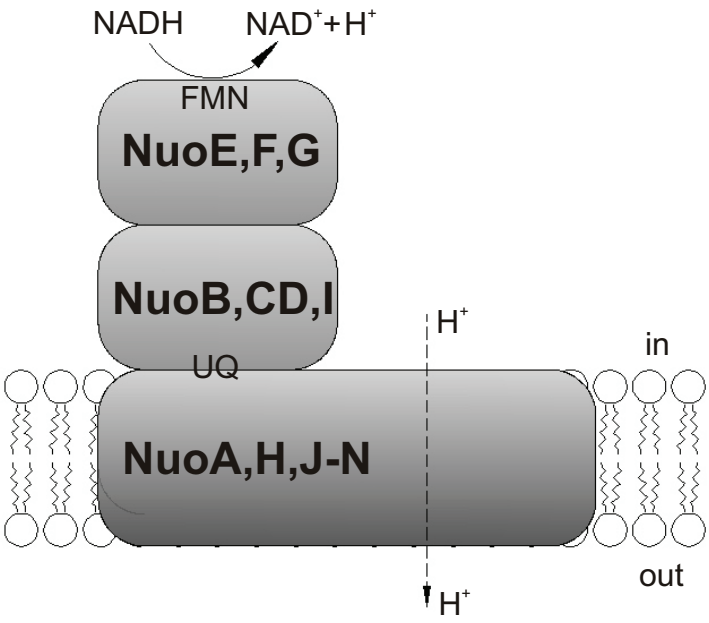

Fig. 4. Proton-pumping NADH-ubiquinone oxidoreductase (NDH-1) in *E. coli*. The three functionally distinct modules and their subunit compositions are shown.

2.4.1.2 Complex I in mammals

Mitochondrial complex I is one of the most complicated enzymes known, with a total molecular mass of approximately 1 MDa (Walker 1992). It is composed of 46 subunits, of which seven are encoded by the mtDNA (ND1-ND6 and ND4L) and the rest by genes residing in the nucleus. At the moment, the amino acid sequences of all except one of the subunits have been determined. Complex I from bovine heart mitochondria is the best-characterized mammalian enzyme, serving as a valuable model for the human enzyme. (Walker 1992, Carroll *et al.* 2003.)

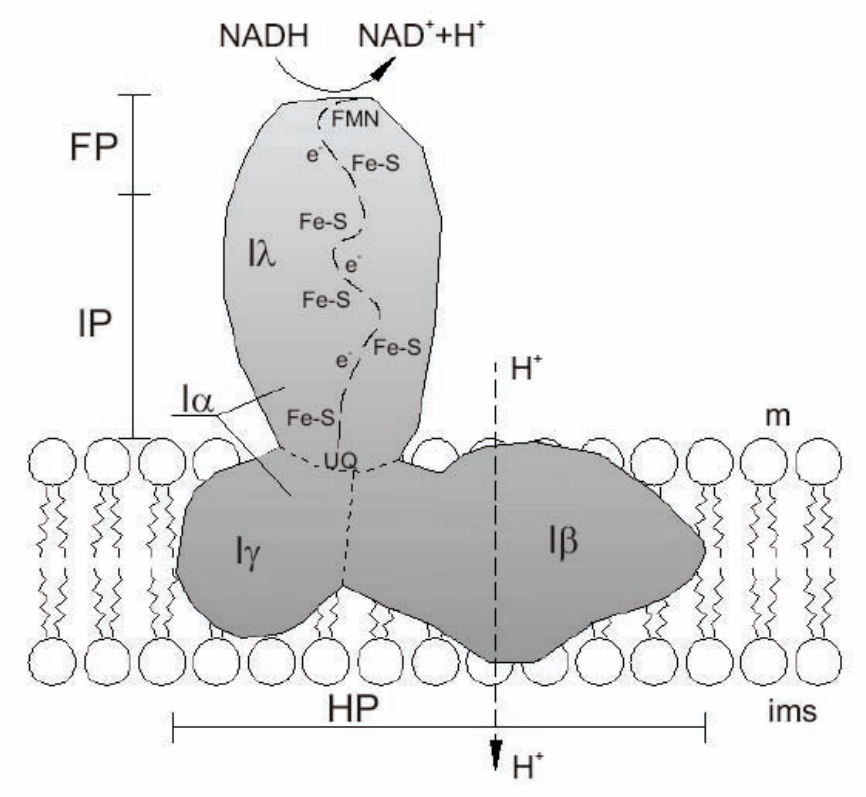

Fig. 5. Structure and function of mammalian complex I in the inner mitochondrial membrane. The fractions obtained by detergent disruption are indicated in the structure. Two electrons from NADH are transferred from FMN through seven iron-sulphur clusters to ubiquinone, at the same time as four protons are pumped from the matrix to the intermembrane space. FP = flavoprotein fraction, IP = iron protein fraction, HP = hydrophobic protein fraction, FMN = flavin mononucleotide, Fe-S = iron-sulphur cluster, e^- = electron, UQ = ubiquinone, H^+ = proton, m = matrix, ims = intermembrane space.

The subunit composition of bovine complex I has been determined by resolving the intact enzyme into a number of fragments with chaotropic agents (Fig. 5). Treatment with perchlorate led to an initial fractionation of the enzyme into three functional parts: a water-soluble flavoprotein (FP), a water-soluble iron protein (IP) and a hydrophobic protein fraction (HP) (Galante & Hatefi 1979). The well-characterized FP fragment is catalytically active, containing a 51 kDa subunit with FMN and one tetranuclear iron-sulphur cluster as cofactors, a 24 kDa subunit with one binuclear iron-sulphur cluster and a 10 kDa subunit (Ragan *et al.* 1982). The IP fragment contains the rest of the iron-sulphur clusters and the HP fragment is formed mostly of membrane-bound subunits. The hydrophilic fragments FP and IP lie within the matrix space constituting the peripheral arm of complex I, whereas the HP fragment forms the membrane arm. (Ragan 1987.) More recently, fractionation of bovine complex I with the mild detergent lauryldimethylamine oxide (LDAO) has led to the separation of two fragments Iα and Iβ

(Finel *et al.* 1992, Carroll *et al.* 2002), and further sucrose gradient centrifugation of fragment Iα has led to the isolation of a smaller fragment Iλ (Finel *et al.* 1994, Fearnley *et al.* 2001). The Iλ fragment includes the seven nuclear-encoded core subunits with all the bound redox cofactors and represents the hydrophilic arm of complex I (Fearnley *et al.* 2001) (Table 1). In addition to the Iλ fragment subunits, fragment Iα contains at least eight subunits which are part of the membrane arm. This subfraction is called Iγ. The Iβ fragment contains at least 13 hydrophobic subunits and forms the major part of the membrane arm. (Carroll *et al.* 2003, Hirst *et al.* 2003.)

The characterization of human complex I has greatly benefited from the work done on bovine complex I. The fractionation of complex I as described above requires large amounts of mitochondria as starting material, which is not easy to obtain from human tissue sources. The subunit composition of human heart complex I has been studied by sucrose gradient fractionation and by immunocapture using monoclonal antibodies against bovine complex I, and as a result, 45 human homologues to the bovine complex I subunits have been identified (Hanson *et al.* 2001b, Murray *et al.* 2003, Taylor *et al.* 2003). The chromosomal localizations and coding sequences of all the bovine subunits characterized at the peptide level now have known counterparts in humans (Loeffen *et al.* 1998b, Smeitink *et al.* 2001b, Hirst *et al.* 2003). The nomenclature of human complex I subunits employed here follows the bovine nomenclature, but the names of the genes are from the human genome (Table 1).

Table 1. Complex I subunits within subcomplexes.

Subcomplex		Subunit (bovine)	Gene (human)	*E. coli*	Specific features
Iα	Iλ	75 kDa	***NDUFS1***	*NuoG*	[2Fe-2S] (N1b), [4Fe-4S] (N4), [4Fe-4S] (N5) [4Fe-4S] (N7)[b]
		51 kDa	***NDUFV1***	*NuoF*	FMN, [4Fe-4S] (N3)
		49 kDa	***NDUFS2***	*NuoCD*[a]	
		30 kDa	***NDUFS3***	*NuoCD*[a]	
		24 kDa	***NDUFV2***	*NuoE*	[2Fe-2S] (N1a)
		PSST	***NDUFS7***	*NuoB*	[4Fe-4S] (N2)
		TYKY	***NDUFS8***	*NuoI*	2 [4Fe-4S] (N6a, N6b)
		AQDQ (18 kDa)	***NDUFS4***	-	
		13 kDa	*NDUFS6*	-	
		10 kDa	*NDUFV3*	-	
		B17.2	*NDUFA12*	-	also called DAP13
		B16.6	*NDUFA13*	-	also called GRIM-19
		B14.7	*NDUFA11*	-	
		B14.5a	*NDUFA7*	-	
		B13	*NDUFA5*	-	
		B8	*NDUFA2*	-	
	Iγ	42 kDa	*NDUFA10*	-	phosphorylated
		39 kDa	*NDUFA9*	-	NADPH, phosphorylated
		15 kDa	*NDUFS5*	-	
		MWFE	*NDUFA1*	-	phosphorylated
		PGIV	*NDUFA8*	-	
		B14	*NDUFA6*	-	
		B9	*NDUFA3*	-	
		ND1	***ND1***	***NuoH***	encoded by mtDNA
at the		ND2	***ND2***	*NuoN*	encoded by mtDNA
interface		ND3	***ND3***	*NuoA*	encoded by mtDNA
between		ND4L	***ND4L***	*NuoK*	encoded by mtDNA
Iγ and Iβ		ND6	***ND6***	*NuoJ*	encoded by mtDNA
		KFYI	*NDUFC1*	-	
		MLRQ	*NDUFA4*	-	cardiolipin
		SDAP	***NDUFAB1***	-	an acyl carrier protein
		B15	*NDUFB4*	-	

[a] *NuoC* and *NuoD* are fused in *E. coli*.

[b] This iron-sulphur cluster is present only in some species of bacteria (*E. coli*) (Nakamaru-Ogiso *et al.* 2005).

Subunits studied in the present work are marked in bold.

Table 1 (continued).

Subcomplex	Subunit (Bovine)	Gene (Human)	*E. coli*	Specific features
Iβ	AGGG	*NDUFB2*	-	
	ASHI	*NDUFB8*	-	
	ESSS	*NDUFB11*	-	also called NP17.3, phosphorylated
	MNLL	*NDUFB1*	-	
	PDSW	*NDUFB10*	-	
	SGDH	*NDUFB5*	-	
	B22	*NDUFB9*	-	
	B18	*NDUFB7*	-	
	B17	*NDUFB6*	-	
	B14.5b	*NDUFC2*	-	
	B12	*NDUFB3*	-	
	ND4	***ND4***	*NuoM*	encoded by mtDNA
	ND5	***ND5***	*NuoL*	encoded by mtDNA
	10.6 kDa	?	-	unresolved protein sequence

Based on Carroll *et al.* (2003), Hirst *et al.* (2003), Holt *et al.* (2003) and Brandt (2006).

2.4.2 Biogenesis of complex I

The assembly of mitochondrial complex I is a multi-step process which involves the correct organization of up to 46 subunits. These are encoded in two distinct cell compartments, imported into the mitochondria and assembled into a holoenzyme embedded in the mitochondrial inner membrane. Before the nuclear-encoded subunits are able to form a functionally active enzyme together with the mtDNA encoded subunits, several post-translational modifications have to occur. In fact, it seems that all except two of the 38 nuclear-encoded complex I subunits sequenced so far are modified during or after cytoplasmic translation (Carroll *et al.* 2005).

2.4.2.1 Post-translational modifications

Most subunits have N-terminal extensions acting as mitochondrial leading sequences, which are removed during the import process. The mitochondrial leading peptide is characterized by positively charged, hydroxylated, hydrophobic amino acids and has the potential to form an amphiphilic alpha helix (Roise & Schatz 1988, von Heijne *et al.* 1989). Subunits with no N-terminal leading peptide have internal targeting sequences that are much less well defined and may be spread over the entire length of the protein. (Truscott *et al.* 2003.) The majority of these subunits have other N-terminal modifications such as removal of the translational initiator methionine and acetylation of the alpha-amino group of the second residue (Carroll *et al.* 2005). Reversible phosphorylation of the subunits has been suggested to regulate the enzyme activity and affect electron transfer or the formation of ROS (Schilling *et al.* 2005). Phosphorylation of the 42 kDa

(Schulenberg *et al.* 2003), 39 kDa (Schilling *et al.* 2005), ESSS (Chen *et al.* 2004) and MWFE subunits (Raha *et al.* 2002) has been observed. The catalytic activity of complex I also seems to be closely dependent on phospholipids, and three classes of interaction have been identified (Sharpley *et al.* 2006). The most tightly bonded is cardiolipin, which may have a functional role or be required for structural integrity, especially for the presence of the MLRQ subunit (Hirst *et al.* 2003).

2.4.2.2 Incorporation of prosthetic groups

Once the seven nuclear-encoded hydrophilic subunits of complex I have reached the mitochondria, incorporation of the prosthetic groups, i.e. FMN and the eight iron-sulphur clusters, occurs (Hirst *et al.* 2003). These prosthetic groups are most likely inserted in an analogous manner to other systems, where the formation of iron-sulphur clusters is catalyzed by ferrochelatases, while FMN may not need any protein factor for insertion (Schulte 2001). Interestingly, a novel complex I subunit Nqo15 was identified when resolving the crystal structure of the peripheral arm in *Thermus thermophilus* (Sazanov & Hinchliffe 2006). The Nqo15 subunit possesses a fold resembling that seen in proteins of the frataxin family, which are thought to be iron chaperones involved in the storage and maturation of proteins containing iron-sulphur and heme (Bulteau *et al.* 2004). Thus a possible role for Nqo15 in complex I of *T. thermophilus* could be iron storage. It seems, however, that the Nqo15 subunit is a unique feature of thermophiles which live in harsh environments. (Sazanov & Hinchliffe 2006.)

As already indicated, the first crystal structure of the bacterial (*T. thermophilus*) complex I peripheral arm has recently been resolved (Hinchliffe & Sazanov 2005, Sazanov & Hinchliffe 2006). In this crystallized structure, nine iron-sulphur clusters were identified that were previously predicted to reside in complex I of certain bacteria (e.g. *E. coli* and *T. thermophilus*). The locations of the redox centres in the crystallized structure of the peripheral arm were in accordance with previous predictions that the 75 kDa subunit binds two tetranuclear clusters, N4 and N5, and one binuclear iron-sulphur cluster, N1b, the 51 kDa subunit contains the non-covalently bound FMN and the tetranuclear iron-sulphur cluster N3, the 24 kDa subunit binds the binuclear iron-sulphur cluster N1a, the TYKY subunit binds the tetranuclear iron-sulphur clusters N6a and N6b, and the PSST subunit binds the tetranuclear iron-sulphur cluster N2. In addition, the ninth iron-sulphur cluster, N7, which is identified only in certain bacteria, was located in the 75 kDa subunit. N7 is located too far away from the other clusters to participate actively in electron transport, but instead it is likely to be an evolutionary remnant. (Hinchliffe & Sazanov 2005, Sazanov & Hinchliffe 2006.)

2.4.2.3 Assembly of complex I

Phylogenetic analyses have revealed that most of the 14 core subunits of complex I have evolved from hydrogenases of different types (Finel 1998). According to this modular evolution theory, three modules, a peripheral NADH dehydrogenase, an amphipathic

hydrogenase and a membrane-bound transporter, with distinct functional properties have united to form the proton-pumping NADH:ubiquinone oxidoreductase. These three modules are also present separately or combined in several other enzymes performing similar functions. (Friedrich 2001.)

The process of complex I assembly has been studied extensively in the fungus *N. crassa* (Friedrich *et al.* 1989, Tuschen *et al.* 1990, Schmidt *et al.* 1992). The findings have supported the modular evolution theory by describing the independent assembly of two modules, a peripheral and a membrane arm. In addition, it seems that assembly of the membrane arm is preceded by the formation of two subcomplexes known as the small and large intermediates. The large intermediate is found to be associated with two complex I intermediate associated proteins, CIA30 and CIA84, which are not present in the final enzyme. (Küffner *et al.* 1998.)

Studies on the process of human complex I assembly have led to two distinct proposals that differ considerably from one another (Antonicka *et al.* 2003, Ugalde *et al.* 2004b). In the model described by Ugalde *et al.* (2004b), which is in accordance with the modular evolution theory, complex I is assembled semi-sequentially. The discrete functional modules are first assembled independently and then joined together in several steps to form a peripheral arm and a membrane arm assembly intermediate. Another connection between the assembly of human and fungus complex I patterns was encountered when NDUFAF1, the human homologue of the fungal complex I chaperone protein CIA30, was identified (Janssen *et al.* 2002), and it has recently been demonstrated that NDUFAF1 is an important protein for the assembly and/or stability of complex I in humans (Vogel *et al.* 2005).

According to the other assembly model, that suggested by Antonicka *et al.* (2003), complex I subcomplexes, identified by 2D Blue native/SDS-PAGE, are composed of parts of both the peripheral and the membrane arm. Similar intermediate subcomplexes have been observed in another study on patients with mutations in nuclear-encoded subunits (Ugalde *et al.* 2004a). These findings led to the proposal of a distinct complex I assembly model in humans, where the peripheral and membrane arms are not assembled in separate ways as has been suggested for *N. crassa.* In addition, a novel molecular chaperone has been identified for complex I assembly using a bioinformatics approach (Ogilvie *et al.* 2005). A candidate gene *B17.2L* was selected by subtracting mitochondrial proteins common in aerobic yeasts but missing in fermentative yeasts (with no complex I). *B17.2L* is a paralogue of *B17.2* (*NDUFS12* in humans), a gene that encodes for a small complex I subunit of the matrix arm, and it seems to be an ancient duplication of the *B17.2* gene (Gabaldon *et al.* 2005). The role of B17.2L in the assembly of complex I was indicated by the finding that it accumulated with complex I subassembly, as seen in patients with a complex I assembly defect, but was missing from the fully assembled complex I in controls. Moreover, a mutation in the *B17.2* gene has been described as leading to a defect in complex I assembly. (Ogilvie *et al.* 2005.)

A number of additional subunits have been added to the 14-subunit “core” in the course of evolution. Complex I in mammalian mitochondria contains 32 additional accessory subunits, the role of which has been studied in various species and by a number of methods. The results indicate that several of them, e.g. AQDQ, B13, B14.7, 20.9, PGIV, PDSW, SDAP and MWFE, are required for the assembly of complex I in the specific species studied (Videira 1998, Videira & Duarte 2001, Yadava *et al.* 2002,

Scacco *et al.* 2003). In bovine tissues, for example, there are 14 accessory subunits with a single transmembrane domain that could be readily inserted into the inner mitochondrial membrane. When anchored to the membrane, these subunits could then promote and organize the assembly of mtDNA-encoded subunits by interacting with their highly hydrophobic transmembrane segments. (Abdrakhmanova *et al.* 2004.)

The question of the assembly of complex I seems to be far from solved at present. In particular, it is not known whether the assembly mechanism seen in *N. crassa* is universal. The mtDNA-encoded subunits in fungus are exclusively located in the membrane arm of complex I, supporting the modular assembly theory, whereas several peripheral arm subunits encoded by the nuclear DNA in the fungus have mitochondrially encoded homologues in other organisms such as plants (Videira 1998).

2.4.3 Function of complex I

As a whole, the functions of complex I are to maintain the NAD^+/NADH ratio in the mitochondrial matrix, to supply ubiquinol to complex III and to contribute to the proton motive force supporting the synthesis of ATP (Saraste 1999).

The overall reaction of complex I

$$NADH + Q + H^+ + 4H^+_{in} \leftrightarrow NAD^+ + QH_2 + 4H^+_{out}$$

achieves the transfer of two electrons from NADH to ubiquinone, coupled to the translocation of four protons across the membrane (Wikström 1984, Galkin *et al.* 1999).

2.4.3.1 Redox reaction

The electron transport chain in complex I is initiated when NADH donates two electrons to FMN in a hydride transfer reaction. FMN transfers the electrons by reducing the iron-sulphur cluster N3 in two single-electron steps (Sled *et al.* 1994). Electrons are further transferred along the linear chain of iron-sulphur clusters at a distance of ~14Å from each other (Hirst 2005) along the following path: N3-N1b-N4-N5-N6a-N6b-N2 (Sazanov & Hinchliffe 2006). It has been proposed that the cluster N2, with the highest pH-dependent midpoint potential, interacts with semiquinone bound in complex I, suggesting that this is the final step in the iron-sulphur chain (Yano *et al.* 2005). The 49 kDa subunit containing the N2 cluster and the neighbouring PSST subunit, which have both evolved from the hydrogen-reducing domain of [NiFe] hydrogenase, are thought to be involved in ubiquinone binding (Kashani-Poor *et al.* 2001). The location of the Ni-binding site in hydrogenase is also conserved in complex I and lies adjacent to the N2 cluster. Furthermore, the site is partly exposed towards an elongated cavity at the interface with the membrane domain (Sazanov & Hinchliffe 2006). Thus the quinone-binding site of complex I may have evolved from the Ni-containing active centre of the hydrogenase. The mechanism of ubiquinone reduction in complex I remains unknown, however.

According to the most recent observations, seven out of the eight iron-sulphur clusters participate in the electron transport chain of complex I. The long distance between clusters N1a and N3 implies that N1a is unlikely to participate directly in the electron transport chain. FMN seems to be located between these two clusters, suggesting that N1a could have a role in preventing excessive reduction of FMN by redistributing the electrons further down the redox chain. This would lead to a rapid oxidation of the flavosemiquinone radicals and minimize the production of ROS during complex I turnover. (Hincliffe & Sazanov 2005, Sazanov & Hinchliffe 2006.)

The overall NADH:ubiquinone oxidoreduction reaction in complex I can be followed by studying differences in light absorption between NADH and NAD+ at a wavelength of 340 nm. The function of complex I can be followed using several naturally occurring inhibitors with highly divergent structures, e.g. rotenone or synthetic chemicals such as *N*-vanillylnonanamide (VNA) (Degli Esposti 1998). In addition to the overall reaction, the oxidation of NADH by FMN can be followed using hexammine ruthenium (HAR) as an electron acceptor (Sled & Vinogradov 1993). NADH:HAR oxidoreductase activity can be used for more robust assessments such as the quantification of complex I.

2.4.3.2 Proton pumping

Three different energy transduction mechanisms have been suggested, mainly based on the two types of functional model seen in other membrane-bound energy-transducing enzymes. The first type of model, direct coupling, involves two proposed mechanisms called the Q-cycle mechanism and the directly coupled mechanism. The Q-cycle mechanism suggests that quinol is directly involved in proton translocation as a mobile proton/electron carrier. An analogous reaction is seen in complex III. (Dutton *et al.* 1998.) The directly coupled mechanism is based on the reaction seen in complex IV, where the proton transfer across the membrane is controlled directly by a gating reaction that is similar to a coupled electron-transfer reaction (Brandt 1997). The other type of model is based on indirect coupling, as seen in complex V. Here the catalytic sector containing the cofactors is spatially separated from the proton transfer sector and energy transduction takes place through interactions with the protein structure. (Belogrudov & Hatefi 1994.) In addition, another model combining both the indirect and direct coupling mechanisms has been suggested in the light of modular evolution (Friedrich 2001). This model implies that complex I contains two energy-coupling sites, the hydrogenase module functions as a redox-driven proton pump and the transporter module may act as a conformation-driven proton pump.

The majority of the evidence at the moment seems to be in favour of the indirectly coupled mechanism, partly because of facts that question the direct coupling mechanism. The most likely scenario is that the redox chemistry of ubiquinone reduction around the iron-sulphur cluster N2 induces specific conformational changes in the structure, which are further transmitted to the hydrophobic subunits in the membrane part of complex I, acting as ion pumps. (Brandt *et al.* 2003.)

2.4.3.3 Role of accessory subunits

The role of the 32 accessory subunits in complex I is believed to be a general one, e.g. to improve structural stability by keeping the redox groups in the right position, or to protect the enzyme from ROS by preventing electron leakage (Friedrich & Weiss 1997). Indeed, it has been suggested that complex I may be a major source of ROS production in mitochondria, which can lead to mtDNA damage and may be one of the causes of ageing (Balaban *et al.* 2005). In addition, accessory subunits may have specific roles in regulating the activity or assembly of complex I, as already described. Furthermore, some of the accessory subunits may have an alternative function within or even outside the mitochondrial complex I. (Hirst *et al.* 2003.)

Two of the accessory subunits of complex I harbour coenzymes related to fatty acid metabolism and exhibit similarities to those enzymes. One of these, SDAP, is closely related to the acyl carrier proteins (ACPs), with phosphopantetheine as a prosthetic group (Runswick *et al.* 1991). ACPs are involved in type II fatty acid synthesis, which is believed to occur in mitochondria (Torkko *et al.* 2001). Recent findings of a soluble form of SDAP in the bovine mitochondrial matrix have provided further evidence for the involvement of SDAP in mitochondrial fatty acid synthesis (Cronan *et al.* 2005). Interestingly, deletion of SDAP in *N. crassa* resulted in impaired complex I assembly and increased lysolipids in the mitochondria (Schneider *et al.* 1995).

Another accessory subunit, the 39 kDa subunit, contains a conserved NADPH-binding motif and is homologous to the family of short-chain acylCoA dehydrogenases. Its role is not clear at present, but it is thought to be involved in intramitochondrial fatty acid synthesis, possibly together with the SDAP subunit. (Hirst *et al.* 2003.)

One of the most recently identified complex I subunits, B16.6, is identical to a product of the human cell death regulatory gene GRIM-19, which is induced by interferon-β and retinoic acid (Fearnley *et al.* 2001). Further studies have shown that GRIM-19 indeed plays pleiotropic roles within a cell and seems to be essential for complex I assembly (Huang *et al.* 2004). This finding provides a new, but still unresolved link between complex I and apoptotic cell death. Another interesting connection between complex I and apoptosis has come from the finding that a lack of apoptosis-inducing factor (AIF) in human or mouse cells causes a severe reduction in complex I activity and abnormal subunit composition. Since AIF itself is not a part of complex I, one additional role that it plays, besides induction of apoptosis and chromatinolysis in the nucleus, could be in the biogenesis and/or maintenance of complex I. (Vahsen *et al.* 2004.) A recent study employing AIF knock-out human colon carcinoma cells has shown that AIF possesses NADH oxidase activity, which was required to restore the complex I activity (Urbano *et al.* 2005).

2.5 Complex I deficiency

OXPHOS system deficiencies are the most frequent causes of inborn metabolic errors, affecting 1 individual in 5000 (Skladal *et al.* 2003, Thorburn *et al.* 2004). Among the group of OXPHOS disorders, isolated complex I deficiency is one of the most frequently

encountered (Bourgeron *et al.* 1995, Loeffen *et al.* 2000). Complex I deficiency is a highly heterogeneous disorder both phenotypically and genotypically. The severity and course of the disease is basically determined by the specific nature of the deleterious mtDNA or nuclear DNA mutation and by the extent of the enzyme defect caused by that mutation. In the case of mtDNA mutations, heteroplasmy, tissue specificity and environmental factors play additional, modifying roles in the course of the disease. Furthermore, the biochemical defect causing the symptoms may also be due to a combined deficiency, with one or more additional OXPHOS complexes affected. (Janssen *et al.* 2004.)

Several clinical syndromes have been associated with complex I deficiency, ranging from lethal neonatal forms (Zheng *et al.* 1989) to neurodegenerative disorders in adult life including Parkinson's disease and dystonia (Beal 2000). Complex I deficiency is also a general feature in several classical mitochondrial syndromes like Leber hereditary optic neuropathy (LHON) (Larsson *et al.* 1991), myoclonic epilepsy with ragged-red fibers (MERRF) (Wallace *et al.* 1988b) and mitochondrial myopathy, encephalopathy, lactic acidosis and stroke-like episodes (MELAS) (Ciafaloni *et al.* 1992).

2.5.1 Clinical phenotypes in children with complex I deficiency

Complex I deficiency (OMIM #252010) was first described in 1979 (Morgan-Hughes *et al.* 1979). Since then, it has become evident that complex I deficiency is an important cause of inborn errors of metabolism in children (Loeffen *et al.* 2000). Symptoms begin at birth or in early childhood in most cases, and generally present as multi-system disorders with a fatal outcome. The majority of the patients die within a few years of the first clinical manifestation. (Triepels *et al.* 2001b, Smeitink *et al.* 2001a.) Complex I deficiency affects most tissues with a high energy demand, such as the brain, heart, kidney and skeletal muscle. The most common clinical phenotypes result from the dysfunction of these organs, and include Leigh syndrome and Leigh-like syndrome, unspecified encephalopathy, fatal infantile lactic acidosis, isolated myopathy, cardiomyopathy, hepatopathy, tubulopathy and macrocephaly with progressive leukodystrophy (Robinson 1998, Kirby *et al.* 1999, Loeffen *et al.* 2000). Other frequent features include ophthalmological signs such as external ophthalmoplegia, ptosis, cataract and retinopathy (Triepels *et al.* 2001b).

2.5.1.1 Leigh syndrome and Leigh-like syndrome

Leigh or Leigh-like syndrome (OMIM #256000) is the most common pediatric phenotype of isolated complex I deficiency (Loeffen *et al.* 2000). Leigh syndrome (LS), first described in 1951 by Denis Leigh, is a progressive neurodegenerative disorder involving encephalopathy with lactic acidosis, occasionally complicated by cardiomyopathy or a multisystemic presentation (van Erven *et al.* 1987, Robinson 1998). The onset is usually in the first year of life, and the children present with developmental delay and failure to thrive. Motor and intellectual retardation, ataxia, dystonia, hypotonia,

and optic atrophy are frequently encountered. Neuroimaging shows symmetrical lesions in the basal ganglia, midbrain and brainstem. On pathological examination, bilateral symmetrical foci of spongy necrosis is detected with myelin degeneration, vascular proliferation and gliosis in the thalami, brain stem and spinal cord. Patients with similar symptoms but with atypical or unknown neuropathology are referred to as Leigh-like cases (LLS). (Rahman *et al.* 1996, Morris *et al.* 1996.)

Besides complex I deficiency, LS or LLS can have several other biochemical causes, and it has been described in association with defects of all OXPHOS system enzymes (Dahl 1998). Mutations in both mtDNA and nuclear DNA may lead to LS or LLS, but a severe form of the disease, called maternally inherited Leigh syndrome (MILS), is caused by mutations in mtDNA. The 8993T>G mutation in the *ATPase 6* gene is a common cause of MILS (Holt *et al.* 1990). The degree of heteroplasmy of the 8993T>G mutation correlates well with the severity of the disease. High levels of mutated mtDNA (>95%) lead to MILS, whereas patients with lower mutation load present an adult-onset, slowly progressive syndrome called neurogenic weakness, ataxia and retinis pigmentosa (NARP). (Tatuch *et al.* 1992, Carelli *et al.* 2002.)

2.5.1.2 Fatal infantile lactic acidosis

Fatal infantile lactic acidosis (FILA) is characterized by a neonatal onset and a fulminant course. The disease presents with hypotonic muscle weakness, lactic acidosis, seizures and failure to thrive (Kirby *et al.* 1999, Loeffen *et al.* 2000). Isolated and combined respiratory chain enzyme deficiencies have been described in patients with FILA (Nagai *et al.* 1993, Bentlage *et al.* 1996). The genetic origin of FILA can either be nuclear (Procaccio *et al.* 1999, Kirby *et al.* 2004b) or mitochondrial (McFarland *et al.* 2004, Blakely *et al.* 2006). Depletion of mtDNA has also been described in association with FILA (Mazziotta *et al.* 1992).

2.5.1.3 Encephalomyopathies

Mitochondrial encephalomyopathies are a heterogeneous group of clinical disorders due to defects in mitochondrial respiratory chain. As already described, organs such as the brain, heart and skeletal muscle are highly energy dependent and thus vulnerable to defects in energy metabolism. Skeletal muscle involvement presents with exercise intolerance, weakness and myalgias, in association with involvement of other organs, most commonly with encephalopathy or cardiomyopathy. (Loeffen *et al.* 2000, Scaglia *et al.* 2004.) Lactic acidosis with an increased lactate/pyruvate ratio is frequently observed. Mitochondrial encephalomyopathies are caused by mutations in both nuclear and mitocondrial DNA. (DiMauro & Gurgel-Giannetti 2005.) Mutations presenting isolated skeletal muscle myopathy are rare (Andreu *et al.* 1999, Seneca *et al.* 2005, Mayr *et al.* 2006).

2.5.1.4 Leber hereditary optic neuropathy

The unique anatomical and physiological features of the optic nerve seem to make it especially vulnerable to complex I deficiency (Bristow *et al.* 2002). This is seen in Leber hereditary optic neuropathy (LHON) (OMIM #535000), where the rather specific clinical phenotype differs greatly from other heterogeneous mitochondrial syndromes. The main phenotype of LHON, described already in 1871 by Theodore Leber, is a tissue-specific, subacute, painless visual loss characterized by central scotomas, abnormal colour vision and optic atrophy. Vision deteriorates over a period of days or weeks in one eye, usually followed by the other eye. LHON typically affects young adults, the average age being 23 years. Men are affected three to four times more often than women. (Simon & Johns 1999, Man *et al.* 2002, Zeviani & Di Donato 2004.)

Approximately 90% of LHON cases carry one of the three mtDNA mutations, 3460G>A (Howell *et al.* 1991), 11778G>A (Wallace *et al.* 1988a) or 14484T>C (Chinnery *et al.* 2001), which all reside in genes encoding complex I subunits. Mutations are usually homoplasmic. Interestingly, only 50% of men and 10% of women harbouring a pathogenic mtDNA mutation develop the optic neuropathy. This marked incomplete penetrance and gender bias imply that additional genetic factors modulate the phenotypic expression of LHON (Hudson *et al.* 2005). (Man *et al.* 2002.) In addition, environmental factors, like tobacco smoke, seem to play a role as risk factors (Tsao *et al.* 1999).

2.5.1.5 Mitochondrial encephalomyopathy with lactic acidosis and stroke-like episodes

Mitochondrial encephalomyopathy with lactic acidosis and stroke-like episodes (MELAS) syndrome (OMIM #540000) is characterized by migraine-like headache, recurrent vomiting, seizures, short stature, normal early development, lactic acidosis and ragged-red fibers in muscle (Pavlakis *et al.* 1984). In general, the classic MELAS phenotype affects children at 5-15 years of age. However, stroke-like episodes seldom occur in early infancy whereas several atypical manifestations, like delayed motor development and failure to thrive are observed (Sue *et al.* 1999, Okhuijsen-Kroes *et al.* 2001). In adults, common clinical manifestations include sensorineural hearing impairment, diabetes, myopathy, cardiomyopathy and cognitive decline. Biochemically, complex I is frequently the most affected respiratory chain enzyme detected in MELAS. (Ciafaloni *et al.* 1992.)

Approximately 80% of patients with MELAS have a heteroplasmic missense mutation 3243A>G in the *tRNA Leu(UUR)* gene (Goto *et al.* 1990). The genotype-fenotype correlation of 3243A>G is rather loose, since the observed clinical manifestations are not restricted solely on MELAS. Identification of mutations in *MTND* genes associated with MELAS or with MELAS/LHON overlap syndrome further address the link between complex I defect and MELAS phenotype (Corona *et al.* 2001). (Zeviani & Di Donato 2004.)

2.5.1.6 Myoclonic epilepsy with ragged-red fibers

Myoclonic epilepsy with ragged-red fibers (MERRF) (OMIM #545000) is a neuromuscular disorder characterized by myoclonus, epilepsy, muscle weakness, cerebellar ataxia, deafness and dementia (Fukuhara *et al.* 1980, Silvestri *et al.* 1993). Eventhough complex IV deficiency is the most prominent biochemical finding in patient's muscle with MERRF, complex I is often also affected. COX-deficient RRFs are invariably detected in muscle. Most patients with MERRF harbor a heteroplasmic 8344A>G mtDNA mutation in *tRNA (Lys)* gene (Shoffner *et al.* 1990). Clinical, biochemical and molecular studies on large pedigrees with 8344A>G mutation have shown a positive correlation between the severity of the disease, age at onset, mtDNA heteroplasmy and reduced activity of respiratory chain complexes in skeletal muscle. (Zeviani & Di Donato 2004.)

2.5.2 Diagnosis

The diagnosis of mitochondrial diseases is usually based on the results of clinical examinations, exercise tests, laboratory investigations, MRIs, histo-enzymological tests, molecular biology and a biochemical study of oxidative phosphorylation based on muscle biopsies. Elevated blood and cerebrospinal fluid lactate and increased ketone body and lactate/pyruvate molar ratios often give a clear indication of a mitochondrial disorder, making these parameters useful for primary laboratory screening. (Munnich *et al.* 1992.) On the cellular level, a decrease in the activity of complex I hampers the oxidation of NADH, leading to an excess of NADH and a lack of NAD^+ in the cells. This then leads to functional impairment of the Krebs cycle, further increasing the blood lactate, pyruvate and ketone body levels. (Triepels *et al.* 2001b.)

The functioning of the respiratory chain can be evaluated by various morphological methods. Although enzyme histochemical stainings for the activities of complexes II, IV and V are reliable, it is difficult to evaluate the activity of complex I by this approach. Furthermore, the deficiency has to be fairly profound in order to be detected by enzyme histochemical stainings. (Larsson & Oldfors 2001.) A characteristic mosaic pattern known as ragged-red fibers (RRFs) is often seen in muscle fiber segments of patients with mtDNA mutations. These fibers are stained red by Gomori trichrome and contain accumulations of mitochondria often with no complex IV activity. The mitochondrial complex I gene *MTND5* has been shown to be a hot spot for mutations causing MELAS and MELAS/MERRF overlap syndromes with RRFs (Santorelli *et al.* 1997, Naini *et al.* 2005). However, in many pediatric cases with complex I deficiency RRFs are absent. (Zeviani & Di Donato 2004.)

A more precise diagnosis of a mitochondrial disorder can be made by reference to biochemical measurements of respiratory chain enzyme activities, often performed on disrupted mitochondria from tissue homogenates (Rustin *et al.* 1991). Spectrophotometric measurement of complex I activity is based on the estimation of rotenone-sensitive NADH:ubiquinone oxidoreductase activity. The functioning of the respiratory chain enzymes can also be measured directly from fresh, intact mitochondria by adding

substrates that enter the respiratory chain at different points and monitoring the consumption of oxygen by oxygraph (Rustin *et al.* 1991). In addition to the activities of individual enzyme complexes, this method also measures the coupling between electron transport through complexes I-IV and ATP production via complex V. The respiratory chain enzyme activities are diagnosed in tissues such as skeletal muscle, cultured fibroblasts, liver, heart and brain. Fresh skeletal muscle tissue or cultured fibroblasts are employed most frequently, although not all complex I deficiencies detected in skeletal muscle are expressed in fibroblasts (Kirby *et al.* 1999, Triepels *et al.* 2001b).

Different strategies using patient-derived cultured cell lines are used to elucidate the underlying genetic defect. Fusions of patient cells lines with mtDNA-depleted rho zero cells can be used to discriminate between mtDNA and nuclear mutations, on the grounds that if the cybrids retain their complex I activity the genetic defect resides in the nuclear DNA and vice versa (Triepels *et al.* 2001b). In addition, chromosomes or parts of chromosomes which can complement the unknown defective gene can be identified by the chromosome transfer technique (Janssen *et al.* 2004).

The availability of antibodies raised against complex I subunits has provided a new tool for elucidating the underlying OXPHOS defect further. Native protein separation methods are used in combination with Western blotting to differentiate between catalytic and assembly problems affecting OXPHOS enzymes in patient cell lines (Hanson *et al.* 2001a). Furthermore, mutations in the same complex I subunit genes have been shown to follow distinct assembly patterns, a feature that can be used to select candidate genes for further mutational analysis (Triepels *et al.* 2001a, Ugalde *et al.* 2004a).

Molecular genetic methods have improved the diagnosis of complex I deficiency and provided more insight into the genetic aspects of the disease (Triepels *et al.* 2001b). Point mutations can be detected by PCR and subsequent sequencing, but due to the heteroplasmic nature of mtDNA, mutations present in muscle or other tissues may be missing from blood (Larsson & Clayton 1995). Elucidation of the underlying genetic defect enables genetic counselling and prenatal diagnosis to be contemplated, and also contributes to our fundamental knowledge of these rare, severe disorders (Rötig *et al.* 2004).

2.5.2.1 Prenatal diagnosis

Reliable prenatal diagnosis of mitochondrial OXPHOS diseases is difficult. Since the molecular defect often remains unclear, the prenatal diagnosis of complex I deficiency mainly relies on enzymatic measurements of complex I activity in chorionic villi or amniocytes. Contradictory results have nevertheless been obtained concerning the reliability of complex I activity measurements in fetal tissues (Faivre *et al.* 2000, Niers *et al.* 2001, Schuelke *et al.* 2002). Mutational analysis is an option only in cases where the genetic cause of the defect is known. Overall, mutation analysis of nuclear-encoded genes in DNA from fetal tissues offers a feasible way of achieving a prenatal diagnosis, but this is difficult in the case of a mtDNA mutation, due to the need for a precise estimation of heteroplasmy and uncertainty about its effects (Niers *et al.* 2001, Rötig *et al.* 2004).

2.5.3 Inheritance of complex I deficiency

Because of the dual genetic origin of complex I, the underlying genetic defect can be either mitochondrial or nuclear. Furthermore, the nuclear genes involved in complex I can be categorized on both a structural and a functional level. Structural genes encode complex I subunits and functional genes encode factors involved in the assembly of complex I, biogenesis of the OXPHOS system and maintenance of the mtDNA. (Janssen *et al.* 2004.) In general, it seems that there are differences in the age at onset, in the outcome and in the severity and intrafamiliar variability of the clinical course between patients affected by OXPHOS defects attributable to nuclear and mtDNA mutations (Rubio-Gozalbo *et al.* 2000). Patients with nuclear mutations become symptomatic at a young age and have a severe clinical course, whereas patients with mtDNA mutations show a wider clinical spectrum of age at onset and severity.

The genetic origin of the complex I deficiency can be resolved in around 40% of pediatric cases at present (Rötig *et al.* 2004). In these cases the defect is at the structural level. The genetic origin of the remaining 60% of cases seems to lie in genes at the functional level. An interesting example of identifying novel causes at the functional level comes from the recent finding of mutations in the *POLG1* gene encoding the catalytic subunit of mitochondrial polymerase γ in patients with multiple oxidative phosphorylation deficiencies (de Vries *et al.* 2006). As already mentioned, only two human complex I assembly factors have been suggested so far, CIA30 and B17.2L (Janssen *et al.* 2002, Ogilvie *et al.* 2005), and many more would presumably be required to build a functional complex I. Until these have been identified, the only possibility for resolving the underlying genetic defect, especially in isolated complex I deficiency, is to study mainly genes at the structural level.

2.5.3.1 Mutations in nuclear DNA

The majority of cases with complex I deficiency seem to follow an autosomal recessive model of inheritance, suggesting a defect of nuclear origin (Loeffen *et al.* 2000). Since the encounter of the first nuclear-encoded complex I mutation by Loeffen *et al.* (1998a), analyses of complex I deficient patients have revealed mutations in ten nuclear-encoded subunit genes and one assembly factor gene (Table 2). These include substitutions of functionally important amino acids, frame shifts and premature stop codon mutations and also large-scale deletions. (Triepels *et al.* 2001b, Ogilvie *et al.* 2005.) The *NDUFV1* and *NDUFS4* genes seem to be hot spots for mutations in nuclear complex I genes.

Table 2. Mutations in nuclear-encoded subunits causing complex I deficiency.

Gene	Amino acid substitution	Clinical features	References
Core subunits			
NDUFS1	Leu231Val	Leigh syndrome	Martin *et al.* 2005
	Arg241Trp/Arg557X	Leigh syndrome	Benit *et al.* 2001
	Asp252Gly/del codon 222	leukodystrophy	Benit *et al.* 2001
	Gln522Lys	leukoencephalopathy	Bugiani *et al.* 2004
	Met707Val/large-scale del	Leigh syndrome	Benit *et al.* 2001
NDUFS2	Ala224Val*	encephalomyopathy	Bugiani *et al.* 2004
	Arg228Gln	hypertrophic cardiomyopathy,	Loeffen *et al.* 2001
	Pro229Gln	and encephalomyopathy	
	Ser314Pro		
NDUFS3	Thr451Ile/Arg199Trp	Leigh syndrome	Benit *et al.* 2004
NDUFS7	Va122Met	Leigh syndrome	Triepels *et al.* 1999
	Arg145His	not specified	Rötig *et al.* 2004
NDUFS8	Pro79Leu/Arg102His	Leigh syndrome	Loeffen *et al.* 1998a
	Pro85Leu/Arg138His	late-onset Leigh syndrome	Procaccio & Wallace 2004
NDUFV1	Arg59X/Thr423Met	encephalomyopathy	Schuelke *et al.* 1999
	Tyr204Cys/Cys206Gly	Leigh syndrome	Benit *et al.* 2001
	Ala211Val	muscle hypotonia and nystagmus	Schuelke *et al.* 2002
	Glu214Lys/IVS8nt+4	Leigh syndrome	Benit *et al.* 2001
	Ala341Val	leukodystrophy and myoclonic epilepsy	Schuelke *et al.* 1999
	Ala432Pro/del nt 989-990	Leigh syndrome	Benit *et al.* 2001
NDUFV2	IVS2+5_+8delGTAA	hypertrophic cardiomyopathy and encephalomyopathy	Benit *et al.* 2003a
Accessory subunits			
NDUFA8	Glu109Lys*	encephalomyopathy	Bugiani *et al.* 2004
NDUFS4	IVSnt-1	Leigh syndrome	Benit *et al.* 2003b
	Trp15X	Leigh-like syndrome	Petruzzella *et al.* 2001
	Trp96X	Leigh-like syndrome	Budde *et al.* 2000
	Arg106X	Leigh-like syndrome	Budde *et al.* 2000
	Lys158fs (5bp dupl)	Leigh syndrome	van den Heuvel *et al.* 1998
	del of exons 3-5	not specified	Rötig *et al.* 2004
NDUFS6	large-scale del	lethal infantile mitochondrial	Kirby *et al.* 2004b
	IVS2nt+2	disease	
Assembly factor			
B17.2L	Arg45X	encephalopathy	Ogilvie *et al.* 2005

*These two heterozygous mutations were identified in one patient

Abbreviations: del = deletion, nt = nucleotide, IVS = inversion, fs = frame shift, dupl = duplication

2.5.3.2 Mutations in mtDNA

Features that distinguish disorders attributable to mitochondrial mutations from others are specific genetic characteristics of mtDNA such as maternal inheritance, polyplasmy, heteroplasmy, the threshold effect and mitotic segregation. Since mtDNA is maternally inherited, pathogenic mutations are transmitted only from mothers to their progeny. Each cell contains thousands of copies of mtDNA, the phenomenon called polyplasmy. If both the wild-type and mutant-type mtDNA coexist in the cell, the situation is called heteroplasmy. When the amount of wild-type mtDNA drops below a certain level (40-20%) the functioning of the OXPHOS system is disturbed and clinical symptoms emerge, a phenomenon called the threshold effect (Lightowlers *et al.* 1997). The level of the threshold effect is tissue-specific, being lower in tissues with a high energy demand, such as the heart, brain, skeletal muscle and retina, which means that these tissues are especially vulnerable to the effects of pathogenic mtDNA mutations. The degree of heteroplasmy can change between cell divisions, since the mtDNA molecules of the dividing cell are distributed randomly among the daughter cells. Because of this mitotic segregation, the amount of mutant mtDNA can drop below the pathogenic threshold level and alter the phenotype of that specific cell line or even the tissue. (DiMauro & Schon 2003.) A combination of these specific features related to mitochondrial genetics leads to a broad spectrum of clinical signs affecting different organs and tissues, and even related to the age or gender of the patients.

The rate of evolution is much faster for mtDNA than for the nuclear genome (Brown *et al.* 1979) partly because the mtDNA is less well protected, especially from ROS generated in its vicinity, and because its repair mechanisms are less efficient (Fernández-Silva *et al.* 2003). Evolution has produced related sets of mtDNA sequences, called haplogroups, that can be recognised by certain sequence changes. Since the mtDNA haplogroups are associated with specific populations on different continents, a great deal has been learned about the evolution of modern humans and about human migrations by studying the haplogroup distributions in different populations. (Herrnstadt & Howell 2004.) It has also been suggested that haplogroup-specific polymorphisms may play a role in the pathogenesis of mitochondrial diseases. The association of a specific haplogroup with the penetrance of the disease has been confirmed in cases of LHON. Two primary LHON mutations, 11778G>A and 14484T>C have been linked to haplogroup J (Torroni *et al.* 1997) and further studies have narrowed the association to the subclades J1c and J2b, which share two specific combinations of amino acid substitutions in the *cytochrome b* gene encoding the subunit of complex III. It has been suggested that changes in cytochrome *b* could have an effect on the activity of complex I through the formation of supercomplexes. (Carelli *et al.* 2006.) In addition, an association between an X-chromosomal locus and LHON was recently discovered which could perhaps explain the variable penetrance and sex bias (Hudson *et al.* 2005).

The higher mutation rate of mtDNA often makes it difficult to distinguish pathogenic mutations from benign polymorphisms (Mitchell *et al.* 2006). Furthermore, there is no clear-cut genotype-phenotype correlation, as the same mutation can cause different phenotypes and the same clinical features can be caused by different mutations. The 11778G>A mutation in *MTND4* was the first known mtDNA mutation to be associated

with a human disease (Wallace *et al.* 1988a). At present, this mutation along with 3460G>A in *MTND1* and 14484T>C in *MTND6* are recognised as the main causes of LHON. In addition to these three primary LHON mutations, over 120 pathogenic mtDNA point mutations have been reported (MITOMAP, www.mitomap.org). Where the majority of pathogenic mtDNA mutations are heteroplasmic, those causing LHON nevertheless seem in general to be homoplasmic. The mtDNA genes encoding subunits of complex I are among the most frequently encountered carriers of pathogenic mutations, and base pair changes causing variable clinical features have been identified in all of them (Table 3). The majority of the pathogenic mutations reported to affect complex I seem to reside in the *MTND6* and *MTND1* genes, which have therefore been called hot spots for mutations causing especially LHON (Chinnery *et al.* 2001, Valentino *et al.* 2004). In addition, mutations in mitochondrial tRNA genes can lead to the absence or misincorporation of certain amino acids during translation and can thereby cause complex I deficiency (DiMauro & Hirano 2005). The effect of tRNA mutation on the functioning of the OXPHOS enzymes varies depending on codon usage, the subunits that are most dependent on the mutant tRNA for protein elongation being more seriously affected (Triepels *et al.* 2001b). In addition, a mutation in a tRNA gene may affect the adjacent subunit gene through changes in the processing of the polycistronic transcript (Bindoff *et al.* 1993).

Table 3. Pathogenic mtDNA mutations causing complex I deficiency.

Mutation	Gene	Amino acid replacement	Clinical Presentation	References
583G>A	*tRNA(Phe)*	n.a.	exercise intolerance	Darin *et al.* 2006
3243A>G	*tRNA(Leu)*	n.a.	MELAS	Goto *et al.* 1990
3250T>C	*tRNA(Leu)*	n.a.	isolated myopathy	Ogle *et al.* 1997
3251T>G	*tRNA(Leu)*	n.a.	myopathy and lactic acidosis	Houshmand *et al.* 1996
3271T>C	*tRNA(Leu)*	n.a.	MELAS	Kirby *et al.* 1999
3302A>G	*tRNA(Leu)*	n.a.	progressive myopathy	Hutchison *et al.* 2005
3303C>T	*tRNA(Leu)*	n.a.	isolated myopathy	Kirby *et al.* 1999
3376G>A	*ND1*	Glu24Lys	LHON/MELAS overlap	Blakely *et al.* 2005
3394T>C	*ND1*	Tyr30His	long QT Syndrome	Matsuoka *et al.* 1999
3460G>A	*ND1*	Ala52Thr	LHON	Huoponen *et al.* 1991
3697G>A	*ND1*	Gly131Ser	MELAS	Kirby *et al.* 2004a
3733G>A	*ND1*	Glu143Lys	LHON	Valentino *et al.* 2004
3796A>G	*ND1*	Thr164Ala	adult-onset dystonia, spasticity and core-type myopathy	Simon *et al.* 2003
3902_3908 inv	*ND1*	in-frame substitution: Asp199Gly, Leu200Lys, Ala201Val	fatal infantile lactic acidosis	Blakely *et al.* 2006
3946G>A	*ND1*	Glu214Lys	MELAS	Kirby *et al.* 2004a
3949T>C	*ND1*	Tyr215His	MELAS	Kirby *et al.* 2004a
5132delAA	*ND2*	Asn222fs	exercise intolerance	Schwartz & Vissing 2002
5693T>C	*tRNA(Asn)*	n.a.	encephalomyopathy	Coulbault *et al.* 2005
7526A>G	*tRNA(Asp)*	n.a.	isolated myopathy	Seneca *et al.* 2005
8344A>G	*tRNA(Lys)*	n.a.	MERRF	Shoffner *et al.* 1990
8363T>A	*tRNA(Lys)*	n.a.	MERRF	Arenas *et al.* 1999
10158T>C	*ND3*	Ser34Pro	Leigh syndrome	Lebon *et al.* 2003
10191T>C	*ND3*	Ser45Pro	progressive epilepsy, stroke-like episodes, optic atrophy and cognitive decline, Leigh syndrome	Taylor *et al.* 2001
11778G>A	*ND4*	Arg340His	LHON	Wallace *et al.* 1988a
11832G>A	*ND4*	Trp358X	exercise intolerance	Andreu *et al.* 1999
12207G>A	*tRNA(Ser)*	n.a.	myopathy and encephalopathy	Wong *et al.* 2006
12706T>C	*ND5*	Phe124Leu	Leigh syndrome	Taylor *et al.* 2002
13042G>A	*ND5*	Ala236Thr	MELAS and MERRF	Naini *et al.* 2005
13084A>T	*ND5*	Ser250Cys	Leigh/MELAS overlap syndrome	Crimi *et al.* 2003
13513G>A	*ND5*	Asp393Asn	Leigh-like syndrome, MELAS and LHON	Santorelli *et al.* 1997
13514A>G	*ND5*	Asp393Gly	MELAS-like	Corona *et al.* 2001
14279G>A	*ND6*	Ser132Leu	LHON	Zhadanov *et al.* 2005
14453G>A	*ND6*	Ala74Val	MELAS	Ravn *et al.* 2001
14459G>A	*ND6*	Ala72Val	LHON and dystonia, Leigh syndrome	Jun *et al.* 1996
14482C>G	*ND6*	Met64Ile	LHON	Howell *et al.* 1998
14482C>A	*ND6*	Met64Ile	LHON	Valentino *et al.* 2002
14484T>C	*ND6*	Met64Val	LHON	Johns *et al.* 1992
14487T>C	*ND6*	Met63Val	Leigh syndrome	Ugalde *et al.* 2003
14495A>G	*ND6*	Leu60Ser	LHON	Chinnery *et al.* 2001
14498C>T	*ND6*	Tyr59Cys	LHON	Wissinger *et al.* 1997
14568C>T	*ND6*	Gly36Ser	LHON	Wissinger *et al.* 1997
14596T>A	*ND6*	Ile26Met	LHON	de Vries *et al.* 1996
14739G>A	*tRNA(Glu)*	n.a.	exercise intolerance	Mayr *et al.* 2006

Abbreviations: n.a. = not applicable, inv = inversion, fs = frame shift

2.5.3.3 Pathogenic mechanisms

Since the 14 highly conserved subunits of complex I are believed to form the functional core of the enzyme, it is not suprising that the majority of the pathogenic mutations related to complex I deficiency are located in them. In general, the pathogenic role of the mutation depends on the nature and location of the base pair exchange that has occurred. As already indicated, the seven mtDNA-encoded ND subunits are all highly hydrophobic membrane proteins, containing a total of as many as 52-59 transmembrane helices (Brandt 2006). The transmembrane regions of the proteins are formed of alpha helices containing hydrophobic amino acids. Helices can be bundled together to form tertiary structures in which only the side chains of the amino acids on the outside of the bundle need to be hydrophobic. The transmembrane helices are connected together by loops and turns formed of residues with polar side chains which are exposed to an aqueous environment on either side of the membrane. (Mathiensen & Hägerhäll 2002, Roth & Hägerhäll 2001, Bernardo *et al.* 2000, Kao *et al.* 2002, Kao *et al.* 2003.) Thus mutations causing changes in the charge or conformation of the amino acids can disturb the secondary structure of the subunit on the membrane arm of complex I, leading to problems affecting structural stability and assembly (Ugalde *et al.* 2003) or the functioning of the holoenzyme (McFarland *et al.* 2004).

In contrast, no transmembrane segments are predicted for the seven remaining core subunits of complex I (Brandt 2006). However, these nuclear-encoded subunits contain the binding motifs for all the redox prosthetic groups and the substrate NADH (Sazanov & Hinchliffe 2006). Amino acid substitutions near or at these binding sites could have a crucial effect on the catalytic activity of complex I. In addition, several nuclear mutations in complex I subunits have been shown to cause altered assembly or stability of the holoenzyme (Ugalde *et al.* 2004a). In the case of non-sense mutations, mRNA translation results in a truncated peptide, the size of which depends on the location of the mutation. A non-sense mutation occurring in the leading peptide of AQDQ resulted in loss of the entire protein subunit, preventing the assembly of complex I (Scacco *et al.* 2003). In addition, mutations in complex I genes can affect the stability of other respiratory chain complexes through the formation of supercomplexes. Decreased levels of complexes I and III were detected in patient cell lines with mutations in the complex I nuclear genes *NDUFS2* and *NDUFS4* (Ugalde *et al.* 2004a) and the *cytochrome b* gene of complex III (Acin-Perez *et al.* 2004).

3 Aims of the present research

Complex I deficiency, the most common cause of OXPHOS disorders, is characterized by a rapidly progressive nature and often fatal course, for which there is no effective treatment. Mutations in nuclear or mtDNA-encoded structural subunits of complex I are currently detected only in about 40% of children with complex I deficiency. Identification of these causative mutations can help us to understand the molecular background of complex I deficiency and to enhance our understanding of the clinical signs and symptoms displayed by patients. Furthermore, molecular diagnosis of nuclear mutations may open the way to prenatal diagnosis. Any efforts to define the origins of these disorders will be of value to families with affected children.

The specific aims of this research were:

1. to identify the underlying genetic defect causing isolated or combined complex I or other OXPHOS enzyme deficiency in 50 children (I-V)
2. to further elucidate the structural and functional consequences of mutations identified in the mtDNA or nuclear DNA genes encoding complex I (I,IV,V)
3. to obtain more information on the structurally and functionally important sites of complex I subunits (I,IV,V)

4 Patients and methods

More detailed descriptions of the patients and methods are presented in the original papers I-V.

4.1 Patients (I-V)

The subjects were 50 children ranging from newborn babies to 18-year-old adolescent from three countries (Table 4). Their clinical features were highly heterogeneous but all of them pointed strongly to a mitochondrial disorder. In most cases a deficiency in an OXPHOS enzyme had been detected; otherwise the selection criteria were based on clinical features suggestive of a mitochondrial disorder.

The nine patients considered in paper I had been described previously (Uusimaa *et al.* 2000) and screening of their mtDNA had not revealed any pathogenic mutations (Uusimaa *et al.* 2004). In paper III three patients with a definitely pathogenic mtDNA mutation were included in the group in addition to those with an unknown genetic defect in order to test the power of conformation-sensitive gel electrophoresis (CSGE) as a mutation screening method.

All the patient samples were examined after obtaining informed consent from the parents of the children.

Table 4. Patients studied in papers I-V.

Study	Nationality	Patients (n)	Inclusion criteria
I	Finnish	13	encephalomyopathy, isolated or combined complex I deficiency, no mtDNA mutations detected
II	Swedish	11	muscle weakness, exercise intolerance, delayed psychomotor development, failure to thrive
III	Dutch	25	isolated (n = 6) or combined (15) complex I deficiency or complex III deficiency (3), decreased pyruvate oxidation and ATP production (1), no mutations in nuclear complex I genes identified
IV	Dutch	1	isolated complex I deficiency and a putatively pathogenic mtDNA mutation identified in paper III
V	Finnish	1	skeletal muscle weakness and short stature

4.2 Clinical investigations (I, II, V)

The patients were evaluated in a standardized manner, including computed tomography (CT) or magnetic resonance imaging (MRI) of the brain, electroencephalography, electroneuromyography, determination of plasma lactate and cerebrospinal fluid lactate (only in paper I) and urinary levels of organic acids. Audiograms and ophthalmological examinations adapted to the age and co-operability of the children were performed. In addition, karyotype analysis, mutation analysis for fragile-X, liver tests, levels of amino acids in urine and plasma and urinary excretion of oligosaccharides were determined for paper I. Patients were eligible for the series in paper I if no definite diagnosis could be established on the basis of these examinations. A muscle or skin biopsy was taken for further histological, biochemical and molecular analyses.

4.3 Morphological analysis of skeletal muscle (II)

Electron microscopy and enzyme histochemical stainings of skeletal muscle samples were performed as described previously (Larsson & Oldfors 2001).

4.4 Tissue culture (I, IV)

For paper I, fibroblast cell lines from the patient and five controls were cultured in 1 x DMEM culture medium (Biochrom AG, Berlin, Germany) containing 5 mg/ml ascorbate, 100 units/ml penicillin, 100 μg/ml streptomycin, 10% (v/v) fetal bovine serum and 0.25 μg/ml amphotericin-B. For paper IV, fibroblasts from a skin biopsy were cultured in M199 medium (Life Technologies, Grand Island, NY, U.S.A.) supplemented with 10% fetal calf serum and antibiotics. The 143B206 TK- rho zero cells were cultured in DMEM (Life Technologies) supplemented with 5% fetal calf serum, antibiotics, 1mM uridine and 100 μg/ml bromodeoxyuridine.

To discriminate between diseases of nuclear and mitochondrial origin in paper IV, transmitochondrial cybrids were constructed by transferring mitochondria from the patient's fibroblasts into 143B206 TK^- rho zero cells (King & Attardi 1996). The cybrids were maintained in a high-glucose DMEM medium (Invitrogen, Gibco, Carlsbad, CA, U.S.A.) supplemented with glutamine, 1 mM sodium pyruvate, 10% fetal calf serum and antibiotics.

4.5 Isolation of mitochondria (I, II, V)

Fractions of disrupted mitochondria were isolated from human tissue homogenates for enzyme activity measurements. Mitochondria were isolated from a 20-100 mg muscle specimen (Wibom *et al.* 2002 in paper II), (Rasmussen *et al.* 1997 in papers I and V) or from cultured fibroblasts (Birch-Machin & Turnbull 2001 in paper I). For papers I and IV,

the inner mitochondrial membrane fraction from cultured fibroblasts and cybrids was isolated for protein separation by Blue native PAGE (Nijtmans *et al.* 2002). The harvested cells were treated with digitonin, which selectively dissolves membranes containing cholesterol, saving the inner mitochondrial membrane. The undissolved inner mitochondrial membrane fraction was then collected by centrifugation and the membrane proteins were solubilized with dodecyl-β-D-maltoside.

4.6 Enzyme activities

4.6.1 Spectrophotometric measurements of OXPHOS enzyme activities (I, II, IV, V)

The spectrophotometric measurements of OXPHOS enzyme activities were carried out in three laboratories (Nijmegen Centre for Mitochondrial Disorders, The Netherlands, Karolinska Institutet, Sweden and University of Oulu, Finland) using nonstandardized protocols. Comparison of the enzyme activities measured in the three laboratories was not performed, since differences in the experimental procedures have been shown to cause high variation of results (Gellerich *et al.* 2004).

For paper I, the enzyme activity of the fibroblast mitochondria was assayed for complex IV and for complex I, I+III and II+III with minor modifications (Rustin *et al.* 1994). Complex I activity was assayed by measuring rotenone-sensitive NADH oxidation in the presence of decylubiquinone. The reaction velocity of cytochrome *c* reduction by NADH was measured in order to determine the activity of complexes I+III. The part of the reaction velocity caused by the activity of complex I was distinguished from the reduction by complex III by adding rotenone to the reaction mixture. The activity of complexes II+III was obtained by measuring the succinate-dependent reduction of cytochrome *c*, and the activity of complex III was measured in terms of ubiquinol-dependent cytochrome *c* reduction. The activity of complex IV was assayed as the oxidation rate of reduced cytochrome *c* (Chrzanowsa-Lightowlers *et al.* 1993). The measurements were performed using the Aminco DW-2 UV-VIS spectrophotometer (American Instrument Co, Silver Spring, MD, U.S.A.) at 37°C. In paper II, the activities of the respiratory chain enzymes glutamate dehydrogenase and citrate synthase were measured by the methods of Wibom & Hultman (1990) and von Döbeln *et al.* (1993) for the patients examined during the period 1995-2000, while the enzyme activites for the patients examined in 2001-2004 were assayed by improved methods (Wibom *et al.* 2002). For paper IV, mitochondrial respiratory chain enzyme activities were measured in muscle samples (quadriceps muscle biopsies), skin fibroblasts and cybrids (Loeffen *et al.* 2000), while for paper V muscle respiratory chain complex activities were assayed for complex I (Vuokila & Hassinen 1988), complexes II+III and I+III (Sottocasa *et al.* 1967) and complex IV (Cooperstein & Lazarow 1951).

4.6.2 Mitochondrial ATP production rate (II)

For paper II, the mitochondrial adenosine triphosphate production rate (MAPR) was measured by the methods of Wibom & Hultman (1990) and von Döbeln *et al.* (1993) in the case of the patients examined in 1995-2000 and by improved methods (Wibom *et al.* 2002) for patients examined later.

4.6.3 Complex I in-gel activity (I)

The in-gel activity assay for complex I was performed according to Nijtmans *et al.* (2002). Mitochondrial inner membrane fractions isolated from cultured fibroblasts were electrophoresed through a 5-15% Blue-native polyacrylamide gradient gel (BN-PAGE) and the activity of complex I in the gel was defined according to the intensity of the colour reaction resulting from the reduction of nitrotetrazolium blue (NTB) by the dehydrogenase portion of the enzyme. Deamino-NADH was used as an electron donor in the reaction.

4.7 Expression and assembly of complex I

4.7.1 Blue native PAGE and second-dimension SDS-PAGE (I, IV)

The assembly and level of expression of complex I in the patient fibroblasts and cybrids was analysed using BN-PAGE and second-dimension (2D) SDS-PAGE. Detergent-solubilized inner mitochondrial membrane enzyme complexes were separated out on a 5-15% acrylamide Blue native gradient gel. The lanes were cut off and the enzyme complexes were separated into subunits by 2D SDS-PAGE (Nijtmans *et al.* 2002, Ugalde *et al.* 2004a).

4.7.2 Western blotting (I, IV)

Proteins from the mitochondrial inner membrane fraction were transferred from the BN-PAGE and 2D SDS-PAGE gels to nitrocellulose filters (BioRad, Hercules, CA, U.S.A.) (Nijtmans *et al.* 2002). Antibodies raised against the complex I subunits 30 kDa, 39 kDa and ND6 (a gift from Prof. Capaldi), the complex III core 2 subunit UQCRC2 (Molecular Probes, Leiden, The Netherlands) and the complex IV subunit COXII (Molecular Probes) were used to identify the respective respiratory chain complexes and their subcomplexes. A secondary antibody, HRP-conjugated goat anti-mouse IgG (Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.), was allowed to react with the primary antibodies, and the reaction was detected by the ECL Western blotting method (Amersham Biosciences, Buckinghamshire, England) on Scientific Imaging Film (Kodak, Rochester, NY, U.S.A.).

4.7.3 Transcription of the TYKY subunit (I)

The *NDUFS8* mRNA was analysed in order to confirm the transcription of both strands and to ensure correct splicing of the introns. Total mRNA was first isolated from the patient fibroblasts using an Oligotex Direct mRNA isolation kit (Qiagen, Valencia, CA, U.S.A.) and transcribed to cDNA using an Omniscript kit (Qiagen). The transcription of cDNA was allowed to occur at 37˚C for 1 h, followed by inactivation of the transcriptase at 93˚C for 5 min. The coding sequence of *NDUFS8* was amplified from the cDNA by hot start PCR using the primers F1 and R3 described by Loeffen *et al.* (1998a). The PCR products were separated on a gel electrophoresis, from which the fragment representing the TYKY coding region was isolated using a Prep-A-Gene DNA purification kit (BioRad). Finally, the primers F1, F2, F3, R1, R2 and R3 were used for cycle sequencing of the *NDUFS8* gene transcript (Loeffen *et al.*1998a).

4.8 Detection of mtDNA and nuclear mutations

4.8.1 Extraction of DNA (I, V)

Total genomic DNA was extracted from the patients' muscle and blood samples using the standard sodium dodecyl sulphate-proteinase K method or a QIAamp DNA Blood Mini Kit (Qiagen).

4.8.2 Amplification of DNA (I-V)

MtDNA was amplified in 63 overlapping fragments, covering nucleotide positions 523 to 16090 of the revised Cambridge reference sequence (rCRS) (Finnilä *et al.* 2000). The template DNA was amplified by PCR in 30 cycles of denaturation at 94°C for 1 minute, annealing at the primer-specific temperature for 1 minute and extension at 72°C for 1 minute, with a final extension step at 72°C for 10 minutes after the last cycle. Amplified PCR fragments of mtDNA were used in cycle sequencing, restriction fragment analysis (RFLP) and conformation-sensitive gel electrophoresis (CSGE).

The hot start or touchdown PCR method was used to amplify the coding regions of nine nuclear-encoded complex I genes *NDUFAB1, NDUFS1, NDUFS2, NDUFS3, NDUFS4, NDUFS7, NDUFS8, NDUFV1* and *NDUFV2*. Intronic primers were designed to flank the coding sequences and exon-intron junctions of the genes. The hot start PCR protocol included an initial denaturation step lasting from 1 min 43 s to 15 min depending on the polymerase used, before proceeding to 30 cycles of amplification. In the touchdown programme the annealing temperature was lowered by 0.5°C per cycle between 66°C and 58°C or 60°C and 52°C and then maintained at 58°C or 52°C, respectively, for the final 20 cycles. The amplified exons of the nuclear complex I genes were used for cycle sequencing and RFLP.

4.8.3 Conformation-sensitive gel electrophoresis (II-V)

Substitutions in the mtDNA coding region were analysed by CSGE. The protocol consisted of analysis of the mtDNA sequence from bp 523 to 16090 according to the rCRS (Andrews *et al.* 1999). MtDNA was amplified in 63 overlapping fragments, which were mixed with a control sample of known sequence. The heteroduplexes were electrophoresed through a 15% polyacrylamide gel and samples with distinct mobility were further analysed by sequencing. (Finnilä *et al.* 2000.) The sensitivity of the CSGE method was tested in paper III by analysing three patients with known mtDNA mutations in a blinded fashion. In addition, five randomly selected DNA samples from the cohort were subjected to direct sequencing and the chromatograms were analysed again in a blinded fashion. The mtDNA sequences were compared with the corresponding sequences obtained with the CSGE protocol in order to estimate the substitution detection rate between the two methods.

4.8.4 Cycle sequencing (I-V)

PCR-amplified fragments were cycle sequenced for identification and confirmation of the base pair substitutions. The PCR products were first purified with 0.5 U shrimp alkaline phosphatase (Amersham Pharmacia Biotech, Freiburg, Germany) and 6 U exonuclease I (New England Biolabs, Beverly, MA, U.S.A.) or with the QIAquick PCR Purification Kit (Qiagen). The fragments were then cycle sequenced using the DYEnamic ET Terminator Cycle Sequencing Kit (Amersham Pharmacia Biotech) with ThermoSequenase II DNA polymerase. The primers used for the sequencing were the same as for amplification. The labelled amplicons were then precipitated with salt/ethanol and analysed with an ABI 377 (Applied Biosystems, Foster City, CA, U.S.A.) sequencing instrument. The resulting sequences were aligned with the rCRS or complex I nuclear gene reference sequences by Sequencher 4.1.4 (Gene Codes Corporation, Ann Arbor, MI).

4.8.5 Assessment of the heteroplasmy levels for mutations in mtDNA (II-V)

The heteroplasmy of the novel and rare mtDNA variants was studied by RFLP of fragments that had been amplified in the presence of 6.3-12.5 μCi of ^{35}S-dATP (Perkin Elmer, Boston, MA, U.S.A.). Mismatch primers were designed for PCR when a specific restriction site was missing. Labelled PCR products were digested with compatible restriction enzymes and the digestion products were separated on a 6% or 10% DNA-PAGE. The amounts of digestion products representing the wild-type and mutant mtDNA were measured and the proportion of the mutation was calculated (Shoffner *et al.* 1990).

4.8.6 Verification of novel variants

All new nuclear and mitochondrial sequence variants were confirmed by restriction fragment analysis (RFLP) or allele-specific PCR, or by sequencing a second PCR amplificant in both directions. Whenever a new variant had been confirmed, RFLP or allele-specific PCR was used to determine its frequency in 96-200 population controls.

4.8.6.1 Restriction fragment analysis (I-V)

Amplified DNA was digested overnight in a 20 µl reaction volume with 5-20 U of restriction enzyme. The digestion products were electrophoresed on a 3% MetaPhor gel (BioWhittaker Molecular Applications, Rockland, ME, U.S.A.) in 1 x TBE buffer (Amresco Inc., Solon, OH, U.S.A.) and stained with ethidium bromide.

4.8.6.2 Allele-specific PCR (I, V)

Allele-specific PCR was used to verify the frequencies of the synonymous Ala430 substitution in the 49 kDa subunit (paper I) and Ile187Thr in the ND1 subunit (paper V). The primers containing a locked nucleic acid (LNA) base at the 3' end (Proligo LLC, Paris, France) were designed to anneal with either the wild-type sequence or the sequence containing the substitution (Braasch & Corey 2001). The presence or absence of a PCR product then determined the specific allele genotype of the subject.

4.8.6.3 Bioinformatics for estimating the pathogenicity of variants (I-V)

All the mtDNA or nuclear complex I gene sequence variants identified were compared with those reported in human mitochondrial genome databases [MITOMAP (www.mitomap.org); mtDB (www.genpat.uu.se/mtDB/); GiiB-JST mtSNP (www.giib.or.jp/mtsnp/index_e.html)] and previous publications (Finnilä *et al.* 2001, Herrnstadt *et al.* 2002, Kivisild *et al.* 2006), or in the human nuclear genome SNP database (www.ncbi.nlm.nih.gov).

The pathogenic potential of the novel non-synonymous substitutions was estimated using information on the nature of the ensuing amino acid exchange and the conservation of the position amongst species. Changes in structure and intracellular localization caused by the amino acid exchange were also taken into account. The predictions were calculated using information deposited in an EMBL database (www.embl-heidelberg.de/predictprotein/predictprotein.html), the GiiB-JST mtSNP database (www.giib.or.jp/mtsnp/index_e.html), WinPep 3.01 (www.ipw.agrl.ethz.ch/~lhennig/winpep.html) (Henning 1999), MitoProt II (ihg.gsf.de/ihg/mitoprot.html) (Claros & Vincens 1996) and PSORT II (psort.nibb.ac.jp). Conservation indices were calculated to denote the conservation of a specific amino acid (Ruiz-Pesini *et al.* 2004).

4.9 Mutagenesis of NDH-1 in *Escherichia coli* (V)

4.9.1 Deletion of NuoH gene

The *NuoH* gene (equivalent to human *MTND1*) was removed from the Nuo-operon of the *E.coli* strain GV102 (Oden *et al.* 1990) using the pKO3 plasmid (Kervinen *et al.* 2004, Kervinen *et al.* 2006) and replaced with a streptomycin resistance gene. The replacement was confirmed by sequencing. The knock-out strain without the chromosomal *nuoH* gene was referred to as HK18.

4.9.2 Mutagenesis and expression of the NuoH gene

Reference and mutant NuoH expression plasmids were prepared for *in trans* complementation. The amplified *NuoH* gene was subcloned into the pETBlue-2 plasmid and point mutations causing the amino acid substitutions Ile201Thr and Ile201Val (equivalent to human Ile187Thr and Ile187Val) were introduced into the *nuoH* gene using the QuikChange XL mutagenesis kit (Stratagene, La Jolla, CA, U.S.A.). The reference *NuoH* or the gene harbouring the mutation was introduced into the *nuoH* knock-out strain HK18 in an expression plasmid (Kervinen *et al.* 2006).

4.9.3 Preparation of bacterial membrane samples

Bacteria for the membrane preparations were grown in Luria-Bertani (LB) medium with appropriate antibiotics and collected when the absorbance reached 0.4-0.7 at 600 nm. The cells were washed and disrupted using a French Press. Finally, the membranes for the enzyme activity measurements were collected by ultracentrifugation at 120 000 x g for 60 min (Kervinen *et al.* 2006).

4.9.4 Activity of NDH-1 in Escherichia coli

The activities of decylubiquinone (DB) reductase, hexammine ruthenium (HAR) reductase and deamino-NADH (d-NADH) oxidase, with and without *N*-vanillylnonanamide (VNA) inhibition, were measured using an Aminco-DW2 or Shimadzu UV-3000 (Shimadzu Corporation, Kyoto, Japan) dual-wavelength spectrophotometer (Kervinen *et al.* 2004, Kervinen *et al.* 2006).

4.10 Statistical analyses (II, III, V)

The MAPR and respiratory chain enzyme activities were compared between the groups of patients analysed in paper II by adjusting the mean values for the assays performed in the control group to 100%. Then the coefficient of variation for each assay was calculated and a mean coefficient of variation was obtained for all the control subjects. Eleven degrees of freedom were used for the calculations of statistical significance (Student's t test) with both the control subjects and patients.

In paper III an evolutionary network was constructed based on the mtDNA variations in the 28 children. A binary data file was created and the calculations for the network were carried out using the reduced median algorithm implemented in the Network 4.106 software (www.fluxus-engineering.com). The numbers of novel non-synonymous sequence variants among the patients were compared with the results of a permutation analysis. Twenty-five sequences of patients were sampled 1000 times with replacements from 617 previously published sequences belonging to European haplogroups (Finnilä *et al.* 2001, Herrnstadt *et al.* 2002). The number of non-synonymous variants present in the sampled sequences and absent from the remaining sequences was counted for each permutation and a 95% confidence interval was derived from these values.

In paper V, the measurements of DB reductase activity were fitted into the Michaelis-Menten one-site saturation kinetics using the SigmaPlot 9 program (Systat Software, Erkrath, Germany). The assays were performed with at least three separate membrane preparations, and a mean for these samples was calculated. The statistical significances of differences in enzyme activity were evaluated using Student's t-test.

5 Results

5.1 Common features of complex I deficiency

5.1.1 Clinical phenotypes (I, II, IV, V)

The patients with complex I deficiency were a clinically heterogeneous group that shared some common features such as early onset of disease, failure to thrive, muscle weakness, muscle hypotonia/hypertonia, exercise intolerance, delayed psychomotor development, ataxia, seizures, optic atrophy, nystagmus, hearing loss, cardiac involvement, respiratory distress, gastrointestinal dysmotility, recurrent infections and a progressive clinical course. The eleven patients with complex I deficiency considered in paper II were divided into four subgroups on the basis of the clinical features: patients with 1) Leigh and Leigh-like syndrome, 2) neonatal lactic acidosis with encephalomyopathy and hypertrophic cardiomyopathy, 3) encephalomyopathy with hearing impairment, optic nerve atrophy and cardiac involvement, and 4) hearing impairment, cataract, muscle weakness and hypertrichosis. These subgroups were also representative of the clinical spectrum of patients in the other series reported in papers I, IV and V.

The most common electroencephalographic abnormalities reported in papers I and II were slowing of the background activity, focal irritation and generalized spike-and-wave discharges. Electroneuromyography showed myopathy, neurogenic degeneration, or lower motor neuron disease. Brain CT or MRI revealed cortical atrophy, intracranial calcifications, basal ganglia lesions or generalized demyelination. Blood lactate was intermittently or constantly elevated in 30% of cases in paper I and 55% of cases in paper II. Electron microscopy of the skeletal muscle revealed increased amounts of mitochondria and variation in their size and shape. The most common abnormal findings in light microscopy were type 2 fiber atrophy, fat accumulation and degenerative and myopathic changes. RRFs were found in 4 patients out of 24 in papers I, II, IV and V.

5.1.2 Biochemistry (II)

The activities of NADH dehydrogenase (complex I) and NADH cytochrome *c* reductase (complex I-III) were found decreased in all eleven patients in paper II, whereas the activities of succinate cytochrome *c* reductase (complex II-III) and cytochrome *c* oxidase (complex IV) were normal. Glutamate dehydrogenase and citrate synthase activities were slightly elevated. The MAPR in isolated mitochondria was reduced, along with the complex I substrates glutamate and malate, whereas the other substrates gave normal ATP production rates. The rate of succinate oxidation was increased both the rotenone-treated normal mitochondria and the untreated mitochondria of 9 out of the 11 complex I-deficient patients. Western blot analyses comparing the proportion of succinate dehydrogenase with the mitochondrial voltage-dependent anion channel (VDAC) in seven muscle tissue samples showed that there was no increased expression of complex II in the patients. Analysis of organic acids in the urine revealed increased secretion of lactate, malate and fumarate in four patients.

5.2 Analysis of nine nuclear-encoded subunits of complex I (I, V)

Sequencing of the nine nuclear genes encoding conserved and functionally important subunits of complex I, *NDUFAB1, NDUFS1-4, NDUFS7-8* and *NDUFV1-2*, in 14 children revealed twelve substitutions (Table 5). Eleven were considered common polymorphisms, either on the grounds of their frequency, which was similar among the patients and 100 controls, or because they had already been reported in a database as single nucleotide polymorphisms (SNPs). Seven of them were synonymous, including a novel 201A>T substitution in *NDUFV2*. A novel non-synonymous substitution was observed in the *NDUFS8* gene, leading to an Arg18Cys substitution in the TYKY subunit. Interestingly, the allele was absent in 202 healthy controls and in the remaining 107 children with unexplained encephalomyopathy.

Table 5. Sequence variation in nine nuclear complex I genes in 14 Finnish patients.

Gene	Variant	Amino acid	refSNP ID
NDUFS1	966G>T	Ala322	rs1127566
	1251A>G	Arg417	rs1801318
NDUFS2	58C>A	Pro20Thr	rs11538340
	1054C>G	Pro352Ala	rs11576415
	1290C>T	Ala430	rs1136207
NDUFS4	12G>C	Val4	rs2279516
	198C>A	Gly66	rs31304
	312G>A	Arg104	rs31303
NDUFS7	68C>T	Pro23Leu	rs1142530
NDUFS8	**52C>T**	**Arg18Cys**	**novel**
NDUFV2	86C>T	Ala29Val	rs906807
	201A>T	Val67	novel

The substitution described further in section 5.2.1 is marked in bold.

5.2.1 52C>T in NDUFS8 (I)

A novel non-synonymous substitution 52C>T was observed in the *NDUFS8* gene of a patient with encephalomyopathy, short stature and nystagmus. The substitution was found in a heterozygous form in the patient and her mother. An analysis of mRNA encoded by the *NDUFS8* gene in patient fibroblasts confirmed that both alleles were expressed. Decreased activity of complex I in the muscle mitochondria of the patient was noted, whereas the activities of the other OXPHOS enzymes were within normal limits. All the OXPHOS enzyme activities and their ratios in fibroblasts, including complex I activity, were within the control range.

The 52C>T substitution leads to an amino acid substitution Arg18Cys in the 34-amino-acid N-terminal leading peptide of the TYKY subunit (Procaccio *et al.* 1997). The amino acid substitution leads to the replacement of a basic arginine18 by a non-polar sulphur-containing cysteine. Structure predictions suggest that this replacement could have an effect on the secondary structure of the TYKY subunit by disrupting the alpha helix of the leading peptide (the Predict protein, WinPEP 3.01). The hydrophobicity properties (MuHd and H_{max}) of the mutant TYKY leading peptide were decreased relative to the wild-type sequence and to a certain mitochondrial-targeting sequence (MitoProt II). In addition, the mutation altered the mitochondrial localization probability values from 52.2% to 47.8% (PSORT II).

The assembly of complex I in patient and control fibroblasts was studied using BN-PAGE and 2D SDS-PAGE. A fully assembled complex I with subcomplexes was seen clearly with an antibody against the 39 kDa subunit and the assembly pattern was similar in the patient and control fibroblasts (Fig. 3 in paper I). In addition, the in-gel activity of complex I in the patient fibroblasts was found to be visually indistinguishable from that in the control fibroblasts, suggesting that complex I NADH-dehydrogenase activity was not decreased in the patient fibroblasts (Fig. 2 in paper I).

5.3 Analysis of mtDNA (II-V)

The mtDNA coding region in 38 patients was resolved by CSGE and subsequent sequencing. Comparison of the sequences with the rCRS revealed a total of 107 synonymous substitutions, 57 non-synonymous substitutions, 36 rRNA or tRNA substitutions, two length variants in homopolymeric cytosine tracks spanning between nucleotides 568-573 and 5895-5899, respectively, and one single-nucleotide deletion. The variants are listed in Table 3 of paper II and Table 2 of paper V, whereas in paper III the 28 sequences were imported into an evolutionary network constructed using mtDNA sequence information on 617 Europeans (Fig. 1 in paper III). All the patients were assigned to one of the European mtDNA haplogroups or subhaplogroups on the basis of the identification of established haplogroup-determining polymorphisms (Torroni *et al.* 1996). The phylogenetic network based on mtDNA coding region variation enabled easy identification of the haplogroup-specific polymorphisms and novel private substitutions.

The majority of the mtDNA variants, including all the synonymous substitutions and most of the non-synonymous ones, were considered polymorphisms. In addition, four

previously reported pathogenic mutations were identified: 3460G>A in *MTND1* (Huoponen *et al.* 1991), 10191T>C in *MTND3* (Taylor *et al.* 2001), 11778G>A in *MTND4* (Wallace *et al.* 1988a) and 14487T>C in *MTND6* (Ugalde *et al.* 2003). Furthermore, four novel non-synonymous substitutions 3866T>C in *MTND1*, 4681T>C in *MTND2*, 9891T>C in *MTCO3* and 14122A>G in *MTND5*, one novel substitution in an rRNA gene, 686A>G in *MTRNR1*, and nine novel synonymous substitutions were discovered (Table 6). Three variants were found that have previously been reported only in a single sequence: 6681T>C in *MTCO1*, 13630A>G in *MTND5*, and 12188T>C in *MTTH* (Herrnstadt *et al.* 2002, Kivisild *et al.* 2006).

Table 6. Novel and previously known pathogenic mtDNA variants.

Synonymous substitutions		Non-synonymous substitutions	Substitutions in rRNA or tRNA genes
Novel variants			
4233T>C	11629A>G	**3866T>C**[#]	686A>G
6665C>T	12924A>G	**4681T>C**	
7954T>C	13608T>C	9891T>C	
8065G>A	13671A>G	14122A>G	
9657C>T			
Pathogenic variants			
n.a.		3460G>A	3243A>G[*]
		8993T>G[*]	7497G>A[*]
		10191T>C	
		11778G>A	
		14487T>C	

n.a. not applicable. The substitutions described further in section 5.3.1 are in bold.

[*]Three children with definite pathogenic mutations were included in the sample set in order to examine the reliability of the CSGE protocol.

[#]Novel at the time of identification.

Before any further molecular analyses were carried out, the pathogenic potential of the novel and rare sequence variants was estimated. The pathogenic role of the two substitutions 14122A>G and 13630A>G in *MTND5* was excluded because of the low conservation of these amino acid positions and the nature of the amino acids exchanged. The 13630A>G substitution had previously been found in the same mtDNA subhaplogroup, indicating that it is a rare haplogroup-specific polymorphism (Herrnstadt *et al.* 2002). Similarly, the 6681T>C substitution in *MTCO1* was considered a rare polymorphism. The rest of the novel and rare substitutions, 686A>G in *MTRNR1*, 9891T>C in *MTCO3* and 12188T>C in *MTTH*, were considered polymorphisms, since the molecular defects in the case of pathogenicity did not correlate with the deficiency seen in the patients. The pathogenic potential of 3866T>C in *MTND1* and 4681T>C in *MTND2* could not be excluded and additional investigations were performed to further establish their role (Table 7).

Table 7. Molecular characteristics of mtDNA mutations identified in children with complex I deficiency.

Paper	Mutation	Subunit	Amino acid change	Heteroplasmy in muscle	Haplogroup	Phenotype
III	3460G>A	ND1	Ala52Thr	100%	J1	myopathy with muscle weakness and exercise intolerance
V	3866T>C	ND1	Ile187Thr	75%	J1c	myopathy, short stature
III,IV	4681T>C	ND2	Leu71Pro	>95%	T2	Leigh syndrome
II	10191T>C	ND3	Ser45Pro	97%	H2	Leigh-like syndrome
II	11778G>A	ND4	Arg340His	100%	J1	encephalomyopathy with hearing impairment, optic nerve atrophy and cardiac involvement
II	14487T>C	ND6	Met64Val	95%	J1	Leigh syndrome

5.3.1 Novel mtDNA mutations

5.3.1.1 3866T>C in MTND1 and mutagenesis of a homologous position in Escherichia coli (V)

An isolated complex I deficiency was detected in the muscle of a Finnish patient with skeletal muscle weakness and short stature. Sequencing of the nine nuclear genes of complex I revealed eight substitutions which had already been reported in the SNP database (Table 2 in paper V). Analysis of the complete mtDNA genome identified 29 base pair substitutions, assigning the patient's mtDNA to subhaplogroup J1c. A novel non-synonymous substitution 3866T>C was discovered, leading to an Ile187Thr amino acid change in ND1. The substitution was heteroplasmic, the proportion of 3866C being 77% in the patient's blood and 75% in the muscle and 34% in his mother's blood. Furthermore, the substitution was absent in 96 healthy Finnish controls.

In order to study the functional and structural consequences of the substitution further, an *E. coli* strain was created carrying the Ile201Thr mutation in the NuoH subunit (homologue to the human Ile187Thr mutation in ND1). d-NADH oxidase and DB reductase activities were slightly lower in the Ile201Thr-mutant strain than in the reference strain (Table 3 in paper V). Interestingly, the activity of HAR reductase, used to quantify NDH-1, was lower in the Ile201Thr strain than that in the reference strain. The sensitivity of the mutant NDH-1 to inhibition by VNA was similar to that in the reference strain. In addition, another mutation Ile201Val was introduced into the *E. coli* NuoH subunit (homologous to Ile187Val in human ND1). This is caused by a 3865A>G mutation in *MTND1* and has previously been detected in a patient with Alzheimer's disease (Tanaka *et al.* 2004). The d-NADH oxidase, DB reductase and HAR reductase

activities of the Ile201Val mutant were similar to those of the Ile201Thr mutant (Table 3 in paper V).

5.3.1.2 4681T>C in MTND2 (III, IV)

A patient with Leigh syndrome showed an isolated complex I deficiency in both fibroblasts and skeletal muscle. The patient suffered from progressive encephalomyopathy and died of respiratory failure at the age of 10 years. To discriminate between a nuclear and mitochondrial origin for the disease, transmitochondrial cybrids were constructed. A significant reduction in complex I activity was measured in the mutant clones, confirming that the genetic defect was located in the mitochondrial genome.

CSGE analysis of the entire mtDNA coding region revealed 24 polymorphisms that assigned the mitochondrial sequence to subhaplogroup T2. Altogether ten substitutions were identified in complex I genes, including the missense mutation 4681T>C in *MTND2*, which was not present in the mitochondrial databases analysed. This mutation, which leads to a Leu71Pro amino acid exchange in the ND2 subunit of complex I, was absent from the father's and mother's blood, whereas it was almost homoplasmic (>95% mutant load) in the blood, fibroblasts and muscle of the patient (Fig. 1B in paper IV). When BN-PAGE and 2D SDS-PAGE were carried out on fibroblasts and cybrid clones from the patient to estimate the assembly and amount of complex I, an impairment of complex I assembly was detected on the strength of two observations. The amount of fully assembled complex I was lower in patient's fibroblasts than in those from the controls (Fig. 2A in paper IV), and low-molecular weight subcomplexes appeared to accumulate in the mutant cells (Fig. 2B in paper IV). Steady-state levels of complex III were not affected by the mutation.

5.3.2 Quality control of the mtDNA sequences (III)

Two approaches were employed in order to evaluate the quality of the sequences. First, three samples harbouring a known mtDNA mutation were included in the material in a blind fashion. Each mutation was correctly identified by the CSGE protocol. Second, the CSGE protocol was compared with direct sequencing in terms of the substitution detection rate. Complete mtDNA was sequenced from five randomly selected patients. Direct sequencing revealed the synonymous substitution 5147G>A, which was not detected by CSGE, and the CSGE protocol revealed the novel heteroplasmic substitution 14122A>G, which was not observed in the sequence chromatograms. These results suggested a sensitivity of 98.8% and a specificity of 100% for both methods.

5.3.3 Statistical analysis of the variation in mtDNA (III)

A permutation analysis was carried out to compare the frequency of novel non-synonymous substitutions between the patients with OXPHOS deficiency and controls. The 25 Dutch patients harboured altogether seven non-synonymous substitutions (4681T>C, 9181A>G, 9891T>C, 14122A>G, 14325T>C, 15287T>C, 15851A>G) that were not found in the 617 control sequences belonging to European haplogroups. A permutation analysis among the 617 sequences indicated that the mean number of such substitutions in a random sample of 25 sequences was 6.5 (95% confidence interval 2 – 12).

6 Discussion

6.1 Mutations causing complex I deficiency

The development of new technologies has made the analysis of complete mtDNA sequences and nuclear genes a feasible diagnostic tool. 50 patients were examined here with either isolated or combined complex I deficiency or other OXPHOS enzyme defect. The coding region of mtDNA had either been analysed previously or was analysed in the present connection in all cases and seven were found to harbour previously known pathogenic mutations. In addition, eight novel or rare mtDNA substitutions were discovered, two of which were considered to have a high pathogenic potential. Sequencing of the nine nuclear-encoded complex I genes in 14 of the present patients and previous sequencing in 25 Dutch patients revealed only one novel, possibly pathogenic mutation. All in all, the frequency of possible mtDNA mutations in our cohort was 18%. If the novel Arg18Cys substitution in TYKY is a pathogenic mutation, the frequency of nuclear mutations in our cohort can be taken to be 3%. Similarly, the entire mtDNA and 11 nuclear-encoded complex I subunits have been analysed in 23 Italian infants or children with isolated complex I deficiency. The underlying genetic defect was resolved in eight cases, or 35% of the patients studied, a figure that is in good agreement with our current knowledge of the prevalence of detected mutations in complex I-deficient children. (Bugiani *et al.* 2004.) In another study, complete analysis of mtDNA and six nuclear DNA-encoded complex I subunit genes in 50 children with complex I deficiency led to determination of the disease-causing mutation in 20% of cases, all the mutations identified being of mitochondrial origin (Lebon *et al.* 2003).

The quality of mtDNA sequences has been a subject of debate during the last few years (Bandelt *et al.* 2001, Herrnstadt *et al.* 2003, Bandelt *et al.* 2005). In the course of the present research CSGE was used to screen for mtDNA sequence changes relative to a reference sample and subsequent sequencing was used in order to identify the nucleotide changes. The resulting mtDNA sequences were then compared with the published sequences of European origin (Finnilä *et al.* 2001, Herrnstadt *et al.* 2002), enabling an easy distinction to be made between haplogroup-specific polymorphisms and substitutions that needed further evaluation. The sensitivity of the CSGE protocol was tested and the results suggested a high sensitivity and specificity for the method.

6.1.1 Definitely pathogenic mutations

Two of the three primary LHON mutations, 3460G>A in *MTND1* and 11778G>A in *MTND4*, were identified among the present patients. The 3460G>A mutation was detected in a Dutch adolescent with isolated complex I deficiency and progressive myopathy as the sole manifestation of her mitochondrial disease. This mutation causes an amino acid exchange Ala52Thr (conservation index 90) in the ND1 subunit of complex I. The amino acid substitution occurs in the loop region between two predicted transmembrane alpha helices on the matrix side of the membrane. The mutation has been shown to lead to a marked decrease in the specific activity of complex I (Majander *et al.* 1991, Howell *et al.* 1991) while not affecting the general mitochondrial ATP synthesis, according to *in vivo* phosphorus magnetic resonance spectroscopy (MRS) (Lodi *et al.* 1997, Cock *et al.* 1999). The functional consequences of the 3460G>A mutation have been studied by mutagenesis in *P. denitrificans* (Zickermann *et al.* 1998), and analyses of bacterial strains carrying mutations homologous to it and adjacent residues have suggested a specific role for this region in ubiquinone reduction.

The other primary LHON mutation 11778G>A detected here was present in four siblings, a girl with complex I deficiency, encephalomyopathy and cardiac involvement and three boys suffering from similar symptoms with additional hearing impairment and optic atrophy. This mutation leads to an Arg340His amino acid substitution in the highly conserved transmembrane region of ND4, which is located close to the matrix side of the membrane and is characterized by positively charged amino acids (Fearnley & Walker 1992). It has been suggested that the domain may be involved in the binding of a ubisemiquinone intermediate, since a reduction in rotenone inhibition has been demonstrated in 11778G>A mitochondria (Degli Esposti *et al.* 1994, Ghelli *et al.* 1997). The biochemical data concerning the pathogenic mechanism of 11778G>A are nevertheless controversial. Reports of normal NADH:ubiquinone oxidoreductase activity in lymphocytes and cybrids (Majander *et al.* 1991, Guy *et al.* 2002) or a mild complex I defect in platelets (Smith *et al.* 1994) have been published. However, studies on intact mitochondria describe a reduced rate of oxygen consumption in the presence of complex I-linked substrates (Larsson *et al.* 1991, Majander *et al.* 1996) and abnormal mitochondrial ATP synthesis (Lodi *et al.* 1997, Guy *et al.* 2002), suggesting a more general role for the 11778G>A mutation in mitochondrial energy metabolism.

Increased expression of the LHON mutations 11778G>A and 14484T>C has been reported in the haplogroup J genome, whereas no such association has been detected between 3460G>A and haplogroup J (Carelli *et al.* 2006). Interestingly, most of the patients with pathogenic mtDNA mutations identified here belonged to haplogroup J1. This was true of both of the patients with the two primary LHON mutations, 3460G>A and 11778G>A, and the patients with two other mtDNA mutations, 3866T>C and 14487T>C, belonging to haplogroups J1 and J1c, respectively. These observations suggest that the combination of haplogroup J1-specific polymorphisms with pathogenic or perhaps only slightly deleterious mutations might be an additional risk factor for mitochondrial dysfunction. (Herrnstadt & Howell 2004.)

Two recently reported pathogenic mutations, 10191T>C in *MTND3* (Taylor *et al.* 2001, Lebon *et al.* 2003, McFarland *et al.* 2003) and 14487T>C in *MTND6* (Lebon *et al.*

2003, Solano *et al.* 2003, Ugalde *et al.* 2003), were identified in Swedish patients with Leigh-like syndrome or Leigh syndrome, respectively. The 10191T>C mutation leads to a Ser45Pro amino acid substitution within the hydrophilic loop between the first and second membrane-spanning alpha helices of ND3 (Fearnley & Walker 1992). The change from serine to proline presumably adjusts the conformation of the peptide bond, leading to altered folding of the ND3 subunit. Studies on mutant complex I in patient fibroblasts have shown a drastic decrease in specific activity, whereas the decrease in the amount of the assembled complex I has been only moderate. It therefore seems that the 10191T>C mutation affects the catalysis of complex I rather than the assembly or stability of the enzyme. (McFarland *et al.* 2004.) The 10191T>C mutation has been identified previously in five children with Leigh syndrome (Lebon *et al.* 2003, Bugiani *et al.* 2004, McFarland *et al.* 2004, Leshinsky-Silver *et al.* 2005) and in an adult patient with a progressive history of epilepsy, stroke-like episodes, bilateral optic atrophy and cognitive decline (Taylor *et al.* 2001). Interestingly, it seems that in some cases the mutation has occurred *de novo* and in others, as in our case, it has been transmitted from the mother. Earlier onset and a more severe phenotype both seem to correlate with a higher mutation load and lower specific activity of complex I.

The 14487T>C mutation leads to a Met63Val amino acid substitution in the third transmembrane helix of ND6. This is evolutionarily the best-conserved region of ND6 (Carelli *et al.* 1999), containing several pathogenic mutations associated with Leigh syndrome (Kirby *et al.* 2000), MELAS (Ravn *et al.* 2001), LHON/dystonia and LHON (Chinnery *et al.* 2001). Studies on the expression and assembly of complex I suggest that the Met63Val amino acid change in ND6 may impair the stability or assembly of the holoenzyme (Ugalde *et al.* 2003). Increased production of ROS has also been detected in transmitochondrial cells harbouring the 14487T>C mutation (Gonzalo *et al.* 2005). The oxidation of lipids and mtDNA was increased without any upregulation of antioxidant enzyme activities. Thus oxidative stress has been considered to contribute to the pathogenesis of the 14487T>C mutation. (Gonzalo *et al.* 2005.) The mutation has been previously reported in three patients with Leigh syndrome (Lebon *et al.* 2003, Ugalde *et al.* 2003, Bugiani *et al.* 2004) and two patients with childhood-onset progressive generalized dystonia and bilateral striatal necrosis (Solano *et al.* 2003). The mutation load of 14487T>C varies among these patients, the highest heteroplasmy level (95%) having been detected in the muscle of our patient and in a child with Leigh syndrome and cardiac involvement (Bugiani *et al.* 2004). No correlation has been detected between age at onset or clinical phenotype and the mutation load.

Interestingly, the same mtDNA mutations have recently been detected in several studies on children from different ethnic backgrounds with mitochondrial encephalopathies and complex I deficiency. Frequently encountered mtDNA mutations in addition to 10191T>C and 14487T>C have been 10158T>C in *MTND3* (Lebon *et al.* 2003, McFarland *et al.* 2003, Bugiani *et al.* 2004, Crimi *et al.* 2004), 11777C>A, affecting the same residue in *MTND4* as the 11778G>A LHON primary mutation (Deschauer *et al.* 2003, Komaki *et al.* 2003, Bugiani *et al.* 2004) and two mutations 13513G>A and 13514A>G affecting the same residue in *MTND5* (Chol *et al.* 2003, Kirby *et al.* 2003, Lebon *et al.* 2003, Bugiani *et al.* 2004, Sudo *et al.* 2004).

6.1.2 Novel mutations

It is often difficult to assess the pathogenic role of an amino acid substitution, especially in the case of mtDNA. Certain criteria can be used to determine the deleterious role of such a mutation (DiMauro & Schon 2003). The probability of the variant being pathogenic increases if the nucleotide change is heteroplasmic, absent in control subjects, phylogenetically highly conserved, or if it co-segregates with the disease in a maternal lineage. In addition, various bioinformatic analyses may be used to predict changes caused by a replacement in the secondary or tertiary structure of the protein. The functional consequences of a mutation can be examined further by means of rho zero cell fusion studies or by introducing the corresponding mutation into a simpler model of complex I. Previous data have shown that bacterial NDH-1 can be used as a model to study the effects of mtDNA mutations on complex I (Zickermann *et al.* 1998, Kervinen *et al.* 2006).

In the present work a novel 4681T>C mutation in the mitochondrial *MTND2* gene was found in a Dutch patient with isolated complex I deficiency and Leigh syndrome. Transfer of the patient's mitochondria to rho zero cells retained the specific complex I defect, confirming the mitochondrial origin of the underlying genetic defect. 4681T>C was heteroplasmic and was not present in 200 healthy controls of the same ethnic background. Since the mutation was not detected in the blood of the mother or father, it seems to have occurred *de novo*. The mutation causes replacement of the hydrophobic Leu71 by proline in the third transmembrane helix of ND2. Even though the conservation index for Leu71 was only 36, the position is moderately conserved for hydrophobic amino acids among species. The replacement of leucine by proline within the alpha helical region may lead to conformational changes that could consequently affect the integrity of the entire complex I. Interestingly, accumulation of specific assembly intermediates of complex I was detected in fibroblasts of this patient, suggesting impaired assembly of complex I. The decreased assembly of complex I would then have led in turn to the decreased complex I activity detected in the patient.

The mtDNA of the patient belonged to the T2 haplogroup. Suprisingly, the mtDNA sequence had only two nucleotide positions that differed from the mtDNA in a previously described Dutch LHON patient, S089, without any of the primary LHON mutations (Howell *et al.* 2003). Our patient harboured 4681T>C, whereas patient S089 had 3338T>C, which causes a relatively rare substitution Val11Ala in ND1. The close similarity between these two mtDNA sequences indicates that they have a recent common ancestor. Moreover, it is interesting that both of these patients had the clinical phenotype of a mitochondrial disorder. It is tempting to speculate that the combination of mtDNA variants in the founder sequence may contribute to the OXPHOS defect in these two patients.

The other novel mtDNA mutation, 3866T>C, was identified in a Finnish patient with isolated complex I deficiency. The mutation leads to a Ile187Thr amino acid replacement in a moderately conserved fifth transmembrane helix of ND1 (Roth & Hägerhäll 2001). Bioinformatics and several features of the consensus criteria set for pathogenic mtDNA mutations supported pathogenicity: the mutation was novel, it was heteroplasmic with a higher mutation load in the patient than in the asymptomatic mother, and it was absent in

96 healthy Finnish controls. Following the identification of 3866T>C in the mtDNA of our patient belonging to haplogroup J1c, the same mutation has been reported in six other mtDNA sequences, but ones belonging to other haplogroups (Ingman *et al.* 2000, Herrnstadt *et al.* 2002, Maca-Meyer *et al.* 2003, Mishmar *et al.* 2003, Kivisild *et al.* 2006, Ingman & Gyllensten 2006), confirming that 3866T>C is not a haplogroup-specific variant. Thus the mutation has arisen several times in different populations, which is a feature that is common to many pathogenic mtDNA mutations.

Additional studies were required to further establish the pathogenic role of 3866T>C. The functional consequences of the Ile187Thr replacement were studied by introducing a homologous substitution in NDH-1 of *E. coli*. Activities measured in the mutant and reference NDH-1 indicated that the replacement did not affect the catalytic activity of the enzyme. However, the amount of NDH-1 with the Ile201Thr mutation was lower than that of the reference, suggesting that the mutation could affect the stability or assembly of the enzyme, as in the case of the two mutations Glu214Lys and Tyr215His in the adjacent loop, causing MELAS (Kirby *et al.* 2004a, Kervinen *et al.* 2006). Interestingly, the mtDNA of the patient belonged to the subhaplogroup J1c, characterized by two mutations 14798T>C and 15452C>A in the cytochrome *b* subunit of complex III. In addition, 4216T>C in the *MTND1* gene is one of the polymorphisms that characterize haplogroup J. This variant leads to an amino acid replacement Tyr304His in the eighth transmembrane helix of ND1. Interestingly, both Tyr304His and Ile187Thr occur in transmembrane helices and at the same approximate depth in the membrane-spanning segment (Roth & Hägerhäll 2001). Even though the haplogroup-specific substitutions are not deleterious by themselves, the outcome could be different in combination with other slightly deleterious mutations such as 3866T>C. Such a combination of the mutations, for instance, could alter the stability of the holoenzyme or the formation of supercomplexes.

One novel non-synonymous substitution was found in the nuclear gene *NDUFS8* leading to Arg18Cys in the TYKY subunit. This gene encodes a 210-amino-acid precursor protein containing a 34-amino-acid N-terminal leading peptide for mitochondrial import (Procaccio *et al.* 1997). Bioinformatic analyses predicted that the Arg18Cys mutation could cause changes in the physicochemical properties of the leading peptide, but our studies did not reveal any differences in complex I assembly or activity between the patient and control fibroblasts. Differences in respiratory chain enzyme activities in muscle and fibroblasts have been described previously, and many OXPHOS-deficient patients express reduced complex I activity in muscle but not in fibroblasts (van den Heuvel *et al.* 2004). In most cases the cause of the differential expression of the OXPHOS defect is unknown. Further studies failed to confirm the pathogenic role of the Arg18Cys replacement in the TYKY leading peptide, but pathogenicity was considered possible if the substitution co-occurred with polymorphisms affecting the protein import machinery of the mitochondria, for instance. Furthermore, only nine of the 39 nuclear-encoded subunits of complex I were sequenced in the patient, so the possibility of inheritance of another deleterious heterozygous mutation remains. A digenic inheritance pattern has been described in a patient with two novel heterozygous mutations, one in the *NDUFS2* gene with paternal origin and the other in the *NDUFA8* gene with maternal origin (Bugiani *et al.* 2004). Further research is clearly needed to establish the pathogenic role of the Arg18Cys replacement in TYKY.

6.2 Common signs of complex I deficiency

6.2.1 Biochemical features related to complex I deficiency

The age at onset, the severity of the clinical course and the outcome in patients with OXPHOS system defect have been suggested to differ between patients with nuclear or mtDNA mutations (Rubio-Gozalbo *et al.* 2000). However, in the present study, no clear pattern in the severity of the disease between nuclear and mitochondrial defect was detected. For instance, patient Y (paper III) with the 3460G>A mtDNA mutation had one of the lowest complex I activities in the group of 25 patients with OXPHOS system deficiency. Since only two of these patients had verified pathogenic mtDNA mutations, the defect in the remaining cases, with higher complex I activities, seems to be due to nuclear defect.

Secondary metabolic alterations were detected in most of the patients with decreased complex I activity examined in connection with paper II, including the patient with the 10191T>C mutation. Increased levels of fumarate and malate in their urine suggested altered regulation of the Krebs cycle. NAD^+ is required in mitochondria for the conversion of malate to oxaloacetate by the Krebs cycle. Decrease in oxidation of NADH caused by complex I deficiency could lead to an increased NADH/NAD^+ ratio, which would in turn have a negative effect on the Krebs cycle. Inhibition of complex I could reduce the amount of NAD^+ and lower the level of oxaloacetate acting as a feed-back inhibitor of complex II. Indeed, markedly increased succinate oxidation rates were found in complex I-deficient mitochondria relative to control mitochondria, suggesting loosening of the feed-back inhibition of complex II by oxaloacetate (Fig. 2 in paper II). (Ernster & Nordenstrand 1967.)

Urinal excretion of lactate, fumarate and malate was not increased in patients with the 14487T>C mutation or the 11778G>A LHON primary mutation. As already described, the biochemical data on the pathogenesis of the 11778G>A mutation are controversial. *In vivo* phosphorus magnetic resonance spectroscopy (MRS) has been used to study the energy metabolism of muscle tissue in patients with LHON (Cortelli *et al.* 1991, Lodi *et al.* 1997), and a decrease in mitochondrial ATP production has been detected, especially in patients with the 11778G>A mutation. Furthermore, a decrease in molecular oxygen consumption with complex I substrates has been demonstrated in intact mitochondria harbouring 11778G>A (Hofhaus *et al.* 1996), but estimates of the severity of the specific complex I enzyme defect vary considerably (Carelli *et al.* 1997, Majander *et al.* 1991, Brown *et al.* 2000, Guy *et al.* 2002). Since there has not been any confirmation of the enzymatic defect in complex I caused by the 11778G>A mutation, other hypotheses for the pathogenesis have been suggested, including apoptosis (Howell 1998) and increased production of ROS (Degli Esposti *et al.* 1994). The production of ROS in association with LHON mutations has been studied by creating cybrids using neuronal precursor cells with mitochondria from patients carrying either the 3460G>A or the 11778G>A mutation (Wong *et al.* 2002). As a result, an increase in mitochondrial superoxide production has been detected in the differentiated neuronal cells. Interestingly, increased ROS production was also detected in transmitochondrial cybrids carrying 14487T>C (Gonzalo *et al.* 2005). In view of the metabolic differences between patients carrying the

mutations 10191T>C, 14487T>C and 11778G>A, it seems that the pathogenic mechanisms differ between 10191T>C and the other two mutations. The pathogenesis of the 10191T>C mutation could be related to a defect in the specific activity of complex I as suggested, where as the latter two mutations could have a pathogenic role further down from the oxidation of complex I-specific substrates. Additional factors such as increased oxidative stress could make a contribution to the pathogenic outcome in these cases.

6.2.2 Clinical features related to complex I deficiency

The phenotypes of the patients included in this research were in general in accordance with previous observations, which have shown the early onset of a similar spectrum of clinical symptoms and high mortality in complex I-deficient children (Kirby *et al.* 1999, Triepels *et al.* 2001b). However, two primary LHON mutations were identified in patients with clinical signs differing considerably from the classical symptoms of LHON. The 3460G>A mutation was identified in a Dutch adolescent with isolated complex I deficiency and progressive myopathy as the sole manifestations of her mitochondrial disease, whereas her younger brother recently developed symptoms of classical LHON. Interestingly, a high sequence similarity was identified between the mtDNA of our patient and that of another Dutch patient, S016, with clinically typical LHON (van Senus 1963, Howell *et al.* 2003), suggesting that both patients originated from the same pedigree. Most family members carrying the homoplasmic primary LHON mutations remain asymtomatic. This incomplete penetrance indicates that mutational burden is not the only determinant of expression and other factors have been suggested to contribute including genetical, both mitochondrial and nuclear, and environmental factors. (Man *et al.* 2002, Hudson *et al.* 2005.) Another primary LHON mutation, 11778G>A, was identified in four siblings with related parents. In their case, a recessive nuclear factor influencing on the atypical phenotype may well be present. One of the siblings was a girl with complex I deficiency, encephalomyopathy and cardiac involvement and three boys suffering from similar symptoms but with additional hearing impairment and optic atrophy. In addition to optic neuropathy, other neurological abnormalities such as movement disorders, peripheral neuropathy, multiple sclerosis-like syndrome and cardiac conduction abnormalities (Nikoskelainen *et al.* 1994, Mashima *et al.* 1996) have been described as being associated with LHON (Nikoskelainen *et al.* 1995). The 11778G>A mutation has been identified in patients with chorea and dementia resembling Huntington disease (Morimoto *et al.* 2004), familial multisystem degeneration with Parkinsonism (Simon *et al.* 1999) and progressive bilateral hearing loss (Ceranic & Luxon 2004), but only a few pediatric cases with LHON primary mutations have been reported to be associated with atypical phenotypes. The 3460G>A, 14459G>A and 14484T>C mutations have been identified in young male patients with neurological disorders resembling Leigh syndrome in addition to LHON (Funalot *et al.* 2002). All in all, the clinical data on our patients suggest that the phenotype caused by the primary LHON mutations is highly variable and that the symptoms may already occur in infancy.

We identified the first mutation in the ND2 subunit to be associated with complex I deficiency and Leigh syndrome. Only two primary disease-causing mutations had

previously been reported in ND2: an amino acid substitution Gly259Ser associated with LHON (Brown *et al.* 1992) and a 2 bp deletion leading to a frame shift at Asn222 identified in a patient with exercise intolerance (Schwartz & Vissing 2002). The 4681T>C mutation induced a more severe clinical phenotype in our patient, most probably due to the high mutant loads (above 95%) found in all the tissues analysed. All in all, the identification of several mtDNA mutations, including 10191T>C in *MTND3* and 14487T>C in *MTND6,* in patients with Leigh or Leigh-like syndrome indicates the frequent association of this phenotype with mutations in *MTND* genes.

7 Conclusions

The underlying genetic defect in about half of all mitochondrial energy metabolism disorders can be traced back to mutations in one of the subunits of complex I. The goal of the present research was to resolve the genetic cause of the disease in 50 patients with isolated or combined complex I deficiency or other OXPHOS system defect. The unique features of mitochondrial genetics and the variable signs of mitochondrial disorders nevertheless guarantee that establishment of the pathogenic role of a mtDNA variant is not an easy task. Two novel mtDNA mutations with pathogenic potential were identified here. A novel 4681T>C mutation in *MTND2* was found in a child with isolated complex I deficiency causing Leigh syndrome. Molecular studies demonstrated that the mutation leads to impaired assembly of complex I, causing decreased activity of the enzyme. In addition, a rare 3866T>C mutation in *MTND1* was identified in a patient with myopathy, leading to an Ile187Thr amino acid substitution in the ND1 subunit of complex I. Activity assays of a mutant *E. coli* NDH-1 bearing the homologous Ile201Thr substitution suggested a defect in the assembly or stability of the holoenzyme. Furthermore, the patient's mtDNA sequence belonged to subhaplogroup J1c, which has been shown to be associated with increased penetrance of LHON. It may be suggested that 3866T>C in combination with the specific mtDNA haplogroup J1c background leads to altered stability of complex I.

Identification of the underlying genetic defect gives information on the structurally and functionally important sites of the complex I subunits. The novel amino acid substitution Leu71Pro caused by 4681T>C occurs in the third transmembrane helix of ND2, where no other disease-associated mutations have been described previously. The replacement presumably leads to conformational changes in the peptide. Since hampered assembly and/or stability of the holoenzyme was detected in mutant cells, it seems that proper folding of ND2 is required for a stable structure of complex I. Another mutation, 14487T>C, leads to Met63Val in the third transmembrane helix of ND6. This and the adjacent transmembrane helix harbour several pathogenic mutations, including the LHON primary mutation 14484T>C. All the mutations are in relatively close spatial proximity, possibly defining two sides of a functionally or structurally important area between the two helices.

All in all, we were able to identify a pathogenic mtDNA mutation in nine children with complex I deficiency. Two LHON primary mutations, 3460G>A and 11778G>A,

were identified in patients with clinical signs differing considerably from the classical symptoms of LHON, suggesting the phenotypes caused by the primary LHON mutations to be more variable than had previously been thought. The analysis of nine nuclear-encoded subunits of complex I resulted in the identification of only one novel heterozygous substitution with pathogenic potential.

Our findings and other recent results emphasize a more frequent contribution of mtDNA mutations to the aetiology of pediatric complex I deficiency than had previously been anticipated. Even though some of them have been discovered for the first time only recently, a set of mtDNA mutations have now been reported to occur in multiple unrelated families. Screening for the mutations 10158T>C, 10191T>C, 11777C>A, 13513G>A, 13514A>G and 14487T>C should be carried out when assessing the underlying genetic defect in children with complex I deficiency. Apart from the clearly pathogenic mutations, the contribution of sequence variation elsewhere in mtDNA cannot be underestimated, as in the case with the 3866T>C mutation.

References

Abdrakhmanova A, Zickermann V, Bostina M, Radermacher M, Schagger H, Kerscher S & Brandt U (2004) Subunit composition of mitochondrial complex I from the yeast Yarrowia lipolytica. Biochim Biophys Acta 1658: 148-156.

Acin-Perez R, Bayona-Bafaluy MP, Fernandez-Silva P, Moreno-Loshuertos R, Perez-Martos A, Bruno C, Moraes CT & Enriquez JA (2004) Respiratory complex III is required to maintain complex I in mammalian mitochondria. Mol Cell 13: 805-815.

Andreu AL, Hanna MG, Reichmann H, Bruno C, Penn AS, Tanji K, Pallotti F, Iwata S, Bonilla E, Lach B, Morgan-Hughes J & DiMauro S (1999) Exercise intolerance due to mutations in the cytochrome b gene of mitochondrial DNA. N Engl J Med 341: 1037-1044.

Alberts B, Johnson A, Lewis J, Raff M, Roberts K & Walter P (2002) Molecular Biology of the Cell. 4th edition, Garland Publishing, New York.

Anderson S, Bankier AT, Barrell BG, de Bruijn MH, Coulson AR, Drouin J, Eperon IC, Nierlich DP, Roe BA, Sanger F, Schreier PH, Smith AJ, Staden R & Young IG (1981) Sequence and organization of the human mitochondrial genome. Nature 290: 457-465.

Andreu AL, Hanna MG, Reichmann H, Bruno C, Penn AS, Tanji K, Pallotti F, Iwata S, Bonilla E, Lach, Morgan-Hughes J & DiMauro S (1999) Exercise intolerance due to mutations in the cytochrome b gene of mitochondrial DNA. N Engl J Med 341: 1037-1044.

Andrews RM, Kubacka I, Chinnery PF, Lightowlers RN, Turnbull DM & Howell N (1999) Reanalysis and revision of the Cambridge reference sequence for human mitochondrial DNA. Nat Genet 23: 147.

Antonicka H, Ogilvie I, Taivassalo T, Anitori RP, Haller RG Vissing J, Kennaway NG & Shoubridge EA (2003) Identification and characterization of a common set of complex I assembly intermediates in mitochondria from patients with complex I deficiency. J Biol Chem 278: 43081-43088.

Arenas J, Campos Y, Bornstein B, Ribacoba R, Martin MA, Rubio JC, Santorelli FM, Zeviani M, DiMauro S & Garesse R (1999) A double mutation (A8296G and G8363A) in the mitochondrial DNA tRNA (Lys) gene associated with myoclonus epilepsy with ragged-red fibers. Neurology 52: 377-382.

Attardi G & Schatz G (1988) Biogenesis of mitochondria. Annu Rev Cell Biol 4: 289-333.

Balaban RS, Nemoto S & Finkel T (2005) Mitochondria, oxidants, and aging. Cell 120: 483-495.

Bandelt HJ, Lahermo P, Richards M & Macaulay V (2001) Detecting errors in mtDNA data by phylogenetic analysis. Int J Legal Med 115: 64-69.

Bandelt HJ, Achilli A, Kong QP, Salas A, Lutz-Bonengel S, Sun C, Zhang YP, Torroni A & Yao YG (2005) Low "penetrance" of phylogenetic knowledge in mitochondrial disease studies. Biochem Biophys Res Commun 333: 122-130.

Beal MF (2000) Energetics in the pathogenesis of neurodegenerative diseases. Trends Neurosci 23: 298-304.

Belogrudov G & Hatefi Y (1994) Catalytic sector of complex I (NADH:ubiquinone oxidoreductase): subunit stoichiometry and substrate-induced conformation changes. Biochemistry 33: 4571-4576.

Benit P, Chretien D, Kadhom N, de Lonlay-Debeney P, Cormier-Daire V, Cabral A, Peudenier S, Rustin P, Munnich A & Rötig A (2001) Large-scale deletion and point mutations of the nuclear NDUFV1 and NDUFS1 genes in mitochondrial complex I deficiency. Am J Hum Genet 68: 1344-1352.

Benit P, Beugnot R, Chretien D, Giurgea I, de Lonlay-Depeney P, Issartel JP, Corral-Debrinski M, Kercher S, Rustin P, Rötig A & Munnich A (2003a) Mutant NDUFV2 subunit of mitochondrial complex I causes early onset hypertrophic cardiomyopathy and encephalopathy. Hum Mutat 21: 582-586.

Benit P, Steffann J, Lebon S, Chretien D, Kadhom N, de Lonlay P, Goldenberg A, Dumez Y, Dommergues M, Rustin P, Munnich A & Rötig A (2003b) Genotyping microsatellite DNA markers at putative disease loci in inbred/multiplex families with respiratory chain complex I deficiency allows rapid identification of a novel nonsense mutation (IVS1nt -1) in the NDUFS4 gene in Leigh syndrome. Hum Genet 112: 563-566.

Benit P, Slama A, Cartault F, Giurgea I, Chretien D, Lebon S, Marsac C, Munnich A, Röting A & Rustin P (2004) Mutant NDUFS3 subunit of mitochondrial complex I causes Leigh syndrome. J Med Genet 41: 14-17.

Bentlage HA, Wendel U, Schagger H, ter Laak HJ, Janssen AJ & Trijbels JM (1996) Lethal infantile mitochondrial disease with isolated complex I deficiency in fibroblasts but with combined complex I and IV deficiencies in muscle. Neurology 47: 243-248.

Bereiter-Hahn J & Voth M (1994) Dynamics of mitochondria in living cells: shape changes, dislocations, fusion, and fission of mitochondria. Microsc Res Tech 27: 198-219.

Berg JM, Tymoczko JL, Stryer L & Clarke ND (2002) Biochemistry. 5th edition, W. H. Freeman & Company, New York.

Bernardo SD, Yano T & Yagi T (2000) Exploring the membrane domain of the reduced nicotinamide adenine dinucleotide-quinone oxidoreductase of Paracoccus denitrificans: characterization of the NQO7 subunit. Biochemistry 39: 9411-9418.

Bindoff LA, Howell N, Poulton J, McCullough DA, Morten KJ, Lightowlers RN, Turnbull DM & Weber K (1993) Abnormal RNA processing associated with a novel tRNA mutation in mitochondrial DNA. A potential disease mechanism. J Biol Chem 268: 19559-19564.

Birch-Machin MA & Turnbull DM (2001) Assaying mitochondrial respiratory complex activity in mitochondria isolated from human cells and tissues. Methods Cell Biol 65: 97-117.

Blakely EL, de Silva R, King A, Schwarzer V, Harrower T, Dawidek G, Turnbull DM & Taylor RW (2005) LHON/MELAS overlap syndrome associated with a mitochondrial MTND1 gene mutation. Eur J Hum Gen 13: 623-627.

Blakely EL, Rennie KJ, Jones L, Elstner M, Chrzanowska-Lightowlers ZM, White CB, Shield JP, Pilz DT, Turnbull DM, Poulton J & Taylor RW (2006) Sporadic intragenic inversion of the mitochondrial DNA MTND1 gene causing fatal infantile lactic acidosis. Pediatr Res 59: 440-444.

Bourgeron T, Rustin P, Chretien D, Birch-Machin M, Bourgeois M, Viegas-Pequignot E, Munnich A & Rotig A (1995) Mutation of a nuclear succinate dehydrogenase gene results in mitochondrial respiratory chain deficiency. Nat Genet 11: 144-149.

Bowmaker M, Yang MY, Yasukawa T, Reyes A, Jacobs HT, Huberman JA & Holt IJ (2003) Mammalian mitochondrial DNA replicates bidirectionally from an initiation zone. J Biol Chem 278: 50961-50969.

Braasch DA & Corey DR (2001) Locked nucleic acid (LNA): fine-tuning the recognition of DNA and RNA. Chem Biol 8: 1-7.

Brandt U (1997) Proton-translocation by membrane-bound NADH:ubiquinone-oxidoreductase (complex I) through redox-gated ligand conduction. Biochim Biophys Acta 1318: 79-91.

Brandt U, Kerscher S, Dröse S, Zwicker K & Zickermann V (2003) Proton pumping by NADH:ubiquinone oxidoreductase. A redox driven conformational change mechanism? FEBS Lett 545: 9-17.

Brandt U (2006) Energy converting NADH:quinone oxidoreductase (complex I). Annu Rev Biochem 75: 69-92.

Braun M, Bungert S & Friedrich T (1998) Characterization of the overproduced NADH dehydrogenase fragment of the NADH:ubiquinone oxidoreductase (complex I) from Escherichia coli. Biochemistry 37: 1861-1867.

Bristow EA, Giffiths PG, Andrews RM, Johnson MA & Turnbull DM (2002) The distribution of mitochondrial activity in relation to optic nerve structure. Arch Ophthalmol 120: 791-796.

Brown WM, George JM & Wilson AC (1979) Rapid evolution of animal mitochondrial DNA. Proc Natl Acad Sci U S A 76: 1967-1971.

Brown MD, Voljavec AS, Lott MT, Torroni A, Yang CC & Wallace DC (1992) Mitochondrial DNA complex I and III mutations associated with Leber's hereditary optic neuropathy. Genetics 130: 163-173.

Brown MD, Trounce IA, Jun AS, Allen JC & Wallace DC (2000) Functional analysis of lymphoblast and cybrid mitochondria containing the 3460, 11778, or 14484 Leber's hereditary optic neuropathy mitochondrial DNA mutation. J Biol Chem 275: 39831-39836.

Budde SM, van den Heuvel LP, Janssen AJ, Smeets RJ, Buskens CA, DeMeirleir L, Van Coster R, Baethmann M, Voit T, Trijbels JM & Smeitink JA (2000) Combined enzymatic complex I and III deficiency associated with mutations in the nuclear encoded NDUFS4 gene. Biochem Biophys Res Commun 275: 63-68.

Bugiani M, Invernizzi F, Alberio S, Briem E, Lamantea E, Carrara F, Moroni I, Farina L, Spada M, Donati MA, Uziel G & Zeviani M (2004) Clinical and molecular findings in children with complex I deficiency. Biochim Biophys Acta 1659: 136-147.

Bulteau AL, O'Neill HA, Kennedy MC, Ikeda-Saito M, Isaya G & Szweda LI (2004) Frataxin acts as an iron chaperone protein to modulate mitochondrial aconitase activity. Science 305: 242-245.

Carelli V, Ghelli A, Ratta M, Bacchilega E, Sangiorgi S, Mancini R, Leuzzi V, Cortelli P, Montagna P, Lugaresi E & Degli Esposti M (1997) Leber's hereditary optic neuropathy: biochemical effect of 11778/ND4 and 3460/ND1 mutations and correlation with the mitochondrial genotype. Neurology 48: 1623-1632.

Carelli V, Ghelli A, Bucchi L, Montagna P, De Negri A, Leuzzi V, Carducci C, Lenaz G, Lugaresi E & Degli Espoti M (1999) Biochemical features of mtDNA 14484 (ND6/M64V) point mutation associated with Leber's hereditary optic neuropathy. Ann Neurol 45: 320-328.

Carelli V, Baracca A, Barogi S, Pallotti F, Valentino ML, Montagna P, Zeviani M, Pini A, Lenaz G, Baruzzi A & Solaini G (2002) Biochemical-clinical correlation in patients with different loads of the mitochondrial DNA T8993G mutation. Arch Neurol 59: 264-270.

Carelli V, Achilli A, Valentino ML, Rengo C, Semino O, Pala M, Olivieri A, Mattiazzi M, Pallotti F, Carrara F, Zeviani M, Leuzzi V, Carducci C, Valle G, Simionati B, Mendieta L, Salomao S, Belfort R, Sadun AA & Torroni A (2006) Haplogroup Effects and Recombination of Mitochondrial DNA: Novel Clues from the Analysis of Leber Hereditary Optic Neuropathy Pedigrees. Am J Hum Genet 78: 564-574.

Carroll J, Shannon RJ, Fearnley IM, Walker JE & Hirst J (2002) Definition of the nuclear encoded protein composition of bovine heart mitochondrial complex I. Identification of two new subunits. J Biol Chem 277: 50311-50317.

Carroll J, Fearnley IM, Shannon RJ, Hirst J & Walker J (2003) Analysis of the subunit composition of complex I from bovine heart mitochondria. Mol Cell Proteomics 2: 117-126.

Carroll J, Fearnley IM, Skehel JM, Runswick MJ, Shannon RJ, Hirst J & Walker JE (2005) The post-translational modifications of the nuclear encoded subunits of complex I from bovine heart mitochondria. Mol Cell Proteomics 4: 693-699.

Ceranic B & Luxon LM (2004) Progressive auditory neuropathy in patients with Leber's hereditary optic neuropathy. J Neurol Neurosurg Psychiatry 75: 626-630.

Chen R, Fearnley IM, Peak-Chew SY & Walker JE (2004) The phosphorylation of subunits of complex I from bovine heart mitochondria. J Biol Chem 279: 26036-26045.

Chinnery PF, Brown DT, Andrews RM, Singh-Kler R, Riordan-Eva P, Lindley J, Applegarth DA, Turnbull DM & Howell N (2001) The mitochondrial ND6 gene is a hot spot for mutations that cause Leber's hereditary optic neuropathy. Brain 124: 209-218.

Chol M, Lebon S, Benit P, Chretien D, de Lonlay P, Goldenberg A, Odent S, Hertz-Pannier L, Vincent-Delorme C, Cormier-Daire V, Rustin P, Rötig A & Munnich A (2003) The mitochondrial DNA G13513A MELAS mutation in the NADH dehydrogenase 5 gene is a frequent cause of Leigh-like syndrome with isolated complex I deficiency. J Med Genet 40: 188-191.

Chrzanowska-Lightowlers ZM, Turnbull DM & Lightowlers RN (1993) A microtiter plate assay for cytochrome c oxidase in permeabilized whole cells. Anal Biochem 214: 45-49.

Ciafaloni E, Ricci E, Shanske S, Moraes CT, Silvestri G, Hirano M, Simonetti S, Angelini C, Donati MA, Garcia C, Martinuzzi A, Mosewich R, Servidei S, Zammarchi E, Bonilla E, DeVivo DC, Rowland LP, Schon EA & DiMauro S (1992) MELAS: clinical features, biochemistry, and molecular genetics. Ann Neurol 31: 391-398.

Claros MG & Vincens P (1996) Computational method to predict mitochondrially imported proteins and their targeting sequences. Eur J Biochem 241: 779-786.

Clayton DA (1992) Replication of animal mitochondrial DNA. Cell 28: 693-705.

Cock HR, Cooper JM & Schapira AHV (1999) Functional consequences of the 3460-bp mitochondrial DNA mutation associated with Leber's hereditary optic neuropathy. J Neurol Sci 165: 10-17.

Cooperstein SJ & Lazarow A (1951) A microspectrophotometric method for the determination of cytochrome oxidase. J Biol Chem 189: 665-670.

Corona P, Antozzi C, Carrara F, D'Incerti L, Lamantea E, Tiranti V & Zeviani M (2001) A novel mtDNA mutation in the ND5 subunit of complex I in two MELAS patients. Ann Neurol 49: 106-110.

Cortelli P, Montagna P, Avoni P, Sangiorgi S, Bresolin N, Moggio M, Zaniol P, Mantovani V, Barboni P, Barbiroli B & Lugaresi E (1991) Leber's hereditary optic neuropathy: genetic, biochemical, and phosphorus magnetic resonance spectroscopy study in an Italian family. Neurology 41: 1211-1215.

Coulbault L, Herlicoviez D, Chapon F, Read MH, Penniello MJ, Reynier P, Fayet G, Lombes A, Jauzac P & Allouche S (2005) A novel mutation in the mitochondrial tRNA Asn gene associated with a lethal disease. Biochem Biophys Res Commun 329: 1152-1154.

Crimi M, Galbiati S, Moroni I, Bordoni A, Perini MP, Lamantea E, Sciacco M, Zeviani M, Biunno I, Moggio M, Scarlato G & Comi GP (2003) A missense mutation in the mitochondrial ND5 gene associated with a Leigh-MELAS overlap syndrome. Neurology 60: 1857-1861.

Crimi M, Papadimitriou A, Galbiati S, Palamidou P, Fortunato F, Bordoni A, Papandreou U, Papadimitriou D, Hadjigeorgiou GM, Drogari E, Bresolin N & Comi GP (2004) A new mitochondrial DNA mutation in ND3 gene causing severe Leigh syndrome with early lethality. Pediatr Res 55: 842-846.

Cronan JE, Fearnley IM & Walker JE (2005) Mammalian mitochondria contain a soluble acyl carrier protein. FEBS Lett 579: 4892-4896.

Dahl HH (1998) Getting to the nucleus of mitochondrial disorders: identification of respiratory chain-enzyme genes causing Leigh syndrome. Am J Hum Genet 63: 1594-1597.

Darin N, Kollberg G, Moslemi AR, Tulinius M, Holme E, Gronlund MA, Andersson S & Oldfors A (2006) Mitochondrial myopathy with exercise intolerance and retinal dystrophy in a sporadic patient with a G583A mutation in the mt tRNA(phe) gene. Neuromuscul Disord 16: 504-506.

Degli Esposti M, Carelli V, Ghelli A, Ratta M, Crimi M, Sangiorgi S, Montagna P, Lenaz G, Lugaresi E & Cortelli P (1994) Functional alterations of the mitochondrially encoded ND4 subunit associated with Leber's hereditary optic neuropathy. FEBS Lett 352: 375-379.

Degli Esposti M (1998) Inhibitors of NADH-ubiquinone reductase: an overview. Biochim Biophys Acta 1364: 222-235.

Deschauer M, Bamberg C, Claus D, Zierz S, Turnbull M & Taylor RW (2003) Late-onset encephalopathy associated with a C11777A mutation of mitochondrial DNA. Neurology 60: 1357-1359.

DiMauro S & Schon EA (2003) Mitochondrial respiratory-chain diseases. N Engl J Med 348: 2656-2668.

DiMauro S & Gurgel-Giannetti J (2005) The expanding phenotype of mitochondrial myopathy. Curr Opin Neurol 18: 538-542.

DiMauro S & Hirano M (2005) Mitochondrial encephalomyopathies: an update. Neuromuscul Disord 15: 276-286.

Dutton PL, Moser CC, Sled VD, Daldal F & Ohnishi T (1998) A reductant-induced oxidation mechanism for complex I. Biochim Biophys Acta 1364: 245-257.

von Döbeln U, Wibom R, Ahlman H, Nennesmo I, Nyctelius H, Hultman E & Hagenfeldt L (1993) Fatal neonatal lactic acidosis with respiratory insufficiency due to complex I and IV deficiency. Acta Paediatr 82: 1079-1081.

Ernster L & Nordenstrand K (1967) Skeletal muscle mitochondria. Methods Enzymol 10: 86-94.

van Erven PM, Cillessen JP, Eekhoff EM, Gabreels FJ, Doesburg WH, Lemmens WA, Slooff JL, Renier WO & Ruitenbeek W (1987) Leigh syndrome, a mitochondrial encephalo(myo)pathy. A review of the literature. Clin Neurol Neurosurg 89: 217-230.

Faivre L, Cormier-Daire V, Chretien D, Chrisoph von Kleist-Retzow J, Amiel J, Dommergues M, Saudubray JM, Dumez Y, Rötig A, Rustin P & Munnich A (2000) Determination of enzyme activities for prenatal diagnosis of respiratory chain deficiency. Prenat Diagn 20: 732-777.

Fearnley IM & Walker JE (1992) Conservation of sequences of subunits of mitochondrial complex I and their relationships with other proteins. Biochim Biophys Acta 1140: 105-134.

Fearnley IM, Carroll J, Shannon RJ, Runswick MJ, Walker JE & Hirst J (2001) GRIM-19, a cell death regulatory gene product, is a subunit of bovine mitochondrial NADH:ubiquinone oxidoreductase (complex I). J Biol Chem 276: 38345-38348.

Fernández-Silva P, Enriquez JA & Montoya J (2003) Replication and transcription of mammalian mitochondrial DNA. Exp Physiol 88: 41-56.

Finel M, Skehel JM, Albracht SP, Fearnley IM & Walker JE (1992) Resolution of NADH:ubiquinone oxidoreductase from bovine heart mitochondria into two subcomplexes, one of which contains the redox centers of the enzyme. Biochemistry 31: 11425-11434.

Finel M, Majander AS, Tyynela J, De Jong AM, Albracht SP & Wikstrom M (1994) Isolation and characterisation of subcomplexes of the mitochondrial NADH:ubiquinone oxidoreductase (complex I). Eur J Biochem 226: 237-242.

Finel M (1998) Organization and evolution of structural elements within complex I. Biochim Biophys Acta 1364: 112-121.

Finnilä S, Hassinen IE, Ala-Kokko L & Majamaa K (2000) Phylogenetic network of the mtDNA haplogroup U in Northern Finland based on sequence analysis of the complete coding region by conformation-sensitive gel electrophoresis. Am J Hum Genet 66: 1017-1026.

Finnilä S, Lehtonen M & Majamaa K (2001) Phylogenetic network for European mtDNA. Am J Hum Genet 68: 1475-1484.

Friedrich T, Hofhaus G, Ise W, Nehls U, Schmitz B & Weiss H (1989) A small isoform of NADH:ubiquinone oxidoreductase (complex I) without mitochondrially encoded subunits is made in chloramphenicol-treated Neurospora crassa. Eur J Biochem 180: 173-180.

Friedrich T & Weiss H (1997) Modular evolution of the respiratory NADH:ubiquinone oxidoreductase and the origin of its modules. J Theor Biol 187: 529-540.

Friedrich T (1998) The NADH:ubiquinone oxidoreductase (complex I) from Escherichia coli. Biochim Biophys Acta 1364: 134-146.

Friedrich T (2001) Complex I: A Chimaera of a redox and conformation-driven proton pump? J Bioenerg Biomembr 33: 169-177.

Fukuhara N, Tokiguchi S, Shirakawa K & Tsubaki T (1980) Myoclonus epilepsy associated with ragged-red fibers (mitochondrial abnormalities): disease entity or a syndrome? Light-and electron-microscopic studies of two cases and review of literature. J Neurol Sci 47: 117-133.

Funalot B, Reynier P, Vighetto A, Ranoux D, Bonnefont JP, Godinot C, Malthiery Y & Mas JL (2002) Leigh-like encephalopathy complicating Leber's hereditary optic neuropathy. Ann Neurol 52: 374-377.

Gabaldon T, Rainey D & Huynen MA (2005) Tracing the evolution of a large protein complex in the eukaryotes, NADH:ubiquinone oxidoreductase (Complex I). J Mol Biol 348: 857-870.

Galante YM & Hatefi Y (1979) Purification and molecular and enzymic properties of mitochondrial NADH dehydrogenase. Arch Biochem Biophys 192: 559-568.

Galkin AS, Grivennikova VG & Vinogradov AD (1999) -->H+/2e- stoichiometry in NADH-quinone reductase reactions catalyzed by bovine heart submitochondrial particles. FEBS Lett 451: 157-161.

Garesse R & Vallejo CG (2001) Animal mitochondrial biogenesis and function: a regulatory cross-talk between two genomes. Gene 263: 1-16.

Gellerich FN, Mayr JA, Reuter S, Sperl W & Zierz S (2004) The problem of interlab variation in methods for mitochondrial disease diagnosis: enzymatic measurement of respiratory chain complexes. Mitochondrion 4: 427-439.

Ghelli A, Degli Esposti M, Carelli V & Lenaz G (1997) Changes in mitochondrial complex I activity and coenzyme Q binding site in leber's hereditary optic neuropathy (LHON). Mol Aspects Med 18: s263-s267.

Gonzalo R, Garcia-Arumi E, Llige D, Marti R, Solano A, Montoya J, Arenas J & Andreu AL (2005) Free radicals-mediated damage in transmitochondrial cells harboring the T14487C mutation in the ND6 gene of mtDNA. FEBS Lett 579: 6909-6913.

Goto Y, Nonaka I & Horai S (1990) A mutation in the tRNA(Leu)(UUR) gene associated with the MELAS subgroup of mitochondrial encephalomyopathies. Nature 348: 651-653.

Gray MW (1993) Origin and evolution of organelle genomes. Curr Opin Genet Dev 3: 884-890.

Gray MW, Burger G & Lang BF (1999) Mitochondrial evolution. Science 283: 1476-1481.

Grigorieff N (1998) Three-dimensional structure of bovine NADH:ubiquinone oxidoreductase (complex I) at 22 A in ice. J Mol Biol 277: 1033-1046.

Guénebaut V, Vincentelli R, Mills D, Weiss H & Leonard KR (1997) Three-dimensional structure of NADH-dehydrogenase from Neurospora crassa by electron microscopy and conical tilt reconstruction. J Mol Biol 265: 409-418.

Guénebaut V, Schlitt A, Weiss H, Leonard K & Friedrich T (1998) Consistent structure between bacterial and mitochondrial NADH:ubiquinone oxidoreductase (complex I). J Mol Biol 221: 1027-1043.

Guy J, Qi X, Pallotti F, Schon EA, Manfredi G, Crelli V, Martinuzzi A, Hauswirth WW & Lewin AS (2002) Rescue of a mitochondrial deficiency causing Leber Hereditary Optic Neuropathy. Ann Neurol 52: 534-542.

Hanson BJ, Marusich MF & Capaldi (2001a) Antibody-based approaches to diagnosis and characterization of oxidative phosphorylation diseases. Mitochondrion 1: 237-248.

Hanson BJ, Schulenberg B, Patton WF & Capaldi RA (2001b) A novel subfractionation approach for mitochondrial proteins: a three-dimensional mitochondrial proteome map. Electrophoresis 22: 950-959.

Hatefi Y, Haavik AG & Griffiths DE (1962) Studies on the electron transfer system. XL. Preparation and properties of mitochondrial DPNH-coenzyme Q reductase. J Biol Chem 237: 1676-1680.

von Heijne G, Steppuhn J & Herrmann RG (1989) Domain structure of mitochondrial and chloroplast targeting peptides. Eur J Biochem 180: 535-545.

Hennig L (1999) WinGene/WinPep: user-friendly software for the analysis of amino acid sequences. Biotechniques 26: 1170-1172.

Herrnstadt C, Elson JL, Fahy E, Preston G, Turnbull DM, Anderson C, Ghosg GG, Olefsky JM, Beal MF, Davis RE & Howell N (2002) Reduced-median-network analysis of complete mitochondrial DNA coding-region sequences for the major African, Asian, and European haplogroups. Am J Hum Genet 70: 1152-1171.

Herrnstadt C, Preston G & Howell N (2003) Errors, phantoms and otherwise, in human mtDNA sequences. Am J Hum Genet 72: 1585-1586.

Herrnstadt C & Howell N (2004) An evolutionary perspective on pathogenic mtDNA mutations: haplogroup associations of clinical disorders. Mitochondrion 4: 791-798.

van den Heuvel, Ruitenbeek W, Smeets R, Gelman-Kohan Z, Elpeleg O, Loeffen J, Trijbels F, Mariman E, de Bruijn D & Smeitink J (1998) Demonstration of a new pathogenic mutation in human complex I deficiency: a 5-bp duplication in the nuclear gene encoding the 18-kD (AQDQ) subunit. Am J Hum Genet 62: 262-268.

van den Heuvel LP, Smeitink JA & Rodenburg RJ (2004) Biochemical examination of fibroblasts in the diagnosis and research of oxidative phosphorylation (OXPHOS) defects. Mitochondrion 4: 395-401.

Hinchliffe P & Sazanov LA (2005) Organization of iron-sulfur clusters in respiratory complex I. Science 309: 771-773.

Hirst J, Carroll J, Fearnley IM, Shannon RJ & Walker JE (2003) The nuclear encoded subunits of complex I from bovine heart mitochondria. Biochim Biophys Acta 1604: 135-150.

Hirst J (2005) Energy transduction by respiratory complex I – an evaluation of current knowledge. Biochem Soc Trans 33: 525-529.

Hofhaus G, Weiss H & Leonard K (1991) Electron microscopic analysis of the peripheral and membrane parts of mitochondrial NADH dehydrogenase (complex I). J Mol Biol 221: 1027-1043.

Hofhaus G, Johns DR, Hurko O, Attardi G & Chomyn A (1996) Respiration and growth defects in transmitochondrial cell lines carrying the 11778 mutation associated with Leber's hereditary optic neuropathy. J Biol Chem 271: 13155-13161.

Holt IJ, Harding AE, Petty RK & Morgan-Hughes JA (1990) A new mitochondrial disease associated with mitochondrial DNA heteroplasmy. Am J Hum Genet 46: 428-433.

Holt I, Lorimer HE & Jacobs HT (2000) Coupled leading- and lagging-strand synthesis of mammalian mitochondrial DNA. Cell 100: 515-524.

Holt PJ, Morgan DJ & Sazanov LA (2003) The location of NuoL and NuoM subunits in the membrane domain of the Escherichia coli complex I: implications for the mechanism of proton pumping. J Biol Chem 278: 43114–43120.

Houshmand M, Larsson NG, Oldfors A, Tulinius M & Holme E (1996) Fatal mitochondrial myopathy, lactic acidosis, and complex I deficiency associated with a heteroplasmic A --> G mutation at position 3251 in the mitochondrial tRNALeu(UUR) gene. Hum Genet 97: 269-273.

Howell N, Bindoff LA, McCullough DA, Kubacka I, Poulton J, Mackey D, Taylor L & Turnbull DM (1991) Leber hereditary optic neuropathy: identification of the same mitochondrial ND1 mutation in six pedigrees. Am J Hum Genet 49: 939-950.

Howell N (1998) Leber hereditary optic neuropathy: respiratory chain dysfunction and degeneration of the optic nerve. Vision Res 38: 1495-1504.

Howell N, Bogolin C, Jamieson R, Marenda DR & Mackey DA (1998) mtDNA mutations that cause optic neuropathy: how do we know? Am J Hum Genet 62: 196-202.

Howell N, Oostra RJ, Bolhuis PA, Spruijt L, Clarke LA, Mackey DA, Preston G & Herrnstadt C (2003) Sequence analysis of the mitochondrial genomes from Dutch pedigrees with Leber hereditary optic neuropathy. Am J Hum Genet 72: 1460-1469.

Huang G, Lu H, Hao A, Ng DCH, Ponniah S, Guo K, Lufei C, Zeng Q & Cao X (2004) GRIM-19, a cell death regulatory protein, is essential for assembly and function of mitochondrial complex I. Mol Cell Biol 24: 8447-8456.

Hudson G, Keers S, Yu Wai Man P, Griffiths P, Huoponen K, Savontaus ML, Nikoskelainen E, Zeviani M, Carrara F, Horvath R, Karcagi V, Spruijt L, de Coo IFM, Smeets HJM & Chinnery PF (2005) Identification of an X-chromosomal locus and haplotype modulating the phenotype of a mitochondrial DNA disorder. Am J Hum Genet 77: 1086-1091.

Huoponen K, Vilkki J, Aula P, Nikoskelainen EK & Savontaus ML (1991) A new mtDNA nutation associated with Leber hereditary optiv neuropathy. Am J Hum Genet 48: 1147-1153.

Hutchison WM, Thyagarajan D, Poulton J, Marchington DR, Kirby DM, Manji SS & Dahl HH (2005) Clinical and molecular features of encephalomyopathy due to the A3302G mutation in the mitochondrial tRNA(Leu(UUR)) gene. Arch Neurol 62: 1920-1923.

Ingman M, Kaessmann H, Paabo S & Gyllensten U (2000) Mitochondrial genome variation and the origin of modern humans. Nature 408: 708-713.

Ingman M & Gyllensten U (2006) mtDB: Human Mitochondrial Genome Database, a resource for population genetics and medical sciences. Nucleic Acids Res 34(Database issue): D749-751.

Janssen R, Smeitink J, Smeets R & van den Heuvel L (2002) CIA30 complex I assembly factor: a candidate for human complex I deficiency? Hum Genet 110: 264-270.

Janssen R, van den Heuvel LP & Smeitink JAM (2004) Genetic defects in the oxidative phosphorylation (OXPHOS) system. Expert Rev Mol Diagn 4: 143-156.

Johns DR, Neufeld MJ & Park RD (1992) An ND-6 mitochondrial DNA mutation associated with Leber hereditary optic neuropathy. Biochem Biophys Res Commun 187: 1551-1557.

Jun AS, Trounce IA, Brown MD, Shoffner JM & Wallace DC (1996) Use of transmitochondrial cybrids to assign a complex I defect to the mitochondrial DNA-encoded NADH dehydrogenase subunit 6 gene mutation at nucleotide pair 14459 that causes Leber hereditary optic neuropathy and dystonia. Mol Cell Biol 16: 771-777.

Kao MC, Di Bernardo S, Matsuno-Yagi A & Yagi T (2002) Characterization of the membrane domain Nqo11 subunit of the proton-translocating NADH-quinone oxidoreductase of Paracoccus denitrificans. Biochemistry 41: 4377-4384.

Kao MC, Di Bernardo S, Matsuno-Yagi A & Yagi T (2003) Characterization and topology of the membrane domain Nqo10 subunit of the proton-translocating NADH-quinone oxidoreductase of Paracoccus denitrificans. Biochemistry 42: 4534-4543.

Kasamatsu H & Vinograd J (1973) Unidirectionality of replication in mouse mitochondrial DNA. Nat New Biol 241: 103-105.

Kashani-Poor N, Zwicker K, Kerscher S & Brandt U (2001) A central functional role for the 49-kDa subunit within the catalytic core of mitochondrial complex I. J Biol Chem 276: 24082-24087.

Kervinen M, Pätsi J, Finel M & Hassinen IE (2004) A pair of membrane-embedded acidic residues in the NuoK subunit of Escherichia coli NDH-1, a counterpart of the ND4L subunit of the mitochondrial complex I, are required for high ubiquinone reductase activity. Biochemistry 43: 773-781.

Kervinen M, Hinttala R, Helander H, Kurki S, Uusimaa J, Finel M, Majamaa K & Hassinen I (2006) The MELAS mutations 3946 and 3949 perturb the critical structure in a conserved loop of the ND1 subunit of mitochondrial Complex I. Hum Mol Genet 15: 2543-2552.

King MP & Attardi G (1996) Mitochondria-mediated transformation of human rho(0) cells. Methods Enzymol 264: 313-334.

Kirby DM, Crawford M, Cleary MA, Dahl HHM, Denneth X & Thornburn DR (1999) Respiratory chain complex I deficiency: an underdiagnosed energy generation disorder. Neurology 52: 1255-1264.

Kirby DM, Kahler SG, Freckmann ML, Reddihough D & Thorburn DR (2000) Leigh disease caused by the mitochondrial DNA G14459A mutation in unrelated families. Ann Neurol 48: 102-104.

Kirby DM, Boneh A, Chow CW, Ohtake A, Ryan MT, Thyagarajan D & Thorburn DR (2003) Low mutant load of mitochondrial DNA G13513A mutation can cause Leigh's disease. Ann Neurol 54: 473-478.

Kirby DM, McFarland R, Ohtake A, Dunning C, Ryan MT, Wilson C, Ketteridge D, Turnbull DM, Thorburn DR & Taylor RW (2004a) Mutations of the mitochondrial ND1 gene as a cause of MELAS. J Med Gen 41: 784-789.

Kirby DM, Salemi R, Sugiana C, Ohtake A, Parry L, Bell KM, Kirk EP, Boneh A, Taylor RW, Dahl HH, Ryan MT & Thorburn DR (2004b) NDUFS6 mutations are a novel cause of lethal neonatal mitochondrial complex I deficiency. J Clin Invest 114: 837-845.

Kivisild T, Shen P, Wall DP, Do B, Sung R, Davis K, Passarino G, Underhill PA, Scharfe C, Torroni A, Scozzari R, Modiano D, Coppa A, de Knijff P, Feldman M, Cavalli-Sforza LL & Oefner PJ (2006) The role of selection in the evolution of human mitochondrial genomes. Genetics 172: 373-387.

Komaki H, Akanuma J, Iwata H, Takahashi T, Mashima Y, Nonaka I & Goto Y (2003) A novel mtDNA C11777A mutation in Leigh syndrome. Mitochondrion 2: 293-304.

Küffner R, Rohr A, Schmiee A, Krüll C & Schulte U (1998) Involvement of two novel chaperones in the assembly of mitochondrial NADH:ubiquinone oxidoreductase (complex I). J Mol Biol 283: 409-417.

Larsson N, Andersen A, Holme E, Oldfors A & Wahlström MD (1991) Leber's hereditary optic neuropathy and complex I deficiency in muscle. Ann Neurol 30: 704-708.

Larsson NG & Clayton DA (1995) Molecular genetic aspects of human mitochondrial disorders. Annu Rev Genet 29: 151-178.

Larsson NG & Oldfors A (2001) Mitochondrial myopathies. Acta Physiol Scand 171: 385-393.

Lebon S, Chol M, Benit P, Mugnier C, Chretien D, Giurgea I, Kern I, Girardin E, Hertz-Pannier L, de Lonlay P, Rötig A, Rustin P & Munnich A (2003) Recurrent de novo mitochondrial DNA mutations in respiratory chain deficiency. J Med Genet 40: 896-899.

Leif H, Sled' VD, Ohnishi T, Weiss H & Friedrich T (1995) Isolation and characterization of the proton-translocating NADH:ubiquinoneboxidoreductase from *Escherichia coli.* Eur J Biochem 230: 538-548.

Leshinsky-Silver E, Lev D, Tzofi-Berman Z, Cohen S, Saada A, Yanoov-Sharav M, Gilad E & Lerman-Sagie T (2005) Fulminant neurological deterioration in a neonate with Leigh syndrome due to a maternally transmitted missense mutation in the mitochondrial ND3 gene. Biochem Biophys Res Commun 334: 582-587.

Lightowlers RN, Chinnery PF, Turnbull DM & Howell N (1997) Mammalian mitochondrial genetics: heredity, heteroplasmy and disease. Trends Genet 13: 450-455.

Lodi R, Taylor DJ, Tabrizi SJ, Kumar S, Sweeney M, Wood NW, Styles P, Radda GK & Schapira AH (1997) In vivo skeletal muscle mitochondrial function in Leber's hereditary optic neuropathy assessed by 31P magnetic resonance spectroscopy. Ann Neurol 42: 573-579.

Loeffen J, Smeitink J, Triepels R, Smeets R, Schuelke M, Sengers R, Trijbels F, Hamel B, Mullaart R & van den Heuvel L (1998a) The first nuclear-encoded complex I mutation in a patient with Leigh syndrome. Am J Hum Genet 63: 1598-608.

Loeffen JL, Triepels RH, van den Heuvel LP, Schuelke M, Buskens CA, Smeets RJ, Trijbels JM & Smeitink JA (1998b) cDNA of eight nuclear encoded subunits of NADH:ubiquinone oxidoreductase: human complex I cDNA characterization completed. Biochem Biophys Res Commun 253: 415-422.

Loeffen JL, Smeitink JA, Trijbels JM, Janssen AJM, Triepels RH, Sengers RCA & van den Heuvel LP (2000) Isolated complex I deficiency in children: clinical, biochemical and genetic aspects. Hum Mutat 15: 123-134.

Loeffen J, Elpeleq O, Smeitink J, Smeets R, Stockler-Ipsiroqlu S, Mandel H, Sengers R, Trijbels F & van den Heuvel L (2001) Mutations in the complex I NDUFS2 gene of patients with cardiomyopathy and encephalomyopathy. Ann Neurol 49: 195-201.

Maca-Meyer N, Gonzalez AM, Pestano J, Flores C, Larruga JM & Cabrera VM (2003) Mitochondrial DNA transit between West Asia and North Africa inferred from U6 phylogeography. BMC Genet 4: 15.

Majander A, Huoponen K, Savontaus ML, Nikoskelainen E & Wikström M (1991) Electron transfer properties of NADH:ubiquinone reductase in the ND1/3460 and the ND4/11778 mutations of the Leber hereditary optic neuroretinopathy (LHON). FEBS Lett 292: 289-292.

Majander A, Finel M, Savontaus ML, Nikoskelainen E & Wikström M (1996) Catalytic activity of complex I in cell lines that possess replacement mutations in the ND genes in Leber's hereditary optic neuropathy. Eur J Biochem 239: 201-207.

Man PY, Turnbull DM & Chinnery PF (2002) Leber hereditary optic neuropathy. J Med Genet 39: 162-169.

Martin MA, Blazquez A, Gutierrez-Solana LG, Fernandez-Moreira D, Briones P, Andreu AL, Garesse R, Campos Y & Arenas J (2005) Leigh syndrome associated with mitochondrial complex I deficiency due to a novel mutation in the NDUFS1 gene. Arch Neurol 62: 659-661.

Mashima Y, Kigasawa K, Hasegawa H, Tani M & Oguchi Y (1996) High incidence of pre-excitation syndrome in Japanese families with Leber's hereditary optic neuropathy. Clin Genet 50: 535-537.

Mathews CK, van Holde KE & Ahern K-G (2000) Biochemistry. 3rd edition. Benjamin Cummings, San Francisco.

Mathiesen C & Hägerhäll C (2002) Transmembrane topology of the NuoL, M and N subunits of NADH:quinone oxidoreductase and their homologues among membrane-bound hydrogenases and bona fide antiporters. Biochim Biophys Acta 1556: 121-132.

Matsuoka R, Furutani M, Hayashi J, Isobe K, Akimoto K, Shibata T, Imamura S, Tatsuguschi M, Furutani Y, Takao A, Ohnishi S, Kasanuki H & Momma K (1999) A mitochondrial DNA mutation cosegregates with the pathophysiological U wave. Biochem Biophys Res Commun 257: 228-233.

Mayr JA, Moslemi AR, Forster H, Kamper A, Idriceanu C, Muss W, Huemer M, Oldfors A & Sperl W (in press) A novel sporadic mutation G14739A of the mitochondrial tRNA(Glu) in a girl with exercise intolerance. Neuromuscul Disord.

Mazziotta MRM, Ricci E, Bertini E, Vici CD, Servidei S, Burlina AB, Sabetta G, Bartuli A, Manfredi G, Silvestri G, Moraes CT & DiMauro S (1992) Fatal infantile liver failure associated with mitochondrial DNA depletion. J Pediatr 121: 896-901.

McFarland R, Kirby DM, Fowler KJ, Ohtake A, Ryan MT, Amor DJ, Fletcher JM, Dixon JW, Collins FA, Turnbull DM, Taylor RW & Thorburn DR (2004) De novo mutations in the mitochondrial ND3 gene as a cause of infantile mitochondrial encephalopathy and complex I deficiency. Ann Neurol 55: 58-64.

Mishmar D, Ruiz-Pesini E, Golik P, Macaulay V, Clark AG, Hosseini S, Brandon M, Easley K, Chen E, Brown MD, Sukernik RI, Olckers A & Wallace DC (2003) Natural selection shaped regional mtDNA variation in humans. Proc Natl Acad Sci USA 100: 171-176.

Mitchell AL, Elson JL, Howell N, Taylor RW & Turnbull DM (2006) Sequence variation in mitochondrial complex I genes: mutation or polymorphism? J Med Genet 43: 175-179.

Montoya J, Gaines GL & Attardi G (1983) The pattern of transcription of the human mitochondrial rRNA genes reveals two overlapping transcription units. Cell 34: 151-159.

Morgan-Hughes JA, Darveniza P, Landon DN, Land JM & Clark JB (1979) A mitochondrial myopathy with a deficiency of respiratory chain NADH-CoQ reductase activity. J Neurol Sci 43: 27-46.

Morimoto N, Nagano I, Deguchi K, Murakami T, Fushimi S, Shoji M & Abe K (2004) Leber hereditary optic neuropathy with chorea and dementia resembling Huntington disease. Neurology 63: 2451-2452.

Morris AAM, Leonard JV, Brown GK, Bidouki SK, Bindoff LA, Woodward CE, Harding AE, Lake BD, Harding BN, Farrell MA, Bell JE, Mirakhur M & Turnbull DM (1996) Deficiency of respiratory chain complex I is a common cause of Leigh disease. Ann Neurol 40: 25-30.

Munnich A, Rustin P, Rotig A, Chretine D, Bonnefont JP, Nuttin C, Cormier V, Vassault A, Parvy P & Bardet J (1992) Clinical aspects of mitochondrial disorders. J Inherit Metab Dis 15: 448-455.

Murray J, Zhang B, Taylor SW, Oglesbee D, Fahy E, Marusich MF, Ghosh SS & Capaldi RA (2003) The subunit composition of the human NADH dehydrogenase obtained by rapid one-step immunopurification. J Biol Chem 278: 13619-13622.

Nagai T, Tuchiya Y, Taguchi Y, Sakuta R, Ichiki T & Nonaka I (1993) Fatal infantile mitochondrial encephalomyopathy with complex I and IV deficiencies. Pediatr Neurol 9: 151-154.

Naini AB, Lu J, Kaufmann P, Bernstein RA, Mancuso M, Bonilla E, Hirano M & DiMauro S (2005) Novel mitochondrial DNA ND5 mutation in a patient with clinical features of MELAS and MERRF. Arch Neurol 62: 473-476.

Nakamaru-Ogiso E, Yano T, Yagi T & Ohnishi T (2005) Characterization of the iron-sulfur cluster N7 (N1c) in the subunit NuoG of the proton-translocating NADH-quinone oxidoreductase from Escherichia coli. J Biol Chem 280: 301-307.

Nass MMK & Nass S (1963) Intramitochondrial fibers with DNA characteristics. J Cell Biol 19: 593-629.

Niers LE, Smeitink JA, Trijbels JM, Sengers RC, Janssen AJ & van den Heuvel LP (2001) Prenatal diagnosis of NADH:ubiquinone oxidoreductase deficiency. Prenat Diagn 21: 871-880.

Nijtmans LGJ, Henderson NS & Holt IJ (2002) Blue Native electrophoresis to study mitochondrial and other protein complexes. Methods 26: 327-334.

Nikoskelainen EK, Savontaus ML, Huoponen K, Antila K & Hartiala J (1994) Pre-excitation syndrome in Leber's hereditary optic neuropathy. Lancet 344: 857-858.

Nikoskelainen EK, Marttila RJ, Huoponen K, Juvonen V, Lamminen T, Sonninen P & Savontaus ML (1995) Leber's "plus": neurological abnormalities in patients with Leber's hereditary optic neuropathy. J Neurol Neurosurg Psychiatry 59: 160-164.

Oden KL, DeVeaux LC, Vibat CR, Cronan JE Jr & Gennis RB (1990) Genomic replacement in Escherichia coli K-12 using covalently closed circular plasmid DNA. Gene 96: 29-36.

Ogilvie I, Kennaway NG & Shoubridge EA (2005) A molecular chaperone for mitochondrial complex I assembly is mutated in a progressive encephalopathy. J Clin Invest 115: 2784-2792.

Ogle RF, Christodoulou J, Fagan E, Blok RB, Kirby DM, Seller KL, Dahl HH & Thorburn DR (1997) Mitochondrial myopathy with tRNA(Leu(UUR)) mutation and complex I deficiency responsive to riboflavin. J Pediatr 130: 138-145.

Ohnishi T (1998) Iron-sulphur clusters/semiquinones in complex I. Biochim Biophys Acta 1364: 186-206.

Ojala D, Montoya J & Attardi G (1981) tRNA punctuation model of RNA processing in human mitochondria. Nature 290: 470-474.

Okhuijsen-Kroes EJ, Trijbels JM, Sengers RC, Mariman E, van den Heuvel LP, Wendel U, Koch G & Smeitink JA (2001) Infantile presentation of the mtDNA A3243G tRNA(leu(UUR)) mutation. Neuropediatrics 32: 183-190.

Pavlakis SG, Phillips PC, DiMauro S, De Vivo DC & Rowland LP (1984) Mitochondrial myopathy, encephalopathy, lactic acidosis, and strokelike episodes: a distinctive clinical syndrome. Ann Neurol 16: 481-488.

Petruzzella V, Vergari R, Puzziferri I, Boffoli D, Lamantea E, Zeviani M & Papa S (2001) A nonsense mutation in the NDUFS4 gene encoding the 18 kDa (AQDQ) subunit of complex I abolishes assembly and activity of the complex in a patient with Leigh-like syndrome. Hum Mol Genet 10: 529-535.

Procaccio V, Depetris D, Soularue P, Mattei MG, Lunardi J & Issartel JP (1997) cDNA sequence and chromosomal localization of the NDUFS8 human gene coding for the 23 kDa subunit of the mitochondrial complex I. Biochim Biophys Acta 1351: 37-41.

Procaccio V, Mousson B, Beugnot R, Duborjal H, Feillet F, Putet G, Pignot-Paintrand I, Lombes A, De Coo R, Smeets H, Lunardi J & Issartel JP (1999) Nuclear DNA origin of mitochondrial complex I deficiency in fatal infantile lactic acidosis evidenced by transnuclear complementation of cultured fibroblasts. J Clin Invest 104: 83-92.

Procaccio V & Wallace DC (2004) Late-onset Leigh syndrome in a patient with mitochondrial complex I NDUFS8 mutations. Neurology 62: 1899-1901.

Ragan CI, Galante YM, Hatefi Y & Ohnishi T (1982) Resolution of mitochondrial NADH dehydrogenase and isolation of two iron-sulfur proteins. Biochemistry 21: 590-594.

Ragan CI (1987) Structure of NADH-ubiquinone reductase (compex I). Curr Top Bioenerget 15: 1-36.

Raha S, Myint AT, Johnstone L & Robinson BH (2002) Control of oxygen free radical formation from mitochondrial complex I: roles for protein kinase A and pyruvate dehydrogenase kinase. Free Radic Biol Med 32: 421-430.

Rahman S, Blok RB, Dahl HH, Danks DM, Kirby DM, Chow CW, Christodoulou J & Thorburn DR (1996) Leigh syndrome: clinical features and biochemical and DNA abnormalities. Ann Neurol 39: 343-351.

Rasmussen HN, Andersen AJ & Rasmussen UF (1997) Optimization of preparation of mitochondria from 25-100 mg skeletal muscle. Anal Biochem 252: 153-159.

Ravn K, Wibrand F, Hansen FJ, Horn N, Rodenburg T & Schwarz M (2001) An mtDNA mutation, 14453G-->A, in the NADH dehydrogenase subunit 6 associated with severe MELAS syndrome. Eur J Hum Genet 9: 805-809.

Robinson BH (1998) Human complex I deficiency: clinical spectrum and involvement of oxygen free radicals in the pathogenicity of the defect. Biochim Biophys Acta 1364: 271-286.

Roise D & Schatz G (1988) Mitochondrial presequences. J Biol Chem 263: 4509-4511.

Roth R & Hägerhäll C (2001) Transmembrane orientation and topology of the NADH:quinine oxidoreductase putative quinine binding subunit NuoH. Biochim Biophys Acta 1504: 352-362.

Rubio-Gozalbo ME, Dijkman KP, van den Heuvel LP, Sengers RC, Wendel U & Smeitink JA (2000) Clinical differences in patients with mitochondriocytopathies due to nuclear versus mitochondrial DNA mutations. Hum Mutat 15: 522-532.

Ruiz-Pesini E, Mishmar D, Brandon M, Procaccio V & Wallace DC (2004) Effects of purifying and adaptive selection on regional variation in human mtDNA. Science 303: 223-226.

Runswick MJ, Fearnley IM, Skehel JM & Walker JE (1991) Presence of an acyl carrier protein in NADH:ubiquinone oxidoreductase from bovine heart mitochondria. FEBS Lett 286: 121-124.

Rustin P, Chretien D, Bourgeron T, Wucher A, Saudubray JM, Rötig A & Munnich A (1991) Assessment of the mitochondrial respiratory chain. Lancet 338: 60.

Rustin P, Chretien D, Bourgeron T, Gerard B, Rötig A, Saudubrau JM & Munnich A (1994) Biochemical and molecular investigations in respiratory chain deficiencies. Clin Chim Acta 228: 35-51.

Rötig A, Lebon S, Zinovieva E, Mollet J, Sarzi E, Bonnefont JP & Munnich A (2004) Molecular diagnostics of mitochondrial disorders. Biochim Biophys Acta 1659: 129-135.

Santorelli FM, Tanji K, Kulikova R, Shanske S, Vilarinho L, Hays AP & DiMauro S (1997) Identification of a novel mutation in the mtDNA ND5 gene associated with MELAS. Biochem Biophys Res Comm 238: 326-328.

Saraste M (1999) Oxidative phosphorylation at the fin de siecle. Science 283: 1488-1493.

Sazanov LA & Hinchliffe P (2006) Structure of the hydrophilic domain of respiratory complex I from Thermus thermophilus. Science 311: 1430-1436.

Scacco S, Petruzzella V, Budde S, Vergari R, Tamborra R, Panelli D, van den Heuvel LP, Smeitink J & Papa S (2003) Pathological mutations of the human NDUFS4 gene of the 18-kDa (AQDQ) subunit of complex I affect the expression of the protein and the assembly and function of the complex. J Biol Chem 278: 44161-44167.

Scaglia F, Towbin JA, Craigen WJ, Belmont JW, Smith EO, Neish SR, Ware SM, Hunter JV, Fernbach SD, Vladutiu GD, Wong LJ & Vogel H (2004) Clinical spectrum, morbidity, and mortality in 113 pediatric patients with mitochondrial disease. Pediatrics 114: 925-931.

Schafer E, Seelert H, Rifschneider NH, Krause F, Dencher NA & Vonck J (2006) Architecture of active mammalian respiratory chain supercomplexes. J Biol Chem 281: 15370-15375.

Scheffler IE (2001) Mitochondria make a come back. Adv Drug Deliv Rev 49: 3-26.

Schilling B, Aggeler R, Schulenberg B, Murray J, Row RH, Capaldi RA & Gibson BW (2005) Mass spectrometric identification of a novel phosphorylation site in subunit NDUFA10 of bovine mitochondrial complex I. FEBS Lett 579: 2485-2490.

Schmidt M, Friedrich T, Wallrath J, Ohnishi T & Weiss H (1992) Accumulation of the pre-assembled membrane arm of NADH:ubiquinone oxidoreductase in mitochondria of manganese-limited grown Neurospora crassa. FEBS Lett 313: 8-11.

Schneider R, Massow M, Lisowsky T & Weiss H (1995) Different respiratory-defective phenotypes of Neurospora crassa and Saccharomyces cerevisiae after inactivation of the gene encoding the mitochondrial acyl carrier protein. Curr Genet 29: 10-17.

Schuelke M, Smeitink J, Mariman E, Loeffen J, Plecko B, Trijbels F, Stockler-Ipsiroqlu S & van den heuvel L (1999) Mutant NDUFV1 subunit of mitochondrial complex I causes leukodystrophy and myoclonic epilepsy. Nat Genet 21: 260-261.

Schuelke M, Detjen A, van den heuvel L, Korenke C, Janssen A, Smits A, Trijbels F & Smeitink J (2002) New nuclear encoded mitochondrial mutation illustrates pitfalls in prenatal diagnosis by biochemical methods. Clin Chem 48: 772-775.

Schulenberg B, Aggeler R, Beechem JM, Capaldi RA & Patton WF (2003) Analysis of steady-state protein phosphorylation in mitochondria using a novel fluorescent phosphosensor dye. J Biol Chem 278: 27251-27255.

Schulte U (2001) Biogenesis of respiratory complex I. J Bioenerg Biomembr 33: 205-212.

Schultz BE & Chan SI (2001) Structures and Proton-Pumping Strategies of Mitochondrial Respiratory Enzymes. Annu Rev Biophys Biomol Struct 30: 23-65.

Schägger H & Pfeiffer K (2000) Supercomplexes in the respiratory chains of yeast and mammalian mitochondria. EMBO J 19: 1777-1783.

Schägger H & Pfeiffer K (2001) The ratio of oxidative phosphorylation complexes I-V in bovine heart mitochondria and the composition of respiratory chain supercomplexes. J Biol Chem 276: 37861-37867.

Schägger H (2002) Respiratory chain supercomplexes of mitochondria and bacteria. Biochim Biophys Acta 1555: 154-159.

Schwartz M & Vissing J (2002) Paternal inheritance of mitochondrial DNA. N Engl J Med 347: 576-580.

Seneca S, Goemans N, Van Coster R, Givron P, Reybrouck T, Sciot R, Meulemans A, Smet J & Van Hove JL (2005) A mitochondrial tRNA aspartate mutation causing isolated mitochondrial myopathy. Am J Med Genet A 137: 170-175.

van Senus AHC (1963) Leber's disease in Netherlands. Doc Ophthalmol 17: 1-163.

Shadel GS & Clayton DA (1997) Mitochondial DNA maintenance in vertebrates. Annu Rev Biochem 66: 409-434.

Sharpley MS, Shannon RJ, Draghi F & Hirst J (2006) Interactions between phospholipids and NADH:ubiquinone oxidoreductase (complex I) from bovine mitochondria. Biochemistry 45: 241-248.

Shoffner JM, Lott MT, Lezza AM, Seibel P, Ballinger SW & Wallace DC (1990) Myoclonic epilepsy and ragged-red fiber disease (MERRF) is associated with a mitochondrial DNA tRNA(Lys) mutation. Cell 61: 931-997.

Silvestri G, Ciafaloni E, Santorelli FM, Shanske S, Servidei S, Graf WD, Sumi M & DiMauro S (1993) Clinical features associated with the A-->G transition at nucleotide 8344 of mtDNA ("MERRF mutation"). Neurology 43: 1200-1206.

Simon DK & Johns DR (1999) Mitochondrial disorders: Clinical and genetic features. Annu Rev Med 50: 111-127.

Simon DK, Pulst SM, Sutton JP, Browne SE, Beal MF & Johns DR (1999) Familiar multisystem degeneration with parkinsonism associated with the 11778 mitochondrial DNA mutation. Neurology 53: 1787-1793.

Simon DK, Friedman J, Breakefield XO, Jankovic J, Brin MF, Provias J, Bressman SB, Charness ME, Tarsy D, Johns DR & Tarnopolsky MA (2003) A heteroplasmic mitochondrial complex I gene mutation in adult-onset dystonia. Neurogenetics 4: 199-205.

Skladal D, Halliday J & Thorburn DR (2003) Minimum birth prevalence of mitochondrial respiratory chain disorders in children. Brain 126: 1905-1912.

Sled VD & Vinogradov AD (1993) Kinetics of the mitochondrial NADH-ubiquinone oxidoreductase interaction with hexammineruthenium(III). Biochim Biophys Acta 1141: 262-268.

Sled VD, Rudnitzky NI, Hatefi Y & Ohnishi T (1994) Thermodynamic analysis of flavin in mitochondrial NADH:ubiquinone oxidoreductase (complex I). Biochemistry 33: 10069-10075.

Smeitink J, van den Heuvel L & DiMauro S (2001a) The genetics and pathology of oxidative phosphorylation. Nat Rev Genet 2: 342-352.

Smeitink J, Seners R, Trijbels F & van den Heuvel L (2001b) Human NADH:ubiquinone oxidoreductase. J Bioenerg Biomembr 33: 259-266.

Smith PR, Cooper JM, Govan GG, Harding AE & Schapira AHV (1994) Platelet mitochondrial function in Leber's hereditary optic neuropathy. J Neurol Sci 122: 80-83.

Solano A, Roig M, Vives-Bauza C, Hernandez-Pena J, Garcia-Arumi E, Playan A, Lopez-Perez MJ, Andreu AL & Montoya J (2003) Bilateral striatal necrosis associated with a novel mutation in the mitochondrial ND6 gene. Ann Neurol 54: 527-530.

Sottocasa GL, Kuylenstierna B, Ernster L & Bergstrand A (1967) An electron-transport system associated with the outer membrane of liver mitochondria. A biochemical and morphological study. J Cell Biol 32: 415-438.

Strachan T & Read A (1999) Human Molecular Genetics 2. 2nd edition, Garland Science, New York and London.

Sudo A, Honzawa S, Nonaka I & Goto Y (2004) Leigh syndrome caused by mitochondrial DNA G13513A mutation: frequency and clinical features in Japan. J Hum Genet 49: 92-96.

Sue CM, Bruno C, Andreu AL, Cargan A, Mendell JR, Tsao CY, Luquette M, Paolicchi J, Shanske S, DiMauro S & De Vivo DC (1999) Infantile encephalopathy associated with the MELAS A3243G mutation. J Pediatr 134: 696-700.

Sutovsky P, Moreno RD, Ramalho-Santos J, Dominko T, Simerly C & Schatten G (2000) Ubiquitinated sperm mitochondria, selective proteolysis, and the regulation of mitochondrial inheritance in mammalian embryos. Biol Reprod 63: 582-590.

Tanaka M, Cabrera VM, Gonzalez AM, Larruga JM, Takeyasu T, Fuku N, Guo LJ, Hirose R, Fujita Y, Kurata M, Shinoda K, Umetsu K, Yamada Y, Oshida Y, Sato Y, Hattori N, Mizuno Y, Arai Y, Hirose N, Ohta S, Ogawa O, Tanaka Y, Kawamori R, Shamoto-Nagai M, Maruyama W, Shimokata H, Suzuku R & Shimodaira H (2004) Mitochondrial genome variation in eastern Asia and the peopling of Japan. Genome Res 14: 1832-1850.

Tatuch Y, Christodoulou J, Feigenbaum A, Clarke JT, Wherret J, Smith C, Rudd N, Petrova-Benedict R & Robinson BH (1992) Heteroplasmic mtDNA mutation (T—G) at 8993 can cause Leigh disease when the percentage of abnormal mtDNA is high. Am J Hum Genet 50: 852-858.

Taylor RW, Singh-Kler R, Hayes CM, Smith PE & Turnbull DM (2001) Progressive mitochondrial disease resulting from a novel missense mutation in the mitochondrial DNA ND3 gene. Ann Neurol 50: 104-107.

Taylor RW, Morris AA, Hutchinson M & Turnbull DM (2002) Leigh disease associated with a novel mitochondrial DNA ND5 mutation. Eur J Hum Genet 10: 141-144.

Taylor SW, Fahy E, Zhang B, Glenn GM, Warnock DE, Wiley S, Murphy AN, Gaucher SP, Capaldi RA, Gibson BW & Ghosh SS (2003) Characterization of the human heart mitochondrial proteome. Nat Biotechnol 21: 281-286.

Thorburn DR, Sugiana C, Salemi R, Kirby DM, Worgan L, Ohtake A & Ryan MT (2004) Biochemical and molecular diagnosis of mitochondrial respiratory chain disorders. Biochim Biophys Acta 1659: 121-128.

Torkko JM, Koivuranta KT, Miinalainen IJ, Yagi AI, Schmitz W, Kastaniotis AJ, Airenne TT, Gurvitz A & Hiltunen KJ (2001) Candida tropicalis Etr1p and Saccharomyces cerevisiae Ybr026p (Mrf1'p), 2-enoyl thioester reductases essential for mitochondrial respiratory competence. Mol Cell Biol 21: 6243-6253.

Torroni A, Huoponen K, Francalacci P, Petrozzi M, Morelli L, Scozzari R, Obinu D, Savontaus ML & Wallace DC (1996) Classification of European mtDNAs from an analysis of three European populations. Genetics 144: 1835-1850.

Torroni A, Petrozzi M, D'Urbano L, Sellito D, Zeviani M, Carrara F, Carducci C, Leuzzi V, Carelli V, Barboni P, De Negri A & Scozzari R (1997) Haplotype and phylogenetic analyses suggest that one European-specific mtDNA background plays a role in the expression of Leber hereditary optic neuropathy by increasing the penetrance of the primary mutations 11778 and 14484. Am J Hum Genet 60: 1107-1121.

Triepels RH, van den heuvel LP, Loeffen JL, Buskens CA, Smeets RJ, Rubio Gozalbo MF, Budde SM, Mariman EC, Wijburg FA, Barth PG, Trijbels JM & Smeitink JA (1999) Leigh syndrome associated with a mutation in the NDUFS7 (PSST) nuclear encoded subunit of complex I. Ann Neurol 45: 787-790.

Triepels RH, Hanson BJ, van den Heuvel LP, Sundell L, Marusich MF, Smeitink JA & Capaldi RA (2001a) Human complex I defects can be resolved by monoclonal antibody analysis into distinct subunit assembly patterns. J Biol Chem 276: 8892-8897.

Triepels RH, van den Heuvel LP, Trijbels JM & Smeitink JA (2001b) Respiratory chain complex I deficiency. Am J Med Genet 106: 37-45.

Truscott KN, Brandner K & Pfanner N (2003) Mechanisms of protein import into mitochondria. Curr Biol 13: R326-337.

Tsao K, Aitken PA & Johns DR (1999) Smoking as an aetiological factor in a pedigree with Leber's hereditary optic neuropathy. Br J Ophthalmol 83: 577-581.

Tuschen G, Sackmann U, Nehls U, Haiker H, Buse G & Weiss H (1990) Assembly of NADH: ubiquinone reductase (complex I) in Neurospora mitochondria. Independent pathways of nuclear-encoded and mitochondrially encoded subunits. J Mol Biol 213: 845-857.

Ugalde C, Triepels RH, Coenen MJ, van den Heuvel LP, Smeets R, Uusimaa J, Briones P, Campistol J, Majamaa K, Smeitink JA & Nijtmans LG (2003) Impaired complex I assembly in a Leigh syndrome patient with a novel missense mutation in the ND6 gene. Ann Neurol 54: 665-669.

Ugalde C, Janssen RJ, van den Heuvel LP, Smeitink JA & Nijtmans LG (2004a) Differences in assembly or stability of complex I and other mitochondrial OXPHOS complexes in inherited complex I deficiency. Hum Mol Genet 13: 659-667.

Ugalde C, Vogel R, Huijbens R, van den Heuvel B, Smeitink J & Nijtmans L (2004b) Human mitochondrial complex I assembles through the combination of evolutionary conserved modules: a framework to interpret complex I deficiencies. Hum Mol Genet 13: 2461-2472.

Urbano A, Lakshmanan U, Choo PH, Kwan JC, Ng PY, Guo K, Dhakshinamoorthy S & Porter A (2005) AIF suppresses chemical stress-induced apoptosis and maintains the transformed state of tumor cells. EMBO J 24: 2815-2826.

Uusimaa J, Remes AM, Rantala H, Vainionpää L, Herva R, Vuopala K, Nuutinen M, Majamaa K & Hassinen IE (2000) Childhood encephalomyopathies and myopathies: A prospective study in a defined population to assess the frequency of mitochondrial disorders. Pediatrics 105: 598-603.

Uusimaa J, Finnilä S, Remes AM, Rantala H, Vainionpää L, Hassinen IE & Majamaa K (2004) Molecular epidemiology of childhood mitochondrial encephalomyopathies in a Finnish population: sequence analysis of entire mtDNA of 17 children reveals heteroplasmic mutations in tRNAArg, tRNAGlu, and tRNALeu(UUR) genes. Pediatrics 114: 443-450.

Vahsen N, Cande C, Briere JJ, Benit P, Joza N, Larochette N, Mastroberardino PG, Pequignot MO, Casares N, Lazar V, Feraud O, Debili N, Wissing S, Engelhardt S, Madeo F, Piacentini M, Penninger JM, Schagger H, Rustin P & Kroemer G (2004) AIF deficiency compromises oxidative phosphorylation. EMBO J 23: 4679-4689.

Valentino ML, Avoni P, Barboni P, Pallotti F, Rengo C, Torroni A, Bellan M Baruzzi A & Carelli V (2002) Mitochondrial DNA nucleotide changes C14482G and C14482A in the ND6 gene are pathogenic for Leber's hereditary optic neuropathy. Ann Neurol 51: 774-778.

Valentino ML, Barboni P, Ghelli A, Bucchi L, Rengo C, Achilli A, Torroni A, Lugaresi A, Lodi R, Barbiroli B, Dotti M, Federico A, Baruzzi A & Carelli V (2004) The ND1 gene of complex I is a mutational hot spot for Leber's hereditary optic neuropathy. Ann Neurol 56: 631-641.

Vellai T & Vida G (1999) The origin of eukaryotes: the difference between prokaryotic and eukaryotic cells. Proc Biol Sci 266: 1571-1577.

Videira A (1998) Complex I from the fungus *Neurospora crassa*. Biochim Biophys Acta 1364: 89-100.

Videira A & Duarte M (2001) On complex I and other NADH:ubiquinone reductases of Neurospora crassa mitochondria. J Bioenerg Biomembr 33: 197-203.

Vogel R, Janssen RJRJ, Ugalde C, Grovenstein M, Huijbens RJ, Visch H-J, van den Heuvel LP, Willems PH, Zeviani M, Smeitink JAM & Nijtmans LGJ (2005) Human mitochondrial complex I assembly is mediated by NDUFAF1. FEBS J 272: 5317-5326.

de Vries DD, Went LN, Bruyn GW, Scholte HR, Hofstra RM, Bolhuis PA & van Oost BA (1996) Genetic and biochemical impairment of mitochondrial complex I activity in a family with Leber hereditary optic neuropathy and hereditary spastic dystonia. Am J Hum Genet 58: 703-711.

de Vries MC, Rodenburg RJ, Morava E, van Kaauwen EP, Ter Laak H, Mullaart RA, Snoeck IN, van Hasselt PM, Harding P, van den Heuvel LP & Smeitink JA (in press) Multiple oxidative phosphorylation deficiencies in severe childhood multi-system disorders due to polymerase gamma (POLG1) mutations. Eur J Pediatr.

Vuokila PT & Hassinen IE (1988) NN'-dicyclohexylcarbodi-imide-sensitivity of bovine heart mitochondrial NADH: ubiquinone oxidoreductase. Inhibition of activity and binding to subunits. Biochem J 249: 339-344.

Walker JE (1992) The NADH:ubiquinone oxidoreductase (complex I) of respiratory chains. Q Rev Biophys 25: 253-324.

Wallace DC, Singh G, Lott MT, Hodge JA, Schurr TG, Lezza AM, Elsas LJ 2nd & Nikoskelainen EK (1988a) Mitochondrial DNA mutation associated with Leber's hereditary optic neuropathy. Science 242: 1427-1430.

Wallace DC, Zheng X, Lott MT, Shoffner JM, Hodge JA, Kelley RI, Epstein CM & Hopkins LC (1988b) Familial mitochondrial encephalomyopathy (MERRF): genetic, pathophysiological, and biochemical characterization of a mitochondrial DNA disease. Cell 55: 601-610.

Weidner U, Geier S, Ptock A, Friedrich T, Leif H & Weiss H (1993) The gene locus of the proton-translocating NADH:ubiquinone oxidoreductase in *Escherichia coli.* Organization of the 14 genes and relationship between the derived proteins and subunits of mitochondrial complex I. J Mol Biol 233: 109-122.

Wibom R & Hultman E (1990) ATP production rate in mitochondria isolated from microsamples of human muscle. Am J Physiol 259: E204-209.

Wibom R, Hagenfeldt L & von Döbeln U (2002) Measurement of ATP production and respiratory chain enzyme activities in mitochondria isolated from small muscle biopsy samples. Anal Biochem 311: 139-151.

Wikström M (1984) Two protons are pumped from the mitochondrial matrix per electron transferred between NADH and ubiquinone. FEBS Lett 169: 300-304.

Wissinger B, Besch D, Bauman B, Fauser S, Christ-Adler M, Jurklies B, Zrenner E & Leo-Kottler B (1997) Mutation analysis of the ND6 gene in patients with Lebers hereditary optic neuropathy. Biochem Biophys Res Commun 234: 511-515.

Wong A, Cavelier L, Collins-Schramm HE, Seldin MF, McGrogan M, Savontaus ML & Cortopassi GA (2002) Differentiation-specific effects of LHON mutations introduced into neuronal NT2 cells. Hum Mol Genet 11: 431-438.

Wong LJ, Yim D, Bai RK, Kwon H, Vacek MM, Zane J, Hoppel CL & Kerr DS (2006) A novel mutation in the mitochondrial tRNA(Ser(AGY)) gene associated with mitochondrial myopathy, encephalopathy, and complex I deficiency. J Med Genet 43: e46.

Yadava N, Potluri P, Smith EN, Bisevac A & Scheffler IE (2002) Species-specific and mutant MWFE proteins. Their effect on the assembly of a functional mammalian mitochondrial complex I. J Biol Chem 277: 21221-21230.

Yagi T, Yano T & Matsuno-Yagi A (1993) Characteristics of the energy-transducing NADH-quinone oxidoreductase of Paracoccus denitrificans as revealed by biochemical, biophysical, and molecular biological approaches. J Bioenerg Biomembr 25: 339-345.

Yano T, Dunham WR & Ohnishi T (2005) Characterization of the delta muH+-sensitive ubisemiquinone species (SQ(Nf)) and the interaction with cluster N2: new insight into the energy-coupled electron transfer in complex I. Biochemistry 44: 1744-1754.

Yasukawa T, Yang MY, Jacobs HT & Holt IJ (2005) A bidirectional origin of replication maps to the major noncoding region of human mitochondrial DNA. Mol Cell 18: 651-662.

Zeviani M & Di Donato S (2004) Mitochondrial disorders. Brain 127: 2153-2172.

Zhadanov SI, Atamanov VV, Zhadanov NI, Oleinikov OV, Osipova LP & Schurr TG (2005) A novel mtDNA ND6 gene mutation associated with LHON in a Caucasian family. Biochem Biophys Res Commun 332: 1115-1121.

Zheng X, Shoffner JM, Lott MT, Voljavec AS, Krawiecki NS, Winn K & Wallace DC (1989) Evidence in a lethal infantile mitochondrial disease for a nuclear mutation affecting respiratory complexes I and IV. Neurology 39: 1203-1209.

Zickermann V, Barquera B, Wikström M & Finel M (1998) Analysis of the pathogenic human mitochondrial mutation ND1/3460, and mutations of strictly conserved residues in its vicinity, using the bacterium Paracoccus denitrificans. Biochemistry 37: 11792-11796.

Original papers

I Hinttala R, Uusimaa J, Remes AM, Rantala H, Hassinen IE & Majamaa K (2005) Sequence analysis of nuclear genes encoding functionally important complex I subunits in children with encephalomyopathy. J Mol Med 83: 786-794.

II Esteitie N, Hinttala R, Wibom R, Nilsson H, Hance N, Naess K, Teär-Fahnehjelm K, von Döbeln U, Majamaa K & Larsson NG (2005) Secondary metabolic effects in complex I deficiency. Ann Neurol 58: 544-552.

III Hinttala R, Smeets R, Moilanen JS, Ugalde C, Uusimaa J, Smeitink JAM & Majamaa K (2006) Analysis of mitochondrial DNA sequences in patients with isolated or combined oxidative phosphorylation system deficiency. J Med Genet 43: 881-886.

IV Ugalde C, Hinttala R, Timal S, Smeets R, Rodenburg RJT, Uusimaa J, van den Heuvel LP, Nijtmans LGJ, Majamaa K & Smeitink JAM (2006) Mutated ND2 impairs mitochondrial complex I assembly and leads to Leigh Syndrome. Mol Genet Metab. In press.

V Hinttala R, Kervinen M, Uusimaa J, Finnilä S, Rantala H, Remes AM, Hassinen IE & Majamaa K (2006) A rare 3866T>C mutation in the mitochondrial ND1 gene and mutagenesis of a homologous position in *Escherichia coli*. Manuscript.

888. Tenhunen, Olli (2006) Mitogen-activated protein kinases and transcription factors during increased cardiac workload and remodelling

889. Elo, Satu (2006) Teoria pohjoissuomalaisten kotona asuvien ikääntyneiden hyvinvointia tukevasta ympäristöstä

890. Vierimaa, Heidi (2006) Salmon cardiac peptide as a model for natriuretic peptide secretion. The role of mechanical load, temperature and endothelin-1

891. Tiitto, Leena (2006) Histopathological features in the progression of idiopathic pulmonary fibrosis/usual interstitial pneumonia with special emphasis on the redox modulating enzymes of the human lung

892. Koskenkari, Juha (2006) Myocardial ischemia-reperfusion injury and systemic inflammatory response in high-risk cardiac surgery. A clinical study of the effects of high-dose glucose-insulin treatment and the use of leukocyte-depleting filter

893. Trias, Tuulikki (2006) Inter-twin and parent-twin relationships and mental health. A study of twins from adolescence to young adulthood

894. Ala-Mursula, Leena (2006) Employee worktime control and health

895. Turpeinen, Miia (2006) Cytochrome P450 enzymes—*in vitro*, *in vivo*, and *in silico* studies

896. Vuorialho, Arja (2006) Costs and effectiveness of hearing aid rehabilitation in the elderly

897. Ala-Kopsala, Minna (2006) Circulating N-terminal fragments of A- and B-type natriuretic peptides: molecular heterogeneity, measurement and clinical application

898. Haho, Annu (2006) Hoitamisen olemus. Hoitotyön historiasta, teoriasta ja tulkinnasta hoitamista kuvaviin teoreettisiin väittämiin

899. Kukkurainen, Marja Leena (2006) Fibromyalgiaa sairastavien koherenssintunne, sosiaalinen tuki ja elämänlaatu

900. Rautio, Katriina (2006) Effects of insulin-lowering drugs in PCOS: endocrine, metabolic and inflammatory aspects

901. Riekki, Riitta (2006) Late dermal effects of breast cancer radiotherapy

902. Leskelä, Hannu-Ville (2006) Human bone marrow stem cells—a novel aspect to bone remodelling and mesenchymal diseases

903. Hörkkö, Tuomo (2006) Growth and progression in colorectal cancer

Book orders:
OULU UNIVERSITY PRESS
P.O. Box 8200, FI-90014
University of Oulu, Finland

Distributed by
OULU UNIVERSITY LIBRARY
P.O. Box 7500, FI-90014
University of Oulu, Finland

UNIVERSITY OF OULU P.O. Box 7500 FI-90014 UNIVERSITY OF OULU FINLAND

ACTA UNIVERSITATIS OULUENSIS

ISBN 951-42-8287-6 (Paperback)
ISBN 951-42-8288-4 (PDF)
ISSN 0355-3221 (Print)
ISSN 1796-2234 (Online)