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# Clinical spectrum and phenotype-genotype correlations in patients with mitochondrial complex I deficiency

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In Dankbarkeit meinen Eltern und meiner Familie gewidmet.

# Abstract

Isolated RCC I deficiency is observed in about one third of patients with mitochondrial disorders. Especially for paediatric patients, the identification of the molecular-genetic basis of the disease is challenging. The large number of possible disease-causing genes hampers fast and effective analyses on the molecular-genetic level. During a screen involving all structural subunits and some assembly factors of RCC I, a pathogenic mutation has been identified in about 20% of patients (previous work at the Institute of Human Genetics, Technische Universität München, Munich and the Helmholtz Zentrum München, Munich).
Within the framework of the research project at hand, five candidate genes have been analysed for pathogenic sequence variants, assuming that other genes for proper RCC I function and assembly exist. As a result, no novel mutations have been identified which could explain the defect of RCC I within the analysed cohort of 150 patients with isolated RCC I deficiency. The large number of patients with unknown disease-causing mutation suggests that other genes may exist or that an inadequate specificity of RCC I enzyme measurements due to the large and relatively vulnerable composition of RCC I is generating false positive enzyme measurements.

A result of the comprehensive mutation screen is the detailed molecular-genetic characterization of the 150 patients with isolated RCC I deficiency. A main topic of the present work was the phenotypic specification of patients' history and the clinical presentation of disease obtained by a standardized questionnaire. This patient cohort is the largest collection of patients with isolated RCC I deficiency so far. Beside the wide range of RCC I residual activities, a heterogeneous spectrum of phenotypes has been observed within these patients. The majority of patients were children and presented with predominant neurological dysfunction in form of encephalomyopathies. By contrast, relatively mild phenotypes were predominantly found in adult patients. Despite the size of the collection of patients, the majority of those did not receive a molecular-genetic diagnosis. Therefore, genotype-phenotype association analyses within the cohort had no significance. Comparing literature reports of patients carrying mutations in nuclear encoded subunits and assembly factors with single patients of the cohort harbouring comparable mutations, the correlation analysis showed more validity. A prioritization of nuclear genes based up on the phenotypes within the patient cohort could facilitate the molecular-genetic diagnostics, but the awareness of other possible disease-causing genes should be kept in mind. Moreover, an extended mutation analysis on the exome level for the patients of the cohort with good clinical characterization and missing molecular-genetic diagnosis is recommended.

The present screen of candidate genes and analysis of phenotypes and genotypes of this thesis were carried out from October 2008 and July 2009 at the Institute of Human Genetics (Helmholtz Zentrum München) and the Paediatric Hospital of München-Schwabing (Technische Universität München).

# Zusammenfassung

Etwa ein Drittel der Patienten mit Mitochondriopathie weisen einen isolierten Defekt des Komplex-I der Atmungskette auf. Die Aufklärung der molekular-genetischen Ursache ist besonders bei pädiatrischen Patienten eine schwierige Aufgabe. Die hohe Zahl möglicher Kandidatengene für die Erkrankung behindert eine schnelle und effektive Diagnostik im Bereich der Molekulargenetik. Durch ein umfassendes Screening der Gene, welche die Struktureinheiten und einige Assemblierungsfaktoren des Komplex-I der Atmungskette kodieren, konnte für 20% der Patienten die pathogene Mutation gefunden werden (vorangegangene Arbeit am Institut für Humangenetik der Technischen Universität München und dem Helmholtz Zentrum München).
Im Rahmen der vorliegenden Arbeit wurden unter der Annahme, dass weitere Gene für die korrekte Zusammensetzung und Funktion des Komplex-I verantwortlich sein können, fünf Kandidatengene auf pathogene Sequenzvarianten untersucht. In den Proben der 150 analysierten Patienten wurde keine neue Mutation gefunden, welche den Defekt des Atmungskettenenzyms erklären könnte. Die verbleibende Zahl der Patienten mit unbekannter molekular-genetischer Ursache der Erkrankung lässt vermuten, dass andere Gene existieren oder die unzureichende Spezifität der Komplex-I-Messungen und die relativ instabile Komposition des größten Atmunskettenkomplexes falsch positive Enzymmessungen für Patienten generieren.

Ein Ergebnis des umfassenden Mutationsscreening ist die erfolgte molekular-genetische Charakterisierung der 150 Patienten mit isoliertem Komplex-I-Defekt. Die Beschreibung der Phänotypen der Patienten mittels eines standartisierten Fragebogens war Hauptaufgabe der vorliegenden Arbeit. Diese Patientenkohorte stellt die bisher größte Sammlung von Patienten mit isoliertem Komplex-I-Defekt dar. Neben der großen Varianz der Komplex-I-Restaktivitäten wurde ein heterogenes Spektrum von Phänotypen bei den Patienten beobachtet. Der größte Teil der Patientenkohorte waren Kinder, welche vorwiegend mit neurologischen Defiziten als Ausdruck einer Enzephalopathie auffielen. Mildere Phänotypen wurden vorwiegend bei erwachsenen Patienten beobachtet. Trotz der großen Patientensammlung und dem umfassenden Mutationsscreening konnte in der Mehrzahl der Fälle keine molekular-genetische Diagnose gefunden werden. Dadurch haben Genotyp-Phänotyp-Analysen innerhalb der Patientenkohorte keine Signifikanz. Der Vergleich mit publizierten Patienten, welche Mutationen in den entsprechenden nukleär kodierten Untereinheiten und Assemblierungsfaktoren aufweisen, hat daher eine bessere Aussagekraft. Durch die Phänotypisierung der Patienten kann eine Priorisierung bestimmter nukleärer Gene die molekular-genetische Diagnostik anleiten. Allerdings muss die Möglichkeit, dass andere Gene Ursache der Erkrankung sein können immer in Betracht gezogen werden. Für die Patienten der untersuchten Kohorte mit guter klinischer Charakterisierung und unklarer genetischer Diagnose sollte ein erweitertes Mutationsscreening auf der Ebene der Exom-Analyse erfolgen.

Die vorliegende Arbeit wurde im Zeitraum von Oktober 2008 bis Juli 2009 am Institut für Humangenetik (Helmholtz Zentrum München) und der Klinik und Poliklinik für Kinder- und Jugendmedizin Schwabing der Technischen Universität München durchgeführt.

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# Abbreviations

| | |
|---|---|
| A | adenine |
| ADP | adenosine-5'-diphosphate |
| AEP | acoustic evoked potentials |
| ATP | adenosine-5'-triphosphate |
| bp | base pair |
| C | cytosine |
| °C | degree Celsius |
| cAMP | cyclic adenosine-5'-monophosphate |
| cDNA | complementary DNA |
| CK | creatine kinase |
| CM | cardiomyopathy |
| cMRI | cranial magnetic resonance imaging |
| CoA | coenzyme A |
| CoQ | ubiquinone |
| $CoQH_2$ | ubiquinol |
| COX | cytochrome c oxidase |
| CS | citrate synthase |
| CSF | cerebral spinal fluid |
| CT | computer tomography |
| cyt c | cytochrome c |
| Da | Dalton |
| dATP | deoxy adenosine-5'-triphosphate |
| dCTP | deoxy cytosine-5'-triphosphate |
| ddNTP | dideoxy nucleotide triphosphate |
| dGTP | deoxy guanosine-5'-triphosphate |
| DHDPS | dihydrodipicolinate synthase |
| DNA | deoxyribonucleic acid |
| dNTP | deoxynucleotide triphosphate |
| dTTP | deoxy thymidine-5'-triphosphate |
| ECG | electrocardiogram |
| ECHO | echocardiogram |
| EDTA | ethylene diamine tetra-acetic acid |
| EEG | electroencephalography |
| FAD | flavine adenine dinucleotide |
| FILA | fatal infantile lactic acidosis |
| FMN | flavine mononucleotide |
| g | gram |
| G | guanine |
| GFP | green fluorescent protein |
| GIT | gastrointestinal tract |
| GRACILE | intrauterine growth retardation, aminoaciduria, cholestasis, iron overload, lactic acidosis and early death |
| $H_2O$ | water |
| IHG | Institute of Human Genetics, TU Munich |
| kDa | kilo Dalton |
| kb | kilo base pairs |
| KSS | Kearns-Sayre Syndrome |
| LHON | Leber's hereditary optic neuropathy |
| LIMD | lethal infantile mitochondrial disease |

| | |
|---|---|
| LS | Leigh syndrome |
| MELAS | mitochondrial encephalopathy with lactic acidosis and stroke-like episodes |
| MERRF | myoclonic epilepsy with ragged red fibers |
| MILS | maternal inherited Leigh syndrome |
| ml | millilitre |
| mmol | millimole |
| mRNA | messenger ribonucleic acid |
| MRS | magnetic resonance spectroscopy |
| MTCYTB | mitochondrial cytochrome b |
| mtDNA | mitochondrial DNA |
| µl | micro litre |
| NADH | nicotine amide adenine dinucleotide |
| NARP | neuropathy, ataxia and retinitis pigmentosa |
| NCBI | National Centre for Biotechnology Information |
| NCP | non-collagen protein |
| ND | NADH ubiquinone dehydrogenase |
| nDNA | nuclear DNA |
| NDUFA | NADH dehydrogenase ubiquinone subcomplex α |
| NDUFB | NADH dehydrogenase ubiquinone subcomplex β |
| NDUFS | NADH dehydrogenase ubiquinone iron sulfur protein region |
| NDUFV | NADH dehydrogenase ubiquinone flavoprotein region |
| nmol | nanomole |
| NPHP | nephronophthisis |
| ORF | open reading frame |
| OXPHOS | oxidative phosphorylation |
| PCR | polymerase chain reaction |
| PDH | pyruvate dehydrogenase |
| PDHC | pyruvate dehydrogenase complex |
| $P_i$ | inorganic phosphate |
| RC | respiratory chain |
| RCC | respiratory chain complex |
| rRNA | ribosomal ribonucleic acid |
| ROS | reactive oxygen species |
| RRF | ragged red fibers |
| SAM | s-adenosylmethionine |
| SD | standard derivation |
| SNP | single-nucleotide polymorphism |
| T | thymine |
| TCA cycle | tricarboxylic acid (Krebs' cycle) |
| tRNA | transfer ribonucleic acid |
| TU | Technical university |
| U | Unit, measurement unit for enzyme activity |
| UQCRB | ubiquinol-cytochrome c reductase binding protein |
| UV-light | ultra violet light |
| VEP | visual evoked potentials |

# 1 Introduction

Mitochondrial diseases are a clinically heterogeneous group of disorders that arise as a result of dysfunction of the mitochondrial respiratory chain (RC) and other metabolic pathways situated within mitochondria. Defects of proper mitochondrial function can potentially affect only a single organ, but more often involve multiple organ systems and often present with predominant neurologic and myopathic features. Because of the clinical variability and the variation of the age of onset, mitochondrial diseases may be difficult to be recognized. For exact diagnosis, a multidisciplinary approach should be followed encompassing clinical, biochemical, morphological and molecular-genetic data collection and evaluation. Even when a diagnosis has been reached, therapy options are rare and to date, there is no way to cure patients with mitochondrial disease.

About one third of the patients with mitochondrial disorders are suffering from respiratory chain complex I (RCC I) deficiency [Kirby et al. 1999; Loeffen et al. 2000; Janssen et al. 2006b; Rodenburg et al. 2010]. The defect can be caused by mutations of nuclear or mitochondrial DNA (mtDNA) and the pattern of inheritance is very variable - there are maternal, dominant, recessive and spontaneous forms of mutations. Although defects in RCC I are frequently observed, the molecular-genetic cause of disease is unknown for the majority of patients. For defects of RCC II-V the mutation detection rate is notably higher. To improve the knowledge about RCC I deficiencies and the genetic background a large sample collection and high-throughput mutation screen has been previously performed at the Institute of Human Genetics (Technical University Munich and the Helmholtz Zentrum). Within my thesis the main topic was to improve the knowledge about the specific phenotypes of patients with isolated RCC I deficiency and to search for genotype-phenotype correlations that may guide molecular-genetic diagnostics. Background knowledge about the genetic cause of disease can help patients and their families regarding the outcome and dealing with the disease beside the possibility of prenatal diagnostics.

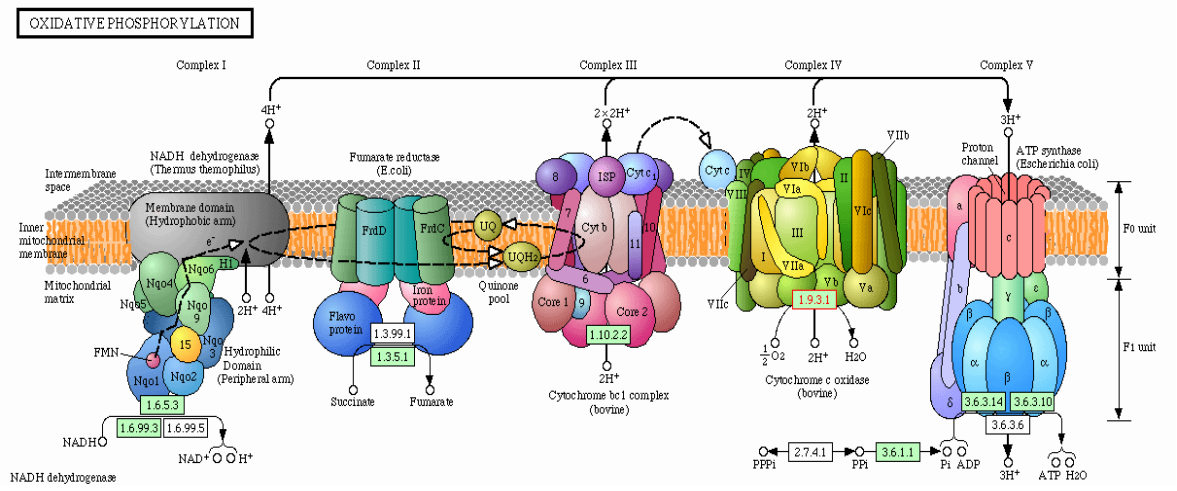
## 1.1 Mitochondria – an essential cell organelle

Mitochondria (Greek: *mito*=thread and *khondrion*=granule) are essential organelles present in the cytoplasm of all eukaryotic cells, with exception of erythrocytes, which live in oxygen-rich environments and are regarded as 'powerhouses' of the cell fulfilling most energy requirement by generating adenosine-5'-triphosphate (ATP) via the oxidative phosphorylation (OXPHOS) system [Schatz 1995]. The endosymbiotic theory assumes that mitochondria are originated as separate prokaryotic organisms, which were taken inside the eukaryotic cell retaining most parts of their autonomy, but also being integrated in functional control mechanisms of the cell [Andersson et al. 2003; Henze et Martin 2003]. In addition to the ATP conversion from adenosine diphosphate (ADP) and inorganic phosphate ($P_i$), mitochondria support other essential cell functions, including calcium signalling, ion homeostasis, synthesis of haem, lipids, amino acids and nucleotides, active transport processes, cell motility, cell proliferation, initiation of programmed cell death (apoptosis) and aging processes, thus demonstrating that mitochondria are involved in numerous processes contributing to proper cell function [Wallace et al. 1997; McBride et al. 2006].

The ATP production from glucose or fatty acids is the result of the collaboration of several metabolic pathways localized in different cell compartments, like the cytosol, peroxisomes and mitochondria, whereas the mitochondrion harbours essential enzymes for the tricarboxylic acid (TCA), so-called Krebs cycle, the fatty acid and pyruvate oxidation. Glucose is initially metabolized in the cytoplasm into pyruvate which is imported into mitochondria and decarboxylated to acetyl-coenzyme A (CoA) by the pyruvate-dehydrogenase (PDH) complex. The TCA cycle metabolizes the energy enriched acetyl-CoA. The resulting reduction equivalents NADH (nicotine amid dehydrogenase) and $FADH_2$ (flavine adenine dinucleotide) constitute the basis for producing ATP by the RC via OXPHOS.
The RC is organised in five multi-protein complexes, which are all embedded in the inner mitochondrial membrane:

- NADH:CoQ(ubiquinone) oxidoreductase (RCC I)
- Succinate:CoQ oxidoreductase (RCC II)
- $CoQH_2$ (ubiquinol):cytochrome c oxidoreductase (RCC III)
- Cytochrome c oxidase (RCC IV)
- ATP synthase (RCC V)

RCC I serves as electron acceptor from the reduced coenzymes NADH, while RCC II is part of the TCA cycle and accepts electrons from $FADH_2$. The transport of these electrons along the electron transport chain (RCC I - RCC IV) and the electron transport molecules CoQ and cytochrome c is coupled with the creation of a proton gradient by RCC I, RCC III and RCC IV (see **Fig. 1**). Finally, the ATP synthase (RCC V) uses the proton movement down its electrochemical gradient (from intermembrane space to mitochondrial matrix) to synthesise ATP from ADP and $P_i$. Although electron transport occurs with great efficiency, a small percentage of electrons are prematurely leaked to oxygen, resulting in the formation of reactive oxygen species (ROS) which can damage the mitochondria and other cell organelles [Victor et al. 2009].



NADH dehydrogenase

**Fig. 1.** Schematic overview of respiratory chain complexes within the inner mitochondrial membrane derived from models from different species. Electron transport and parallel proton pumping is also demonstrated for the single complexes. (www.proteomics.jhu.edu/.../Mito%20Diagram%201.JPG)

### 1.1.1 Mitochondrial respiratory chain complexes

#### 1.1.1.1 NADH:CoQ oxidoreductase – RCC I

The composition and topology of mammalian RCC I has been intensively investigated in bovine heart mitochondria [Walker 1992; Walker et al. 1992; Walker et al. 1995; Carroll et al. 2002; Carroll et al. 2003; Hirst et al. 2003; Carroll et al. 2006]. RCC I is an L-shaped protein complex, which is build up of two arms perpendicular to each other: a peripheral hydrophilic arm, which protrudes into the mitochondrial matrix and a hydrophobic arm, embedded in the inner mitochondrial membrane containing the mtDNA encoded subunits (**Fig. 2**, **A**) [Grigorieff 1998; Sazanov et Walker 2000; Efremov et al. 2010].

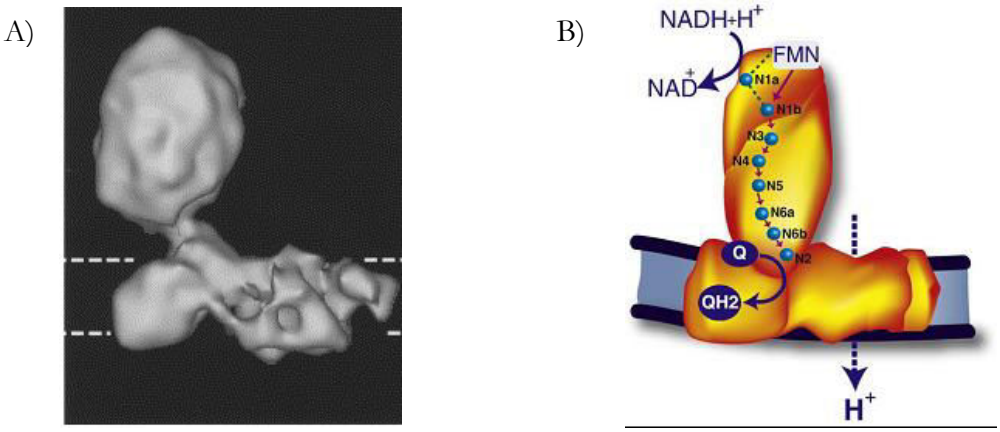



**Fig. 2**. **A)** Reconstruction of bovine RCC I from Grigorieff [1998, p.1035-36]. The reconstruction is oriented with the membrane domain horizontal and the matrix arm pointing up. The approximate position of the lipid bilayer is indicated by two broken lines. A thin stalk is visible which connects the globular domain of the matrix arm with the membrane domain. **B)** Within the peripheral arm of RCC I electrons, which are transferred from NADH to the flavine mononucleotide (FMN), are passed through a series of iron-sulfur (Fe-S) clusters to the final acceptor ubiquinone (Q) which is reduced to ubiquinol ($QH_2$). In parallel four protons ($H^+$) are pumped into the intermembrane space. [Lazarou et al. 2009, p.80].

The nomenclature of subunits is based upon the molecular weight in kDa (**Fig. 3**). Containing 45 subunits RCC I is the largest enzyme complex of the RC with a molecular weight of ~980 kDa [Fernandez-Vizarra et al. 2009]. It encompasses seven structural subunits encoded by the mtDNA and 38 by the nuclear DNA (nDNA). The nuclear encoded subunits and assembly factors have to be transported into the mitochondrion. Because of the complex membrane construction of mitochondria, several transport proteins (translocases) are essential for the importing processes [Herrmann et Neupert 2000].

Fourteen of the structural subunits are highly conserved across evolution, which means that all of them are found in prokaryotes and have orthologous subunits in the eukaryotic counterpart [Gabaldon et al. 2005; Brandt 2006]. Therefore, it is assumed that they create the catalytic core and are essential for the correct redox and proton pumping activity [Walker 1992; Friedrich et Bottcher 2004; Papa et al. 2009]. These 14 core subunits encompass all mitochondrial encoded subunits (ND1-ND6, ND4L) and the nuclear encoded subunits NDUFS1[1], NDUFS2, NDUFS3, NDUFS7, NDUFS8, NDUFV1 and NDUFV2 [Lazarou et al. 2009]. The remaining subunits, which have no prokaryotic counterpart, are thought to be accessorial

[1] Nuclear encoded subunits are termed NADH dehydrogenase ubiquinone ('NDU') followed by the abbreviation of function/localization (FS – iron sulfur protein region; FV – flavoprotein region; FA – subcomplex α).

components which do not play a significant role in the transfer of electrons or protons. They may have general functions like the stabilisation of the complex, building a construct isolating the essential subunits to avoid loss of electrons, preventing the increased generating of cell damaging ROS or protecting the complex from oxidation damage [Hirst et al. 2003]. Another theory postulated that the accessorial subunits can act as docking stations for other RCC by building highly efficient catalytic super complexes [Budde et al. 2000].

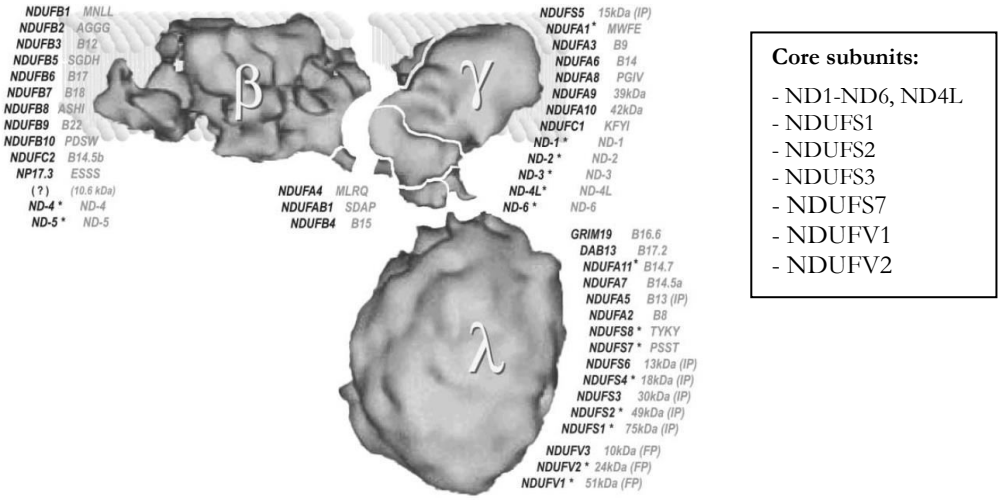



**Fig. 3.** Schematic representation of human RCC I subunits and their putative topology [Ugalde et al. 2004, p.2462]. Bovine homologues are written in gray and human subunits in which mutations have been identified are marked with asterisks.

So far, the assembly of the 45 subunits to RCC I mature holoenzyme is poorly understood. Imported nuclear encoded subunits must be assembled with the hydrophobic mitochondrial subunits. This process requires regulation and signalling between the mitochondrion and nucleus [Lazarou et al. 2009]. In recent years new insights into the biogenesis of RCC I were gained. The required assembly factors are genes necessary for proper RCC I function, but are not structural subunits. By disease gene mapping a number of new assembly factors, namely NDUFAF1, NDUFAF2, C3ORF38[2], C3ORF60, C6ORF66, C20ORF7 and Ecsit have been identified, but the relevance of them is not certainly clear [Ogilvie et al. 2005; Vogel et al. 2005; Vogel et al. 2007; Pagliarini et al. 2008; Saada et al. 2008; Sugiana et al. 2008; Saada et al. 2009]. A general consensus of the subunit assembly pathway can be drawn from the different patient cell line studies. The membrane embedded subunits, especially the ND1 subunit, anchors an early subassembly, including the NDUFS2, NDUFS3, NDUFS7 and NDUFS8 subunit, which is followed by the addition of subunits and subassemblies. NDUFAF1 in conjunction with Ecsit may act at an early assembly stage of membrane subcomplex [Dunning et al. 2007; Vogel et al. 2007]. Sugiana and colleagues suggested that C20ORF7 is required for RCC I assembly as well [Sugiana et al. 2008]. NDUFAF2 and perhaps C6ORF66 are involved in the assembly of the two membrane arms. Afterwards a subassembly, containing NDUFV1, NDUFV2, NDUFV3, NDUFS1, NDUFS4, NDUFS6 and other subunits, is added. The assembly process is completed by the addition of ND4 and ND5 subunits, which finalizes assembly of the membrane-embedded transporter molecule [Dunning et al. 2007; Lazarou et al. 2007]. The role of the recently identified C8ORF38 is unknown [Pagliarini et al. 2008]. Most of the models suggest a progressive way for assembly of RCC I, but additionally a

[2] Abbreviation means chromosome 3 open reading frame 28.

dynamic model exists, in which subunits or subassemblies may be exchanged by pre-existing ones [Lazarou et al. 2007].
The main entry point for electrons from the electron donators is RCC I. The complex receives two electrons from the oxidized NADH, which are passed to a non-covalently bound FMN and through a series of Fe-S clusters electrons transferred to CoQ. CoQ is thereby reduced to $CoQH_2$. In parallel four protons are transported from the mitochondrial matrix towards the intermembrane space between the inner and outer mitochondrial membrane as illustrated in **Fig. 2**, B) [Lazarou et al. 2009].
RCC I deficiencies (OMIM 252010) cause about one third of mitochondrial disorders and are the most frequently encountered OXPHOS deficiencies, which can be explained by the intricate enzyme complex with its numerous subunits, the twofold genetic background and the complicate assembly process [Kirby et al. 1999; Loeffen et al. 2000; Janssen et al. 2006b; Rodenburg et al. 2010]. RCC I deficiency in patients and the related phenotypes will be the main part of this work and therefore detailed information about previously known RCC I defect aspects in mitochondrial disease are mentioned in 1.3.

#### 1.1.1.2 Succinate:CoQ oxidoreductase – RCC II

The OXPHOS can be alternatively started by RCC II receiving electrons from $FADH_2$. RCC II enzyme complex is composed out of four nuclear encoded subunits – two soluble proteins, the flavoprotein (SdhA) and the Fe-S protein (SdhB), which are anchored to the inner mitochondrial membrane by the subunits SdhC and SdhD [Rustin et Rotig 2002]. It contains three different prosthetic groups, namely FAD, Fe-S cluster and cytochrome $b_{560}$. They transfer electrons from succinate to fumarate and finally pass them to CoQ which is reduced to $CoQH_2$. The electron transport is not linked to any proton pumping [Hatefi 1985].

#### 1.1.1.3 CoQH2:cytochrome c oxidoreductase – RCC III

RCC III catalyzes the electron transfer from reduced $CoQH_2$ to mitochondrial cytochrome c and thereby recycles CoQ which can function as electron acceptor again. RCC III is a dimer and each monomer is composed of ten nuclear-encoded subunits and the mtDNA encoded subunit mitochondrial cytochrome b (MTCYTB) [Fernandez-Vizarra et al. 2007]. Prosthetic groups, required for the electron transport, are cytochrome $b_{562}$, $b_{566}$, $c_1$ and two Fe-S clusters. For each transported electron, two protons are transferred from the matrix to the mitochondrial intermembrane space contributing to the formation of an electrochemical gradient [Hatefi 1985].

#### 1.1.1.4 Cytochrome c oxidase – RCC IV

RCC IV catalyzes the final step within the RC – the reduction of molecular oxygen ($O_2$) to water molecules ($H_2O$). The electron donator is cytochrome c. Cytochrome c contains two identical haemes, which are associated with an atom of copper. RCC IV accepts electrons from four molecules of cytochrome c and in parallel pumps four protons into the intermembrane space [Hatefi 1985]. In mammals, the RCC IV monomer is composed of 13 subunits, but the active form of the enzyme works as a dimer in vivo. Mitochondrial cytochrome c oxidase subunits 1 (MT-CO1), MT-CO2 and MT-CO3 are encoded by the mitochondrial genome and build the catalytic centre of the complex. The remaining ten

smaller subunits are nuclear encoded and imported into mitochondria [Grossman et Lomax 1997].

#### 1.1.1.5 ATP synthase – RCC V

The ATP synthase has a molecular weight of ~500 kDa and is build up of two major protein parts: i) a membrane bound $F_O$ fraction and ii) a water-soluble part $F_1$ with ATPase activity. The electrochemical proton gradient, which is created by RCC I, RCC III and RCC IV, is utilized by RCC V promoting the condensation of ADP and $P_i$ to ATP. The RCC V is like an engine composed out of two opposing rotary motors which are connected in series [von Ballmoos et al. 2009].

### 1.1.2 Characteristics of the mitochondrial genome

Mitochondria are unique cell organelles under the dual genetic control of both their own genome and the nDNA. Human mtDNA is a circular, double stranded molecule with 16.569 bp (see **Fig. 4**). It contains 37 genes: two ribosomal RNA (rRNA) genes, 22 transfer RNA (tRNA) genes and 13 messenger RNA (mRNA) genes which encode structural subunits of the RCC I, III, IV and V. Copy numbers of the mitochondrial genome vary in a tissue specific way. Genetics of the mitochondrial genome is different in almost every aspect from Mendelian genetics[3]. Three principles of mitochondrial genetics are important to know for understanding the pathogenic function of mutations in mtDNA and the consequences for patients and their families.

1) Inheritance of mtDNA
   At fertilization, all mitochondria derive from the mother because paternal mitochondria fail to enter the oocyte. The transmission of mtDNA is only through the maternal line to all her children, male and female, but only her daughter will transmit it to their progenies. Only one exception has been described with paternal inheritance of a mtDNA mutation, but this seems to be an extremely rare variant [DiMauro et Davidzon 2005; Mancuso et al. 2007].
2) Homoplasmy and heteroplasmy
   Each cell contains hundreds or thousand copies of mtDNA. The term 'homoplasmy' of a variant indicates that all copies of mitochondrial genome are identical at a specific position. In contrast a mixture of different mitochondrial genotypes is observed in cases of heteroplasmy. The vast majority of pathogenic mutations are present in only several numbers of copies (heteroplasmic mutations). The ratio of mutated and normal mtDNA copies is crucial for the clinical expression of the disease and the biochemical defect. A critical number of mutated mtDNA copies is required to cause mitochondrial dysfunction in a particular tissue or organ, the so-called 'threshold effect', which is unique for each tissue, mutation and genetic background. This explains observations that some tissues are affected when having a mtDNA mutation while others do not present signs of dysfunction harbouring the same mutation [DiMauro et Davidzon 2005; Taylor et Turnbull 2005].

[3] Mendelian genetics assign tenets about the inheritance of characteristics from parent organisms to their children (published by Gregor Mendel in 1866) and resume that half the genetic material of a fertilized zygote derives from each parent.

3) Mitotic segregation

The mtDNA copies of each cell are randomly distributed among the daughter cells at cell division. Therefore, the proportion of mutated mtDNAs in daughter cells can shift and according to this the clinical phenotype can change as well. This phenomenon explains how patients with mtDNA-related disorder can shift from one clinical phenotype to another symptom manifestation when they grow older [DiMauro et Davidzon 2005; Taylor et Turnbull 2005].

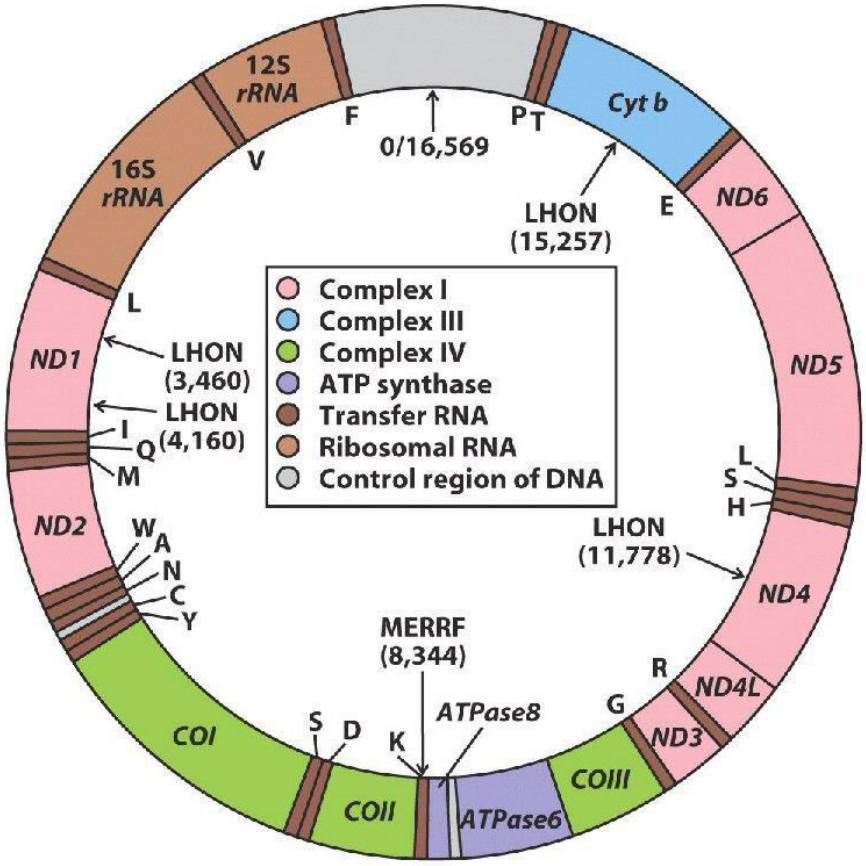



**Fig. 4.** Human mtDNA (http://chemistry.umeche.maine.edu/CHY431/Code4.html) with special mutation localizations for LHON (Leber hereditary optic neuropathy) and MERRF (myoclonic epilepsy with ragged red fibers).

## 1.2 Mitochondrial disorders

Strictly speaking, the term 'mitochondrial disorder' describes defects hampering proper function of the OXPHOS system. Defects of the energy production and other metabolic pathways have been associated with a plenty of human diseases [Munnich et al. 1996a; Schapira 1998; Shapira 2002a; Zeviani 2004; Bender et al. 2006]. With a minimum birth prevalence of one in 5000 live births, mitochondrial disorders can be regarded as a common cause of inborn errors of metabolism, assuming the likely underestimation of the diagnosis of disease [Skladal et al. 2003; Schaefer et al. 2004; Thorburn 2004]. In 1959, the first patient with mitochondrial disease was diagnosed [Ernster et al. 1959]. By elucidating the mitochondrial genome sequence in 1981, Anderson et al. formed the basis for genetic research in mitochondrial disorders [Anderson et al. 1981]. Since that time mitochondrial research underwent a comeback and numerous working groups focused on intensive investigations of the mitochondrial secrets. Up to now the number of mutations of subunits maintaining the RCC and assembly factors associated with different symptoms or syndromes, is rising yearly. Indeed, all cells with the exception of erythrocytes contain mitochondria and are dependent upon the energy providence in form of ATP generated by these cell organelles. Therefore, any symptom, in any organ or tissue, at any age and with any mode of inheritance, can be a hint for a mitochondrial disorder which is truly very unspecific [Munnich et al. 1996a]. The tissues with high energy demand are most vulnerable to mitochondrial defects, including the central nervous system (CNS), skeletal and heart muscle. Furthermore, normal function of endocrine organs, gastrointestinal tract (GIT) organs, liver and kidneys can be disturbed [Munnich et al. 1996a; Zeviani et al. 1996]. Typical symptoms and presentations seen in patients with mitochondrial disorders are assigned in 1.2.1.

Establishing a diagnosis for patients with mitochondrial disorders is often a challenge caused by the heterogeneous clinical picture of phenotypes. The knowledge about the clinical spectrum and the number of disease-causing molecular genetic defects are continuously expanding, but there are relatively poor genotype-phenotype correlations [Rodenburg et al. 2010]. A comprehensive diagnostic workout (see 1.2.2) is necessary and the evaluation of obtained results is performed in a multidisciplinary way including laboratory, morphological, biochemical and molecular-genetic findings in context with the clinical phenotype of patients and the family history [Zeviani 2004; Haas et al. 2007; Kirkman et al. 2008].

### 1.2.1 Clinical presentations

In patients with mitochondrial disease, any organ system can be affected when the complex construction of metabolic pathways for the energy generating processes and function of required mitochondrial enzymes is interrupted. Frequent symptoms observed within the different organ systems are illustrated by **Fig. 5**.

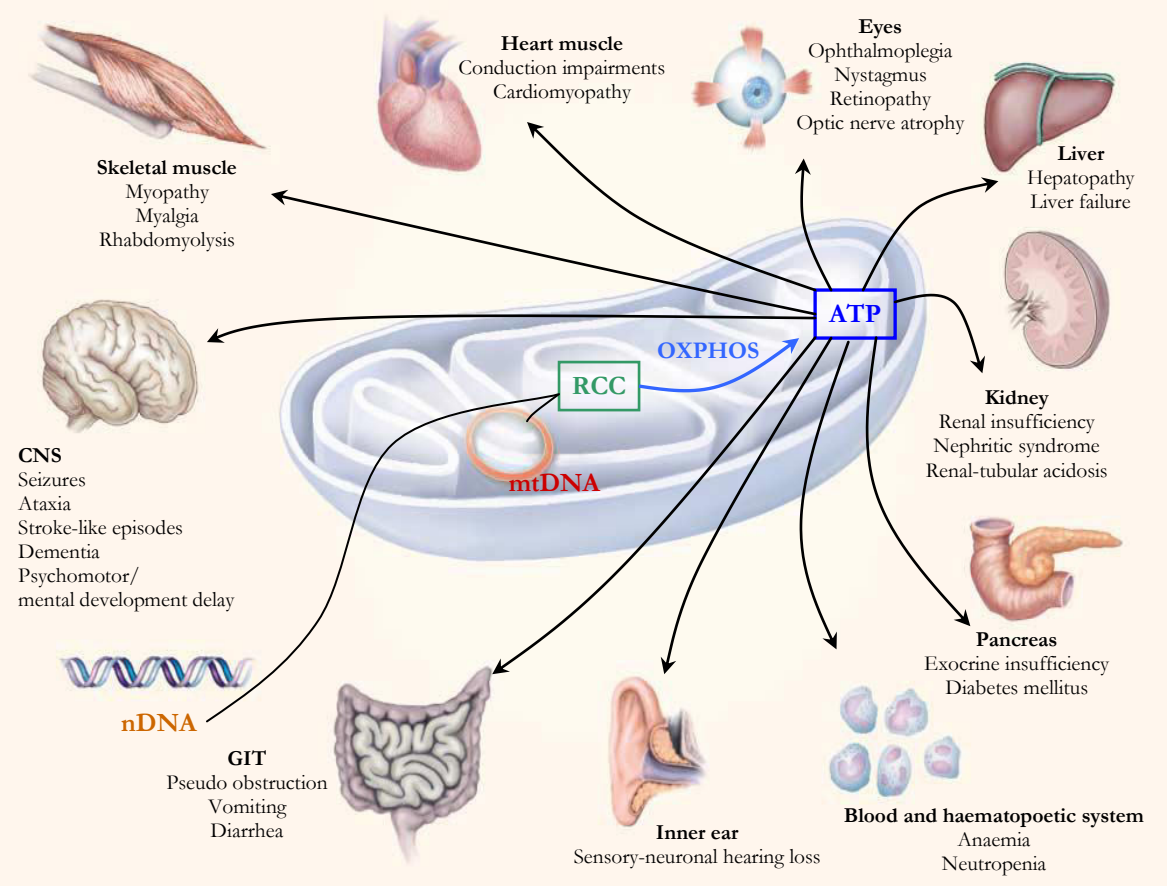



**Fig. 5.** Affected organ systems and symptoms observed in patients with mitochondrial disorders [modified from Johns 1995, p.639].

The numerous presentation forms demonstrate the wide variability of phenotypes in patients with mitochondrial disease. Despite the increasing understanding and recognition of mitochondrial disorders, common phenotypes in children remain difficult to define and they frequently present with a wide heterogeneous spectrum of symptoms. In contrast, adult patients often present with phenotypes suggestive for a 'mitochondrial syndrome' caused by mutations within the mitochondrial genome (**Appendix. 1**) [Mancuso et al. 2007; Koenig 2008].

The nervous system has been reported to be the most commonly affected organ system, followed by the skeletal muscle tract. Symptoms vary from epilepsy, stroke-like episodes, ataxia and dementia to generalized manifestation forms like muscular hypotonia, peripheral neuropathy, psychomotor and mental development delay or regression [Munnich et al. 1996a]. Patients may also suffer from respiratory difficulties that are caused by brainstem lesions affecting the respiratory regulation centre. Skeletal muscle manifestations observed are myopathy with myalgia, muscular weakness or exercise intolerance often leading to muscular atrophy as consequence of the defect. Rhabdomyolysis segregating with highly increased creatine kinase (CK) levels in blood has been reported in patients with severe muscle pain and weakness. Inadequate energy supply of heart muscle cells can result in either hypertrophic or dilative cardiomyopathy and patients may present with conduction impairments (e.g. Wolff-

Parkinson-White syndrome (WPW)[4]). Manifestations within the ophthalmological tract include symptoms like optic nerve atrophy, loss of vision, fields of vision loss, reduction in visual acuity, ophthalmoplegia, ptosis and nystagmus. Furthermore, cataract and retinal pigment degeneration (retinopathy) have been observed. Visceral involvement of the liver includes symptoms of hepatocellular dysfunction (elevated liver enzymes), chronic liver insufficiency or valproate-triggered hepatic failure for example. Reduced function of the renal system has been described to result in tubular acidosis, nephritic syndrome and renal insufficiency. Symptoms representing dysfunction of the GIT can be recurrent episodes of vomiting, diarrhoea or obstructions and exocrine pancreatic insufficiency with malabsorption resulting in failure to thrive. Signs of bone marrow suppression including panzytopenia (affecting all cell lines), hypodegenerative anaemia or single neutropenia have been observed, resulting in an immunological-deficient status that increases the affinity for all kind of infections (viral, bacterial, fungal or protozoan origin). Deterioration of the clinical status is often reported during infectious episodes, because of the increasing imbalance of extended energy demand of the body and the decreased energy provided by dysfunctional mitochondria. Less energy in neuronal transmitter cells, which regulate hormone secretion and synthesis, and endocrine organs, like the thyroid, pancreas, and adrenal gland, can result in decreased hormone levels and clinical present as hypothyroidism, diabetes mellitus, pubertas tarda and other manifestations. Moreover, so-called 'indicative soft signs' encompassing short stature (‹ 3. percentile), facial dysmorphism and malformations, hair and skin abnormalities like hypertrichosis, alopecia or mottled pigmentation, migraine-like headaches and others can be found in patients and their family members. A high incidence of mid- and late pregnancy loss is also a common occurrence that often goes unrecognized. [Munnich et al. 1996a; Zeviani et al. 1996; DiMauro et Davidzon 2005; Taylor et Turnbull 2005; Koenig 2008]

### 1.2.2 Diagnostic workout in mitochondrial disorders

As mentioned above, the challenge of diagnosing patients with mitochondrial disorders is mostly the result of the heterogeneous spectrum of clinical manifestations. Several guidelines and sets of diagnostic criteria have been developed over the last years [Rodenburg 2010]. However, no generally agreed guideline has been established and the diagnostic routine tests for patients suffering from mitochondrial disease are still controversial. First reported criteria, encompassing major and minor criteria, have been developed by Walker and are handled like 'guidelines' [Walker et Byrne 1996]. Bernier evaluated these criteria in the paediatric population in form of the 'modified Walker criteria' (**Table. 1**) [Bernier et al. 2002]. The establishment of a diagnosis for patients with clinical symptoms suggestive for a mitochondrial disease is a multidisciplinary approach involving a broad spectrum of laboratory tests, including metabolite analysis (1.2.2.1), enzymatic measurements (1.2.2.3) and molecular genetic analysis (1.2.2.4) [Freisinger et al. 2007; Haas et al. 2008]. There are different findings that point towards the evidence of a mitochondrial disease, but most parameters described in the following are not specific for this kind of disease and seen under other conditions as well, including other metabolic or neuronal diseases besides inadequate sample handling. Although, some parameters are frequently altered in patients with mitochondrial disease, they have been

[4] Syndrome of pre-excitation of the heart ventricles due to an accessory pathway, which is an abnormal electrical communication from the atria to the ventricles. WPW is a type of atrioventricular reentry tachycardia.

reported to be in normal ranges in some patients with a definitive diagnosis as well [Debray et al. 2007b]. Finally, the combination of the clinical presentation and results of performed investigations leads towards the diagnosis.

**Table. 1.** Criteria for diagnosing mitochondrial disorders in children [Skladal et al. 2003a]

| Presentation | Major criteria | Minor criteria |
|---|---|---|
| Clinical | Clinically complete RC encephalomyopathy or mitochondrial cytopathy | Symptoms compatible with a RC defect |
| Histology | >2% RRF in skeletal muscle | smaller number of RCC or widespread electron microscopic abnormalities of mitochondria |
| Enzymology | COX-negative fibers (>2% if <50 years or >5% if >50 years) or residual activity of RCC <20% in tissue, <30% cell line in two or more tissues | antibody-based demonstration of a RCC defect 20-30% in tissue, 30-40% in cell line or two or more tissues |
| Functional | fibroblast ATP synthesis rate >3 SD* below mean | fibroblast ATP synthesis rates 2-3 SD* below mean or fibroblasts unable to grow in galactose media |
| Molecular | nuclear or mtDNA mutation of undisputed pathogenicity | mutation of probable pathogenicity |
| Metabolic | - | one or more metabolic indicators of impaired RC function |

*standard derivation; RRF=ragged red fibers; COX= cytochrome c oxidase

#### 1.2.2.1 Analysis of metabolic parameters

Analysis of blood samples obtained in a minimal-invasive way can help categorizing the metabolic disorder and should be done subsequently to the evaluation of the medical history of patients and the physical examination. Parameters of the standard haemogram performed in the clinics (including cell counts, haemoglobin, electrolytes and base-excess) are not specific for mitochondrial diseases. The most recognized abnormality in patients with mitochondrial disease is an elevated lactate level in blood. But it can be elevated under other conditions, including inadequate sample handling or collection and further systematic disorders as well [Koenig 2008]. Increased lactate/pyruvate ratios point towards defects in mitochondrial metabolism [Rodenburg 2010]. Dysfunction of the RCC causes less ATP production and results in up-regulation of glycolysis as compensation for the insufficient energy supply. But resulting increased pyruvate cannot be passed through the PDH in order to be converted to energy-enriched acetyl-CoA, the primary substrate for the TCA cycle, because of the ATP deficit. Alternatively, pyruvate is reduced into lactate and elevated levels can be found in both blood and cerebral spinal fluid (CSF). A more sensitive indicator for mitochondrial diseases is the elevation of lactate levels in CSF. However, it can be normal in patients with mitochondrial defects or increased in CNS infection, stroke, malignancy, inflammation and seizures [Munnich et al. 1996a; Chow et al. 2005; Haas et al. 2008; Koenig 2008].

Elevated alanine levels in blood or CSF point towards increased pyruvate generation (via up-regulated glycolysis) resulting in the transformation of alanine when an amino acid is transferred on pyruvate. Urine amino acid analysis is typically performed when low serum bicarbonate is present [Haas et al. 2008]. Amino acid quantification can be performed on blood (plasma or serum), urine and CSF, although testing of urine is typically only helpful in diagnosing a mitochondrial disease-associated tubulopathy. Other amino acids whose elevations have been associated with mitochondrial dysfunction are proline and glycine. Increased levels of acyl-carnitines in blood plasma are suggestive for disrupted fatty acid oxidation. Increased CK levels caused by the damage of muscle cells are frequently seen in patients with myopathy. Organic acids are byproducts of protein, carbohydrate and fat catabolism and screening in urine is preferred. Increased excretion of TCA cycle

intermediates, ethylmalonic acid, and 3-methyl glutaconic acid commonly occurs in mitochondrial disease, but is rarely a diagnostic hint towards a specific mitochondrial disorder [Haas et al. 2008]. Another common finding on urine organic acid analysis in individuals with mitochondrial disease is dicarboxylic aciduria, arising as a result of microsomal fatty acid metabolism.

#### 1.2.2.2 Neuroimaging investigations

Cranial computer-tomography (CCT) and cranial magnetic resonance imaging (cMRI) are important modalities in the evaluation of anatomical structures in neurometabolic disorders. Mitochondrial disorders represent a class of diseases in which cMRI findings are relatively non-specific or change over time, greatly lowering the diagnostic sensitivity of cMRI. However, it is a valuable investigation for the follow-up of patients [van der Knaap et al. 1996; Valanne et al. 1998]. A special neuroimaging technique, proton magnetic resonance spectroscopy (MRS)[5], has evolved from which important metabolic information can be derived utilizing the same acquisition parameters needed for the cMRI within the same examination session. Proton MRS is the most commonly used spectroscopy technique for neurometabolic evaluations. Several compounds involved in mitochondrial physiology are detectable by MRS, based upon variations in their chemical properties within electrical fields, thus making MRS a useful adjunct in the evaluation of suspected mitochondrial disease [Lin et al. 2003; Bianchi et al. 2003; Dinopoulos et al. 2005].

#### 1.2.2.3 Invasive tissue biopsy

In patients with reasonable suspicion on the evidence of mitochondrial disease on the basis of clinical, metabolic and imaging findings previously described, invasive tissue biopsy of the clinically most affected organ system should be performed. Tissue biopsies offer samples for morphological, biochemical and molecular-genetic analysis and can clarify the suspected diagnosis [Koenig 2008]. Patients with suspected mitochondrial disease should be referred to experienced diagnostic centres to ensure optimal preservations of collected tissues [Rodenburg 2010]. In the majority of cases with mitochondrial disorders, skeletal muscle is affected and combined biopsies, including skin and muscle, can be taken in one session. Therefore, a muscle biopsy provides an optimal opportunity to detect possible aberrations in the functionality of mitochondria and its metabolic enzymes [Rodenburg 2010]. While this has made skeletal muscle the most widely used tissue for OXPHOS enzyme studies, it also has limitations. Patients with detectable defects in liver, renal or cardiac tissue may display normal enzymatic function in skeletal muscle tissue [Garcia-Cazorla et al. 2006]. In the case of an affected CNS morphological investigations are only possible post mortem to prove the suspected defect. Alternatively, muscle biopsies are frequently performed in these patients. It is also recommended to investigate enzyme activities in a second tissue, e.g. fibroblasts (cells cultured from skin biopsy) to minimize the possibility of overlooking an enzyme defect and to ensure the exclusion of accidental low enzyme activity results due to inadequate sample handling [Loeffen et al. 2000].

[5] MRS detects the ability of small molecules to emit radio waves based on the nuclear spin of protons, neutrons, and atomic nuclei.

*Functional investigations*

The biochemical investigation of the functional state of mitochondria is regarded as a cornerstone within the diagnostic procedure of patients with suspected mitochondrial disease [Rodenburg 2010]. Functional investigations of the tissues biopsied include measurements of enzyme activities of the OXPHOS system, consisting of RCC I-V and can be performed on tissue homogenate or mitochondria-enriched fractions. Measurements in fresh muscle samples should be preferred and performed within 2-3 hours following the collection. The investigations on fresh tissue offer the opportunity to evaluate the entire mitochondrial energy-generating system, including the analysis of oxygen consumption in the presence of various mitochondrial substrates, such as pyruvate, malate and glutamate, or to determine the ATP production rate in intact mitochondria [Rustin et al. 1994; Janssen et al. 2006a]. The different assays for measurements of the mitochondrial energy-generating system provide specific information about the mitochondrial functional state and are more sensitive to detect mitochondrial defects [Janssen et al. 2008]. Beside the opportunity to measure enzyme activities in freshly collected tissue frozen samples (snap-frozen immediately after collection) can be analysed, which is less sensitive [Janssen et al. 2003; Kirby et al. 2007]. Different procedures for the evaluation of functionality of the RCC are well-described [Rustin et al. 1994; Janssen et al. 2006a; Haas et al. 2008]. Polarographic and radiolabelled assays measure mitochondrial substrate oxidation in fresh tissue samples and give an overall estimate of RCC function. The measurements of enzyme activities by spectrophotometric methods can be performed in isolated mitochondria from tissues or cultured cells, in tissue homogenates or in whole cells and give valuable information about maximal enzyme activities of the catalytic component of the various RCC following either detergent- or freeze-thaw disruption of the inner mitochondrial membrane and are generally quite easy to reproduce and interpret within a given laboratory [Chretien et Rustin 2003; Thorburn et al. 2004; Mayr et al. 2004]. Activity measurements are reported in relation to a marker enzyme, such as citrate synthase (CS), or as internal ratios rather than relative to protein concentration [Haas et al. 2008]. The interpretation of results should be handled with care, since there is only a small margin between patient and control ranges for the OXPHOS enzyme activities [Rustin et al. 1994]. Furthermore, it has been shown that there are age-dependent differences [Sperl et al. 1992; Honzik et al. 2008].

*Histological investigations*

Histochemical, immune-histochemical and ultrastructural studies of biopsies are used to evaluate the evidence of a mitochondrial disorder or other differential diagnosis. Structural alterations of mitochondria in affected tissues can be seen in both light and electron microscopy, whereas the latter method is best to find ultrastructural changes in the mitochondrion itself. Increased number or size of mitochondria, subsarcolemmal accumulation of mitochondria in the cells which are stained with Gomori-trichrome and visualized as ragged red fibers (RRF) or succinate dehydrogenase (SDH) which evaluates RCC II, cytochrome-c oxidoreductase (COX)-negative fibers which is seen in RCC IV deficiencies, glycogen droplets or lipids, abnormal cristae or paracrystalline inclusions are findings described in patients suffering from mitochondrial disease [DiMauro et al. 2002; Bourgeois et al. 2004]. These findings can give rise to a mitochondrial disease. However, they are not

pathognomonic for a defect of the RCC and can also be observed under other conditions, including muscle dystrophies, neurogenic atrophies, inflammatory myopathies, chronic steroid use, aging or other metabolic disorders [Jackson et al. 1995; Lindal et al. 1992]. Mitochondrial diseases have also been described in patients, especially children, who showed no pathological alterations in mitochondrial structures, meaning that normal results of histological investigations do not exclude a defect [Patterson 2004; Rollins et al. 2001].

#### 1.2.2.4 Genetic diagnostics

The mitochondrial genome encompasses 37 genes coding for specific subunits of RCC I, III, IV and V (mRNA), rRNA and tRNA. The nDNA encodes numerous proteins involved in mitochondrial function, including subunits and assembly factors of the RCC and those involved in mitochondrial signalling, mtDNA replication and maintenance. Mutations in either mtDNA or nDNA can result in a RCC defect or can lead to global mitochondrial dysfunction [Koenig 2008]. In any case, a detailed evaluation of the family history is of high diagnostic value. The information derived may give a hint whether the disorder is inherited in a maternal, autosomal recessive, autosomal dominant or an X-linked trait. In case of a maternal inheritance, the diagnostic strategy will focus on the analysis of the mtDNA to identify pathogenic base substitutions, deletions, rearrangements or missense mutations [Bauer et al. 1999]. Several well-defined syndromes have been associated with specific mtDNA mutations (see **Appendix. 1**) and in most of these cases the underlying mutation can be diagnosed using extracted DNA from blood samples. Since the mutation load is tissue-specific the most affected tissue should be analysed for mtDNA mutations [DiMauro 2007].

In patients with 'unclassified' syndromes or a heterogeneous spectrum of symptoms associated with RCC dysfunction a mutation of the nDNA should be considered. The large number of possible disease-causing genes limits focused molecular-genetic diagnostics [Bernier et al. 2002; Taylor et al. 2004]. There are no clear genotype-phenotype correlations which may lead the molecular-genetic diagnostic way. In routine diagnostics the entire mtDNA is screened for mutations in addition to some nuclear genes that have been associated with RCC I deficiency, like NDUFS1, NDUFS2, NDUFS3, NDUFS4, NDUFS6, NDUFS8, NDUFV1, NDUFV2, NDUFA1 for patients with RCC I deficiency [Koenig 2008]. Establishing a molecular-genetic diagnosis is a special challenge in children with mitochondrial disease. They present less frequently with clinics suggesting a 'classical mitochondrial syndrome' and it has been reported that about 90% of them harbour mutations in the nuclear genome [Loeffen et al. 2001].

## 1.3 Genetics of RCC I deficiency

In 1988 the first mutations in mtDNA causing RCC I deficiency have been described [Holt et al. 1988; Wallace et al. 1988]. Over the last two decades many mutations and a related heterogeneous phenotype spectrum have been associated to patients with RCC I deficiency. Mutations in the mitochondrial genome often cause phenotypes with the picture of 'classical mitochondrial syndromes' (see **Appendix. 1**) mainly affecting adult patients. Mutations in nDNA are more frequently observed in children. Frequently observed symptoms of infantile patients with RCC I deficiency have been categorized into six main phenotype groups: Leigh syndrome, lethal infantile mitochondrial disease (LIMD) or fatal infantile lactic acidosis (FILA), cardiomyopathy, leukencephalopathy, pure myopathy and combined hepatopathy and tubulopathy. However, several presentations cannot be assigned to any of these groups and are therefore defined as mitochondrial encephalomyopathy with RCC I deficiency. An overlap of symptoms is also very common [Pitkanen et al. 1996; Kirby et al. 1999; Loeffen et al. 2000; Bugiani et al. 2004].

Pathogenic mutations in either mtDNA or nDNA can be detected in approximately 15-20% of paediatric patients with RCC I deficiency within molecular-genetic routine diagnostics [Loeffen et al. 2001; Thorburn et al. 2004]. In adult patients the detection rate is higher with approximately 40-50% [Loeffen et al. 2001; Scaglia et al. 2004].

### 1.3.1 MtDNA mutations associated with RCC I deficiency

More than 200 pathogenic point mutations, deletions, insertions and rearrangements within the mitochondrial genome have been described to date [Anderson et al. 1981; Koenig 2008]. In all seven mtDNA encoded subunits mutations have been identified to cause RCC I deficiency (references and phenotypes see **Table. 2**). The variety of clinical symptoms and signs that accompany mtDNA-related disorders is the result of the complexity of mitochondrial genetics (see 1.1.2) [DiMauro et al. 2002]. Specifically, the differences of mutational load, surpassing the pathogenic threshold in some tissues but not in others, contribute to the different severity of symptoms, the variable age of onset and the heterogeneous phenotypes observed [Mancuso et al. 2007]. However, some mitochondrial symptoms are defined and summarized as syndromes with the remark on possible variations and overlaps (**Appendix. 1**). These syndromes have been reported in patients with mtDNA mutations and are not particular for RCC I deficiency patients.

The onset of disease in case of mtDNA mutations is most often observed in late childhood or adulthood, but may occur in infancy as well [Bugiani et al. 2004; McFarland et al. 2004; Janssen et al. 2006b; Berger et al. 2008].

**Table. 2.** mtDNA genes encoding RCC I subunits and related phenotypes in case of disease (OMIM; http://www.ncbi.nlm.nih.gov/omim)

| Gene | OMIM | Disease | References |
|---|---|---|---|
| MT-ND1 | 516000 | LHON, MELAS | Valentino et al. 2004<br>Kirby et al. 2004a |
| MT-ND2 | 516001 | Leigh syndrome | Ugalde et al. 2007 |
| MT-ND3 | 516002 | Leigh syndrome, LIMD | Crimi et al. 2004<br>McFarland et al. 2004 |
| MT-ND4 | 516003 | LHON, Leigh syndrome | Hofhaus et al. 1993<br>Brown et al. 2000<br>Komaki et al. 2003 |
| MT-ND4L | 516004 | LHON | Brown et al. 2002 |
| MT-ND5 | 516005 | Leigh syndrome,<br>MELAS, LHON | Hofhaus et al. 1995<br>Kirby et al. 2003<br>Lebon et al. 2003<br>Bourges et al. 2004 |
| MT-ND6 | 516006 | Leigh syndrome,<br>LHON, MELAS | Kirby et al. 2000<br>Ugalde et al. 2003<br>Bugiani et al. 2004 |

Abbreviations: LHON=Leber's hereditary opticus atrophy; MELAS=mitochondrial encephalopathy with lactic acidosis and stroke like episodes; LIMD= lethal infantile mitochondrial disease.

### 1.3.2 Mutations in nuclear encoded subunits and assembly factors

The nuclear genome provides the main part of proteins required for proper function and assembly of RCC I. Furthermore, it encodes numerous proteins which are involved in the 'intergenomic communication'. These proteins have crucial roles in the cross-talk between the nucleus and mitochondria, and regulate the integrity and quantity of mtDNA [Hirano et Vu 2000]. The majority of patients with RCC I deficiency in infancy or early childhood do not harbour mtDNA mutations [Loeffen et al. 2001]. Therefore, it is suspected that main part of RCC I deficiency in children is the result of mutations in the nuclear genome. The clinical presentation of disease in patients with nDNA mutations includes a wide heterogeneous spectrum, whereas the tendency of a progressive neurodegenerative disease with fatal course and early death is often observed. First nuclear mutation associated with RCC I deficiency was described by Loeffen in a patient with Leigh syndrome who harboured a compound heterozygous mutation in NDUFS8 [Loeffen et al. 1998]. So far, pathogenic mutations in twelve nuclear subunits have been described to cause RCC I deficiency (see **Table. 3**). Beside the nuclear encoded structural subunits, some mutations in assembly factors have been associated with RCC I deficiency as well. The first assembly factor - NDUFAF1 - was identified in 2005 by Janssen and Vogel who found that knockdown of the assembly factor using RNA interference led to reduced levels of both enzymatic activity and fully assembled RCC I [Vogel et al. 2005]. The first patient harbouring a pathogenic mutation in this gene was described two years later [Dunning et al. 2007]. Currently, five additional assembly factors have been associated with incomplete RCC I assembly (**Table. 3**, below double line).

**Table. 3.** Nuclear encoded subunits and assembly factors and related phenotypes and references (OMIM; http://www.ncbi.nlm.nih.gov/omim)

| Gene | OMIM | Disease | References |
|---|---|---|---|
| NDUFA1 | 300078 | Leigh syndrome, progressive neurodegenerative disorder | Fernandez-Moreira et al. 2007<br>Potluri et al. 2009 |
| NDUFA2 | 602137 | Leigh syndrome | Hoefs et al. 2008 |
| NDUFA11 | 612638 | LIMD, encephalocardiomyopathy | Berger et al. 2008 |
| NDUFS1 | 157655 | Leigh syndrome, leukodystrophy | Bénit et al. 2001<br>Bugiani et al. 2004<br>Martin et al. 2005<br>Pagniez-Mammeri et al. 2009<br>Hoefs et al. 2010 |
| NDUFS2 | 602985 | Cardiomyopathy, encephalopathy | Loeffen et al. 2001<br>Tuppen et al. 2010 |
| NDUFS3 | 603846 | Leigh syndrome, unspecific encephalomyopathy | Bénit et al. 2004a<br>Pagniez-Mammeri et al. 2009 |
| NDUFS4 | 602694 | Leigh syndrome, cardiomyopathy | van den Heuvel et al. 1998<br>Budde et al. 2000<br>Petruzzella et al. 2001<br>Bénit et al. 2003b<br>Anderson et al. 2008<br>Leshinky-Silver et al. 2009 |
| NDUFS6 | 603848 | LIMD | Kirby et al. 2004b<br>Spiegel et al. 2009 |
| NDUFS7 | 601825 | Leigh syndrome | Triepels et al. 1999<br>Lebon et al. 2007a+b |
| NDUFS8 | 602141 | Leigh syndrome | Loeffen et al. 1998<br>Procaccio et Wallace 2004 |
| NDUFV1 | 161015 | Leigh syndrome, leukodystrophy | Schuelke et al. 1999<br>Bénit et al. 2001<br>Laugel et al. 2007<br>Breningstall et al. 2008<br>Zafeiriou et al. 2008 |
| NDUFV2 | 600532 | Cardioencephalomyopathy | Bénit et al. 2003a<br>Pagniez-Mammeri et al. 2009 |
| NDUFAF1 | 606934 | Cardioencephalomyopathy | Dunning et al. 2007 |
| NDUFAF2 | 609653 | Encephalomyopathy (acute episodes) | Ogilvie et al. 2005<br>Barghuti et al. 2008<br>Hoefs et al. 2009 |
| C6ORF66 | 611776 | Encephalomyopathy | Saada et al. 2008 |
| C8ORF38 | 612392 | Leigh syndrome | Pagliarini et al. 2008 |
| C20ORF7 | 612360 | LIMD | Sugiana et al. 2008<br>Gerards et al. 2009 |
| C3ORF60 | 612911 | LIMD | Saada et al. 2009 |

## 1.4 Mutation analysis in patients with RCC I deficiency

### 1.4.1 Mutation screen

The Institute of Human Genetics (TU Munich and the Helmholtz Zentrum München) has a focus on mitochondria and its relevance in human diseases. In routine molecular-genetic diagnostics, only the mtDNA encoded subunits of RCC I are currently analysed. Only a small number of paediatric patients with mitochondrial disorders receive a molecular-genetic diagnosis. Other genes known to play important roles in proper function and assembly of RCC I have not been screened. As part of an high-throughput DNA mutation screen by melting profile analysis, 150 patients with isolated RCC I deficiency have been analysed for sequence variants in all structural RCC I subunits, four assembly factors and the mitochondrial tRNA encoding genes. The sequence variants identified are listed in **Appendix 2** and **3**. A molecular diagnosis has been established in additional 18% (27/150) of the patients which passed through previously performed routine molecular-genetic diagnostics without the identification of a causative mutation. This leads to the conclusion that there must be additional components involved in the aetiology of RCC I deficiencies [Prokisch et al., unpublished].

### 1.4.2 New candidate genes for RCC I deficiency

Several approaches have been previously described with the aim of identifying new putative candidate genes which might be causative in RCC I deficiency. One approach was homozygosity mapping, whereby several new assembly factors have been identified during the last few years [Saada et al. 2008; Gerards et al. 2009; Saada et al. 2009]. In an alternative approach, bioinformatic tools have been used in order to identify new mitochondrial proteins. Pagliarini and colleagues used the combination of biomolecular, bioinformatic and phylogenetic approaches to identify specifically factors necessary for proper RCC I function [Pagliarini et al. 2008]. They performed mass spectrometry on isolated mitochondria to create a compendium of 1098 mitochondrial proteins. By virtue of shared evolutionary history they predicted 19 proteins to be important for proper RCC I function. Two of these genes have been confirmed to be involved in RCC I function. Based on conclusive evidence of the top four genes, namely C20ORF7, C7ORF10, C10ORF65 and C3ORF60 (NDUFAF3), these genes were considered as candidate genes. The function of these four genes is mainly unknown. A fifth putative gene, XPNPEP3, has been associated with RCC I deficiency in patients with nephronophthisis [O'Toole et al. 2010].

Together, these five candidate genes were analysed within the present work in the collected cohort of patients with RCC I deficiency using the established high-throughput mutation screen approach (1.4.1). Detailed information about the candidate genes is given in **Appendix. 4**.

## 1.5 Motivation and concept

With a minimum birth prevalence of about one in 5000 live births, mitochondrial disorders must be regarded as one of the most common group of inborn errors of metabolism. Although knowledge and understanding of pathogenesis has constantly improved during last years for the majority of patients with isolated RCC I deficiency, the disease causing molecular-genetic defect has not been identified. For patients with defects in RCC II-V, the detection rate of mutated genes is higher. With the aim to elucidate the molecular-genetic basis of patients with isolated RCC I deficiency, a comprehensive mutation screen has been previously performed (1.4.1).

One aim of the present work was to screen new candidate genes, namely XPNPEP3, C20ORF7, C7ORF10, C10ORF65 and C3ORF65 (NDUFAF3), for pathogenic mutations in 150 DNA samples of RCC I deficiency patients using the previously established method for high-throughput DNA melting profile analysis.

The collection and analysis of the clinical phenotypes of analysed patients with isolated RCC I deficiency was the second topic of the present work. Therefore, a standardized questionnaire was developed to collect the information of patients about family history, clinical symptoms, imaging, metabolic, histological and biochemical results in a retrospective manner. In addition to the clinical charts of patients, the correspondence with the referring clinicians and laboratories was analysed to improve the data collection. The cohort of patients is the largest study on patients with isolated RCC I deficiency. Purpose of the study is to give an overview of present signs and symptoms in this special entity of patients with mitochondrial disorders. Furthermore, it is an aim to search for genotype-phenotype correlations for patients with RCC I deficiency. Statistical analysis and manual comparisons from identified patients with nDNA mutations with previously reported patients will be performed. The establishment of genotype-phenotype correlations can provide an information basis for affected patients and their families on disease course, expected quality of life with the disease, therapy options and prenatal diagnostics. In addition, they can guide the molecular-genetic diagnostics by pin-pointing genes for sequence analysis.

# 2 Material and methods

The work with samples and information from patients with mitochondrial disease mentioned within here has been approved by the ethic commission of the TU Munich ('Fakultät für Medizin'; research project 'Aufklärung genetischer Ursachen und Mechanismen von mitochondrialen Erkrankungen', project number 2341/09).

## 2.1 Biomolecular methods

### 2.1.1 Reagents, equipment and software

| Reagents | Description | Company |
|---|---|---|
| Ultra pure $H_2O$ | Milli-Q ultra pure $H_2O$ purification system | Millipore, Germany |
| Whole-genome DNA amplification kit | illustra GenomiPhi V2 DNA Amplification Kit | GE Healthcare, UK, |
| High Performance Buffer | High Performance Buffer PCR buffer 10% | Thermo Scientific, Germany |
| dNTPs set (dATP, dGTP, dTTP, dCTP) | conc. 2 mM (10 µl of each diluted in 460 µl $H_2O$) | Fermentas Life Science, St.Leon-Rot, Germany |
| Magnesium Chloride Solution | Thermo Scientific conc. 25 mM | Abgene, Germany |
| 5× Q-solution | Q-Solution, 5× conc. | Qiagen, Germany |
| LCGreen I Dye | LCGreen I Dye 10x (in TE buffer) | Idaho Technology Inc., Salt Lake City, USA |
| Thermo-Start Hotstart DNA Polymerase | Thermo Scientific Taq-Polymerase conc. 5 U/µl | Abgene, Germany |
| DNA polymerase | DNA polymerase, conc. 5 U/µl | Qiagen, Germany |
| PCR buffer | PCR buffer 10x (containing 15 mM Magnesium Chloride) | Qiagen, Germany |
| Primer, forward and reverse | primer, conc. 20 ng/µl | Metabion, Germany |
| PCR test DNA | Test DNA from a colleague conc. 10 ng/µl | From a colleague |
| TE buffer (pH 7.5) | Tris(hydroxymethyl)aminomethane conc. 10 mM<br>EDTA (ethylenediaminetetraacetic acid) conc. 1 mM | Merck, Germany<br>Roth, Germany |
| 15% Ficoll solution | 1.5 g Ficoll™ PM 400 in 10 ml TE buffer (pH 7.4) | Amersham Biosciences, UK |
| Orange G (Sodium Salt) | Orange G (Sodium Salt) | Sigma, USA |
| Agarose | DNA Agarose for gel electrophoresis | Biozym, Germany |
| 1x TBE buffer | 102 g Tris(hydroxymethyl)aminomethane conc. 840 mM<br>55 g EDTA conc. 20 mM<br>7.5 g Boric acid conc. 900 mM<br>filled up to 2 l ultra pure $H_2O$ | Merck, Germany<br>Roth, Germany<br>Roth, Germany<br>Millipore, Germany |
| Ethidium bromide | Ethidium bromide 1% conc. 10 mg/ml | Roth, Germany |
| DNA ladder GeneRuler | DNA ladder GeneRuler, 1 kpb conc. 0.5 µg/µl | Fermentas Life Science, Germany |
| Oil | Sigma mineral oil | Sigma-Aldrich, Germany |
| BigDye® Terminator v3.1 cycle Sequencing Kit | Sequencing Standard Kit v3.1 encompass ready reaction mix and 5x sequencing buffer | Applied Biosystems, Germany |
| HPLC $H_2O$ LiChrosolv® | HPLC (high performance liquid chromatography) $H_2O$ | Merck, Germany |
| Ethanol | Ethanol 100% | Merck, Germany |

| Equipment/software | Description | Company |
|---|---|---|
| Pipettes | Gilson pipetman one channel (2, 20, 200, 1000 µl) | Gilson, Middleton, USA |
| | Gilson pipetman ultra 12 multi-channel (1-20, 20-300 µl) | |
| | Brand 8 multi-channel pipette (0.5-10, 10-20 µl) | Brand, Germany |
| | Rainin 12 multi-channel for sequencing (1-10, 20-200 µl) | Mettler-Toledo GmbH, Germany |
| Pipetting robotics | Freedom EVO pipetting robotics | Tecan Trading AG, Männerdorf, Switzerland |
| Spectrophotometer | ND-1000 Spectrophotometer NanoDrop | Thermo Scientific Abgene, Germany |
| Software spectrophotometer | NanoDrop ND-1000 Version 3.5.2 | Thermo Scientific Abgene, Germany |
| LightScanner Primer Design software | LightScanner Primer Design software | Idaho Technology, Texas, USA |
| Primer design software | ExonPrimer | T.Strom, Helmholtz Zentrum München, Germany |
| Tape pads | Qiagen Tape Pads for PCR plates 1018104 (AB-0558) | Thermo Scientific Abgene, Germany |
| PCR plates 96-well | PCR Plates Thermofast® non skirted (AB-0600) | Thermo Scientific Abgene, Germany |
| PCR plates 384-well | PCR-Plates Thermofast® White 384-well (TF-0384/w) | Thermo Scientific Abgene, Germany |
| PCR plates 96-well for purification | NucleoFast® 96 PCR Plates (Cat. No. 743100.10) | Macherey-Nagel, Germany |
| PCR plates 96-well for sequencer bar-coded | Thermo-Fast® 96 Detection Plate bar-coded | Abgene Applied Biosystems, Germany |
| Genetic Analyser Plate Septa 96-Well ABI Prism | Genetic Analyser Plate Septa 96-Well ABI Prism | Abgene Applied Biosystems, Germany |
| DNA thermal cycler | MJ Research PTC-225 Thermal Cycler (96er system, tube system, 384er system) | GMI, Minnesota, USA |
| Centrifuge and vortexer | FVL-2400 CombiSpin | PeqLab, Foreham, UK |
| Centrifuge | Centrifuge Sigma 4K15 | Sigma-Aldrich, Germany |
| Microwave | Microwave | Siemens, Germany |
| Power supply unit | Power-Pac® | Bio-Rad Lab Equipment, California, USA |
| Gel electrophoresis chamber | Sub cell GT system for horizontal gel electrophoresis | Bio-Rad Lab Equipment, California, USA |
| Combs and gel retainer | combs and gel retainer different sizes | |
| UV-light system | UVT-40M; UV-light | Herolab, Germany |
| Camera | E.A.S.Y. 440K camera | |
| Vacuum filtration system | Millipore Manifold 384 vacuum filtration system | Millipore, Germany |
| Sequencer 48-capillary system | ABI 3730 DNA Analyser | Applied Biosystems/Hitachi, USA |
| Software for sequence analysis | GAP-Assembler Version 4.11 | http://staden.sourceforge.net/staden_home.html |
| Software statistical analysis of data from patients | SPSS16.0 version | SPSS Inc, Chicago, USA |

### 2.1.2 DNA sample preparation

#### 2.1.2.1 Determination of DNA quality and concentration

For the quantification of DNA samples a spectrophotometer was used. The DNA sample absorbance was measured at 230 nm, 260 nm and 280 nm using a cuvette with 1 µl of DNA. The ratio of absorbance at 260 nm and 280 nm was used to assess the purity of DNA, whereas a ratio of ~ 1.8 was accepted as 'pure'.

#### 2.1.2.2 Whole-genome DNA amplification

In general a small amount of isolated DNA from muscle or blood was sent to the Institute of Human Genetics (IHG) from the patients' responsible doctors. But with regard to high-throughput mutation screening and the project of analyzing a multitude of RCC I encoding genes, these small amounts were insufficient. For this reason isolated DNA samples from patients were amplified using the GenomiPhi V2 DNA Amplification Kit protocol. One micro litre of template DNA (10 ng) was mixed up with 9 µl of sample buffer and heated up to 95°C for three minutes. After cooling on ice to 4°C, 10 ml of premixed enzyme (Phi29 DNA polymerase) and buffer (1:9) was added to the samples and incubated at 30°C for two hours with a post-amplification step for enzyme inactivation at 65°C for ten minutes. Subsequent to cooling to 4°C 60 µl of TE buffer (Tris 10 nM and EDTA 1 mM, pH 7.5) were added. The yield after amplification was estimated to 5 µg DNA in 80 µl reaction mix and subsequently diluted to 50 ng/µl to ensure proper DNA conservation. To analyse the quality of amplification, a test polymerase chain reaction (PCR) with 1 ng DNA/50 µl was performed (see 2.1.4).

#### 2.1.2.3 Dilution of amplified DNA and preparation of 384-well screening plates

The amplified stock-DNA with a concentration of 50 ng/µl was diluted 1:10 and 1:5 to prepare 96-deep well plates with 800 µl of 1 ng/µl. These plates were the basis for the preparation of 384-well screening plates using a standardized program of the Freedom EVO® pipetting robotics. Cleaning program with 0.12% hypochlorite, 96% ethanol was started before. For the mutation screening 5 ng of each DNA sample was loaded twice on the 384-well plates. DNA on prepared plates dried up and the plates were stored at room temperature without loss of DNA quality for approximately one year.

### 2.1.3 Primer design

Primers[6] for DNA amplification were designed with standard settings according to the reference sequences of human genome 18 (hg18) with the LightScanner Primer Design software or the ExonPrimer program and ordered from Metabion, Germany. See **Appendix. 5** for forward and reverse sequences, PCR conditions and amplified product sizes.

[6] A primer is a strand of nucleic acid that serves as a starting point for DNA synthesis, because the DNA polymerases that catalyze replication can only add new nucleotides to an existing strand of DNA.

### 2.1.4 Polymerase chain reaction

#### 2.1.4.1 Principle of DNA amplification

The PCR is a common method in molecular biology research and diagnostic procedures for DNA amplification. Using a heat-stable polymerase, e.g. the taq polymerase which is an enzyme originally isolated from the bacterium thermos aquaticus, DNA pieces can be amplified with an in vitro enzymatic replication strategy. The initialization step (heating the reaction up to 95°C) is required for DNA polymerases activation. The heating to 94-98°C causes DNA melting of the DNA template by disrupting the hydrogen bonds between complementary bases, yielding single-stranded DNA molecules (denaturation step). The reaction temperature is lowered to 50-65°C to allow annealing of the primers to the single-stranded DNA templates. Annealing temperatures ($T_A$) are primer specific. The DNA polymerase binds to the primer-template hybrid and begins DNA synthesis (extension or elongation step). Commonly a temperature of 72°C is used for the taq polymerase which synthesizes a new DNA strand complementary to the DNA template strand by adding dNTPs (deoxy nucleotide triphosphates) that are complementary to the template in 5' to 3' direction, condensing the 5'-phosphate group of the dNTPs with the 3'-hydroxyl group (phosphodiester bonds) at the end of the extending DNA strand. The extension time depends both on the DNA polymerase used and on the length of the DNA fragment to be amplified. The DNA polymerase polymerizes a thousand bases per minute at its optimum temperature. Under optimum conditions DNA is amplified exponentially. The following single step is performed to ensure that any remaining single-stranded DNA is fully extended (final elongation). Typically, PCR consists of a series of 20-40 cycles as described above.

#### 2.1.4.2 Optimization of PCR settings

Ordered primers were tested according to the standard reaction mix and protocol with 63°C, 65°C and 68°C annealing temperatures (see **Table. 4** and **Table. 5**). In 80% the PCR was successful under these conditions by the first time. For the remaining cases different settings regarding the reaction mix (**Table. 4**) and PCR protocols (TouchDown protocol **Table. 6**) were tested. A PCR temperature gradient of 10°C for detecting optimal $T_A$ (58-68°C) was tested alternatively. The addition of dye was necessary for the following mutation screening by melting curve analysis and was therefore part of the standard reaction mix. The dye stabilizes double-stranded DNA molecules and optimal $T_A$ for the primers is increased 1-3°C. For some primers the PCR was only successful without dye and the LCGreen I Dye was added subsequent to DNA amplification (see **Table. 9**). Q-solution simplifies the amplification step of difficult templates by modifying the DNA melting behaviour and optimizes PCR for DNA with high G/C-content[7] (Qiagen® 2004: QIAGEN® Multiplex PCR Handbook). Primer tests with a different taq DNA polymerase from Qiagen and according buffers were performed when standard settings failed. Furthermore, magnesium and primer concentration titrations, beside the variation of PCR cycle numbers, were tested to find optimal PCR settings.
Detailed information about finally used PCR settings is listed in **Appendix. 5**. Primers were newly designed and ordered when these different settings failed to get a PCR product.

[7] =guanosine/cytosine content is the percentage of nitrogenous bases on a DNA molecule. The GC pair is bound by three hydrogen bonds and more stable than DNA with low G/C-content.

**Table. 4.** Different reaction mixes for primer test PCR

| Reagents | standard | without Dye | Q-solution | Q-solution without Dye | Qiagen |
|---|---|---|---|---|---|
| Ultra pure $H_2O$ | 11.5 µl | 12.5 µl | 7.5 µl | 8.5 µl | 10.72 µl |
| High Performance Buffer 10% | 2 µl | 2 µl | 2 µl | 2 µl | - |
| 10x PCR buffer Qiagen | - | - | - | - | 2 µl |
| dNTPs conc. 2 mM | 2 µl | 2 µl | 2 µl | 2 µl | 2 µl |
| Magnesium Chloride Solution conc. 25 mM | 2 µl | 2 µl | 2 µl | 2 µl | - |
| LCGreen I Dye 10x | 1 µl | - | 1 µl | - | - |
| 5x Q-solution | - | - | 4 µl | 4 µl | 4 µl |
| Thermo Start DNA Polymerase conc. 5 U/µl | 0.2 µl | 0.2 µl | 0.2 µl | 0.2 µl | - |
| DNA polymerase conc. 5 U/µl Qiagen | - | - | - | - | 0.08 µl |
| *Primer, forward and reverse conc. 20 ng/µl | 0.8 µl | 0.8 µl | 0.8 µl | 0.8 µl | 0.8 µl |
| Test DNA (50 ng/µl) | 0.5 µl | 0.5 µl | 0.5 µl | 0.5 µl | 0.5 µl |
| **Total reaction mix volume** | **20 µl** | **20 µl** | **20 µl** | **20 µl** | **20 µl** |

*forward and reverse primers were diluted in ultra pure $H_2O$ (8:1:1) and vortexed before

**Table. 5**. PCR standard protocol

| Step | Temperature | Time | |
|---|---|---|---|
| Initialization | 95°C | 15 min | 40 times |
| Denaturation | 94°C | 30 s | |
| Annealing | X°C (different temperatures tested: 63°C, 65°C, 68°C) | 30 s | |
| Extension | 72°C | 1 min | |
| Denaturation | 94°C | 30 s | |
| Cooling | 25°C | 30 s | |

**Table. 6**. PCR TouchDown protocol

| Step | Temperature | Time | |
|---|---|---|---|
| Initialization | 95°C | 15 min | |
| Denaturation | 95°C | 30s | 24 times |
| Annealing | 70°C<br>-0.5°C per cycle | 30s | |
| Elongation | 72°C | 30 s | |
| Denaturation | 95°C | 30s | 24 times |
| Annealing | 54°C | 30s | |
| Elongation | 72°C | 60s | |
| Final elongation | 72°C | 10 min | |
| Cooling | 25°C | 30s | |

### 2.1.5 Agarose gel electrophoresis

#### 2.1.5.1 Principle

The agarose gel electrophoresis is a method for separating linear DNA fragments to determine fragment sizes of the amplified PCR products. This is achieved by moving negatively charged nucleic acid molecules through an agarose matrix with an electric field (electrophoresis), where the DNA fragments move from the negative charged cathode to the positive anode. Shorter molecules move faster and migrate further than longer ones. In comparison to a standard 1 kb DNA ladder product sizes can be evaluated (DNA ladder bands 1000, 750, 500, 250 bp).

#### 2.1.5.2 Procedure of agarose gel preparation

For detecting the DNA bands under UV-light ethidium bromide, which fluoresces under UV-light when intercalated into DNA, was added to the agarose gel (**Table. 7**). The agarose was boiled up with 400 ml 1x TBE buffer in the microwave. After cooling to 70°C, 8 µl of ethidium bromide were added to the agarose mixture. The gel retainer with the according comb was filled up with the agarose gel mix to approximately 0.5 cm of height.

**Table. 7.** Applied reagents for agarose gel electrophoresis

| Reagents | Volume/amount |
|---|---|
| 15% Ficoll™ PM 400 | 1.5 g to 19 ml TE buffer |
| Orange G (Sodium Salt) | One spatula point |
| DNA Agarose for gel electrophoresis | 6 g |
| 1x TBE buffer | 400 ml |
| Ethidium bromide 1% (10 mg/ml) | 10 µl |
| DNA ladder GeneRuler, 1 kb | 5 µl loaded on gel (diluted 1:1 in $H_2O$) |

Agarose gel mix

The solid gel was embedded in the electrophoresis chamber and covered with 1x TBE buffer. Pre-mixtured PCR products with 15% Ficoll dilution (5 µl of each) were loaded into the gel pockets flanked by a 1 kb DNA ladder on each side. After an average running time of 30 minutes with 130 V and 400 mA, the gel was photographed under UV-light. The predicted product size was compared with the observed size of fragments in the agarose gel (**Fig. 6**).

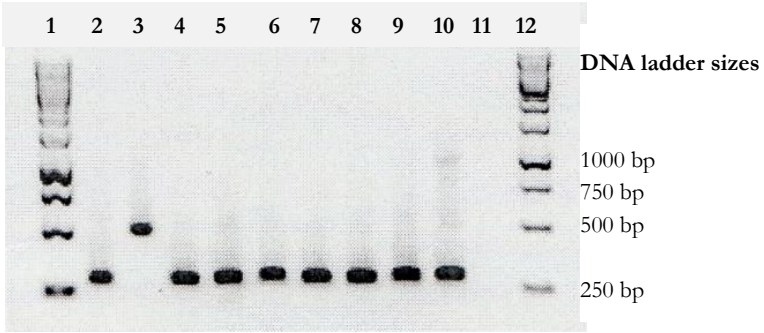



**Fig. 6.** Quality of PCR products. Photographed agarose gel after electrophoresis for PCR products of amplified exon 2 (product size 561 bp, lane 3) and exon 10_11 of C7ORF10 (273 bp, lane 2, 4-9) demonstrate that predicted sizes and observed sizes fit well. Lane 10: positive control using test-DNA; Lane 11: negative control (all reagents without test DNA)

### 2.1.6 DNA melting profile analysis with the Idaho LightScanner

#### 2.1.6.1 Idaho LightScanner - principle

**Fig. 7.** LightScanner system

The Idaho LightScanner is a technology to detect DNA variants by high-resolution analysis of melting curves (**Fig. 7**). The Idaho Technology LightScanner system is suitable for high-throughput mutation analysis, because 384 samples can be analysed in parallel. Techniques based on fluorescence measurements are now the most common approach for DNA melting curve analysis. The temperature-dependent dissociation between two DNA-strands can be measured using a DNA-intercalating fluorophore (LCGreen dye) which detects places of heteroduplexes. Within the last hybridization step of the PCR heterozygous variants in the sequence of strands causes the formation of heteroduplexes displaying a different melting profile, because single strand separation occurs earlier than in matched homoduplexes (**Fig. 8**).

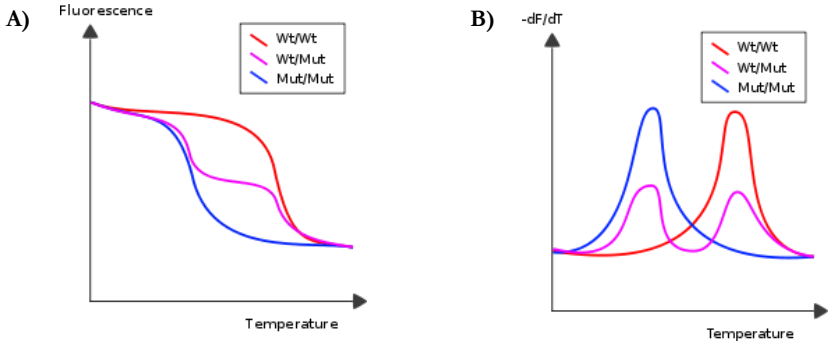



**Fig. 8. A)** Relation between fluorescence and temperature for labeled probe designed for homozygous wild-type (Wt), heterozygous Wt and mutant (Mut) and homozygous Mut situations.
**B)** The graph of the negative first derivative of the melting-curve may make it easier to pin-point the temperature of dissociation (defined as 50% dissociation), by virtue of the peaks thus formed.

The sensitivity of mutation detection is lower in cases of homozygous variants, because they match completely and display more comparable melting profiles to wild-type samples than mismatched heteroduplexes caused by heterozygous mutations. (www.idahotech.com)
Control experiments showed a sensitivity of >90% for the detection of known heterozygous mutations. For homozygous mutations the sensitivity is lower.

#### 2.1.6.2 Protocol for Idaho LightScanner mutation screen

To screen patients' DNA samples for mutations each exon[8] of a gene was amplified. Using the 384-well plates 192 DNA samples were screened in parallel. The following reaction batches (**Table. 8**) were used with the PCR protocols described above (**Table. 5** and **Table. 6**). To prepared 384-well plates (each well with 5 ng of amplified DNA) 5 µl of the master mix were pipetted per well. A tape pad was fixed on top of the plates to avoid condensation during the PCR process and plates were centrifuged before the PCR. Subsequent to the PCR 8 µl mineral oil were added per well before the automatically analysis by the LightScanner was started.

[8] An exon encompasses a nucleic acid sequence that is represented in the mature form of an RNA molecule after introns (portions of precursor DNA) have been removed by splicing.

**Table. 8.** Master mix for PCR of DNA samples using a 384-well plate

| Reagents | standard | standard without Dye | Q-solution | Q-solution without Dye | Qiagen |
|---|---|---|---|---|---|
| Ultra pure $H_2O$ | 1470 µl | 1592.3 µl | 490 µl | 612.5 µl | 1313.2 µl |
| High Performance Buffer 10% | 245 µl | 245 µl | 245 µl | 245 µl | - |
| 10x buffer (Qiagen) | - | - | - | - | 245 µl |
| dNTPs conc. 2 mM | 245 µl | 245 µl | 245 µl | 245 µl | 245 µl |
| Magnesium Chloride Solution conc. 25 mM | 245 µl | 245 µl | 245 µl | 245 µl | - |
| 5x Q-solution | - | - | 980 µl | 980 µl | 490 µl |
| LCGreen I Dye 10x | 12.5 µl | - | 122.5 µl | - | - |
| Thermo Start DNA Polymerase conc. 5 U/µl | 24.5 µl | 24.5 µl | 24.5 µl | 24.5 µl | - |
| DNA polymerase, conc. 5 U/µl | - | - | - | - | 9.8 µl |
| Primer, forward and reverse (8:1:1) conc. 20 ng/µl | 98 µl | 98 µl | 98 µl | 98 µl | 98 µl |
| Patient DNA (5 ng/µl), 2 samples on each plate | | | | | |
| **Total** | **2450 µl** | **2450 µl** | **2450 µl** | **2450 µl** | **2450 µl** |

In protocols where the initial PCR was performed without the fluorescent dye it was added subsequent to DNA amplification (2 µl per well from mixture of 245 µl LCGreen I Dye and 735 µl ultra pure $H_2O$) and labelled with the amplified PCR products within the following PCR cycler programme (**Table. 9**).

**Table. 9.** PCR cycler protocol for belated binding of LCGreen I Dye

| Step | Temperature | Time |
|---|---|---|
| Denaturation | 95°C | 20 s |
| Annealing | 55°C | 30 s, repeat 6 times |
| Denaturation | 94°C | 30 s |
| Cooling | 10°C | 1 min |

#### 2.1.6.3 Analysis of the melting profiles

Amplicons were slowly heated from 77°C to 96°C (complete denaturation) while the fluorescence was monitored by the Idaho LightScanner. Melting curves were analysed by the LightScanner software with normalized, temperature-shifted curves displayed as difference plots (-dF/dT). The normalization was achieved by the definition of three points, where all curves had to pass through (see **Fig. 9**, 1-3). A normal variance of ±0.05 for Δfluorescence was defined and when the peak of curve was >0.1 a variant in sequence was assumed to cause the altered melting curve (lower right picture). Samples with altered melting curves compared with the average of multiple wild-types were directly sequenced using the BigDye Cycle sequencing kit as described in 2.1.7. [Meisinger et al. 2009]

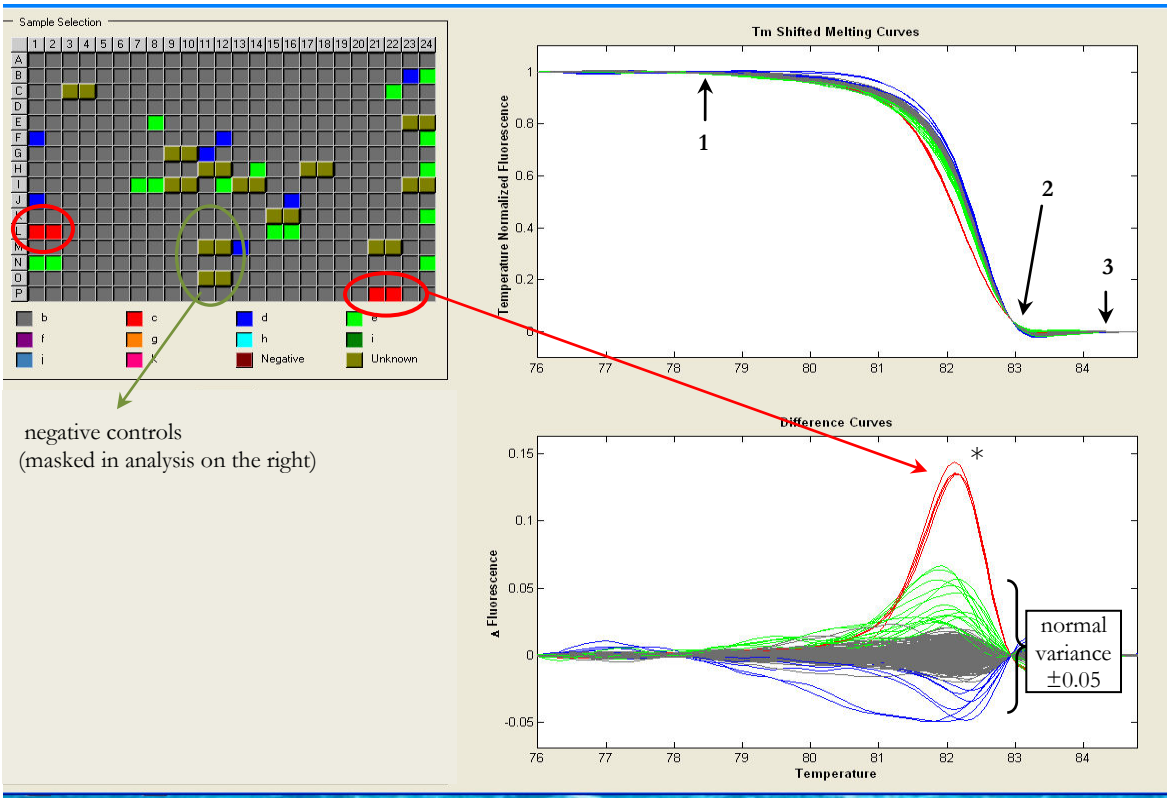



**Fig. 9.** Example of DNA melting curve profile analysis for amplified exon 6 of C20ORF7 (screen-shot). *****Red curves displayed conspicuous profiles and samples of the two patients (each patient two-time presentation per plate next to each other, see red boxes by 'sample selection' L1,2 and P21,22) have been identified to harbor a single-nucleotide polymorphism (SNP) at coding position 486 not resulting in an exchange of bases. Control sequencing for blue and green samples did not reveal any changes in sequences.

### 2.1.7 Genome DNA sequencing of candidate genes

#### 2.1.7.1 The principle of DNA sequencing

The term 'DNA sequencing' refers to methods that determine the order of the nucleotide bases (adenine, guanine, cytosine and thymine) in a molecule of DNA.

The chain-termination method developed by Sanger and co-workers in 1975 soon became the method of choice, owing to its relative ease and reliability. The key principle is the use of dideoxynucleotide triphosphates (ddNTPs, including ddATP, ddGTP, ddCTP, or ddTTP) as DNA chain terminators. The ddNTPs lack a 3'-OH group, which is normally required for the formation of a phosphodiester bond between two nucleotides, thus terminating DNA strand extension. The resulting DNA fragments have various lengths. It requires a single-stranded DNA template, a DNA polymerase, DNA primers (forward and reverse), dNTPs and ddNTPs. The ddNTPs are labelled with a base specific fluorescent dye (ddATP=green, ddGTP=black, ddTTP=red, ddCTP=blue). This is the basis for automatically analysis by the sequencer which can evaluate the four different bases due to their different spectral characteristics. The newly synthesized and labelled DNA fragments are heat-denatured and separated by size using automatically gel electrophoresis (ABI3730 sequencer). The fluorescently labelled DNA fragments move through the path of a laser beam that causes the dyes on the fragments to fluoresce (**Fig. 10**). Subsequently the data collection software

converts the fluorescence signal to digital data. Each dye emits light at a different wave-length when excited by the laser and therefore the four bases (labelled with different colours) can be detected and distinguished in one capillary injection.

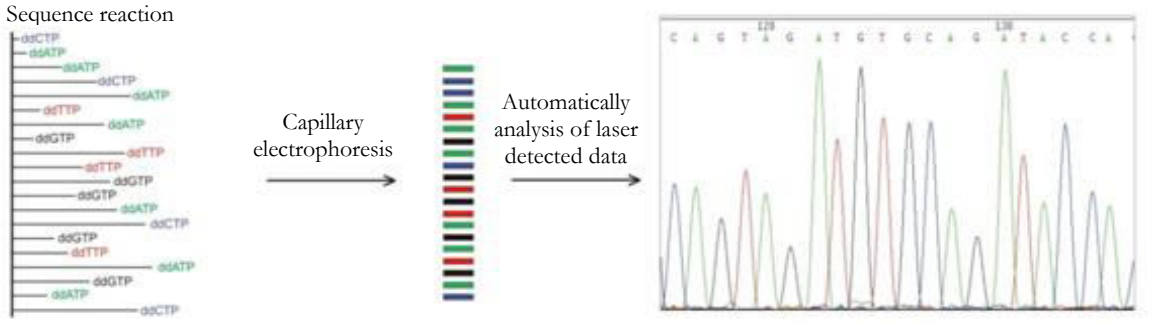



**Fig. 10.** Scheme of sequencing steps. During sequence reaction a DNA polymerase synthesizes DNA fragments with varying sizes by assembly of fluorescent labelled ddNTPs. Separation of fragments by capillary gel electrophoresis and the translation of detected fluorescence data into base sequences are performed automatically by the ABI3730 sequencer.

Limitations include non-specific binding of the primer to the DNA, affecting accurate read-out of the DNA sequences, and DNA secondary structures affecting the fidelity of the sequence. Further limitations include dye effects due to differences in the incorporation of the dye-labelled chain terminators into the DNA fragment, resulting in unequal peak heights and shapes in the electronic DNA sequence trace. This problem has been addressed with the use of modified DNA polymerase enzyme systems and dyes that minimize incorporation variability in addition to methods for the elimination of 'dye blobs'[9]. With the assistance of several software packages, which can trim low-quality DNA traces automatically, low-quality base sequences are removed. The accuracy of such algorithms is below visual examination by a human operator, but sufficient for automated processing of large sequence data sets.

#### 2.1.7.2 High quality purification of the PCR product

The clean-up of the amplified PCR product is a prerequisite for sequencing analysis. Ultra filtration technology is an efficient method to remove undesired components like detergents, primers or additives from PCR reactions. The NucleoFast 96 PCR plates seals to the vacuum block without additional push down steps. 8 µl of PCR samples were mixed with 100 µl of ultra pure water and centrifuged. Afterwards 100 µl of the mix was loaded onto the NucleoFast 96 PCR plate. Vacuum was applied for 10-15 min. Finally, 20 µl of HPLC water were dispensed onto the membrane. After some mixing steps purified samples were recovered from the membrane and loaded onto a new 96-well PCR plate.

#### 2.1.7.3 Sequencing reaction

The sequence reaction (reaction mix see **Table. 10**) has been performed with the DNA thermal cycler according to the protocol described in **Table. 11**. Each purified and amplified DNA fragment was mixed with listed reagents and either forward or reverse primers.

[9] Means dissociated primer dyes which can mask true data.

**Table. 10.** Reaction batch for sequence reaction

| Reagents | Volume |
|---|---|
| Purified PCR product | 1 µl |
| HPLC $H_2O$ | 1 µl |
| Primer, forward conc. 20 ng/µl (diluted 1:9 in ultra pure $H_2O$) | 0.5 µl |
| Primer, reverse conc. 20 ng/µl (diluted 1:9 in ultra pure $H_2O$) | 0.5 µl |
| BigDye3.1 ready reaction mix | 0.5 µl |
| BigDye3.1 reaction buffer 5x | 1.5 µl |
| **Total volume** | **5 µl** |

**Table. 11.** Sequence reaction protocol for MJ Research PTC-225 Thermal Cycler

| Step | Temperature | Time | |
|---|---|---|---|
| Denaturation | 96°C | 1 min | |
| Denaturation | 96°C | 10 s | 25-35 times |
| Annealing | 50°C | 5 s | |
| Elongation | 60°C | 4 min | |
| Cooling | 10°C | 1 min | |

#### 2.1.7.4 Ethanol precipitation

To obtain clean sequencing data, the sequence reaction products were precipitated with ethanol. In the first step, 25 µl of 100% ethanol were added to each sample. After an incubation time of 15 minutes at room temperature samples were centrifuged at 3000 RCF (relative centrifugal force) for 30 minutes at 4°C. Subsequent samples were headfirst centrifuged again for ten seconds at 100 RCF, followed by adding 125 µl of 75% ethanol and centrifuging at 2000 RCF for 14 minutes at 10°C. Afterwards, the sample plate was carefully beaten headfirst on a Kleenex and finally centrifuged headfirst at 600 RCF for one minute. Ethanol evaporated from samples during 15 minutes at room temperature. Finally, 50 µl HPLC water were added and 25 µl of the precipitated sample water mix loaded onto the barcode plate and covered with a plate septum.

#### 2.1.7.5 Sequence analysis

Subsequent to purification of amplified DNA, sequence reaction and ethanol precipitation samples were analysed by the ABI3730 DNA Analyser automatically (2.1.7.1). Afterwards, row sequence data were analysed and checked for sequence variants with the GAP-Assembler Version 4.11 from Staden (http://staden.sourceforge.net/staden_home.html) in comparison to the reference sequences from the Genome Bioinformatics Human Genome Browser [Kent et al. 2002; Meyer et al. 2010; http://genome.ucsc.edu/]. Homozygous mutations affect both DNA strands, whereas heterozygous sequence variants are only observed in one allele. In comparison to the reference sequences, mutations can be observed as single base exchanges (point mutation), insertions or deletions of one or more bases. Homozygous mutations display a different base at the according nucleotide position in comparison to the reference sequence (**Fig. 11**). Heterozygous point mutations are seen as two small overlaid peaks (**Fig. 12**), whereas heterozygous insertions or deletions were observed as following overlap of mutated and wild type strand (**Fig. 13**).

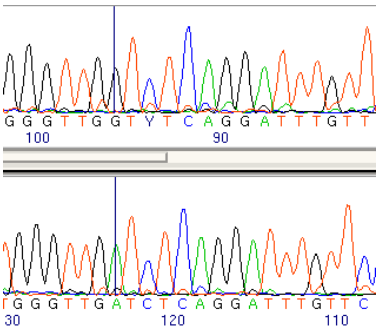



**Fig. 11.** A) Reference sequence from UCSC Genome Browser. B) Patient with homozygous point mutation (c.9G>A; p.W3X) in NDUFA12L tagged by the blue line.

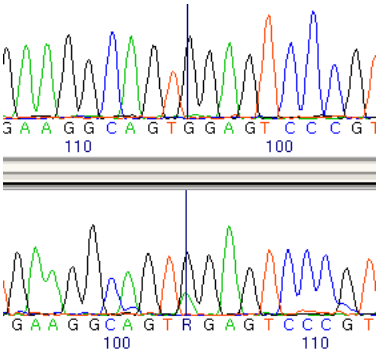



**Fig. 12.** A) Reference sequence from UCSC Genome Browser. B) Patient with heterozygous point mutation (c.946G>A, p.G316R) in C7ORF10 tagged by the blue line.

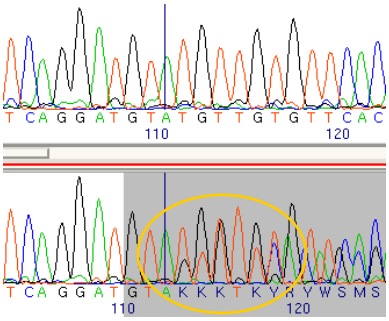



**Fig. 13.** A) Reference sequence from UCSC Genome Browser. B) Patient with heterozygous 71-74delTGTT in exon 2 of XPNPEP3 and the resulting overlap of sequences, where one allele is similar to the reference sequence and the other one continues with GTGTT… in parallel (right from blue line).

## 2.2 Patient cohort

150 DNA samples of patients with RCC I deficiency were included in the mutation screen previously performed at the IHG (1.4.1). Inclusion criteria for the complex mutation screen of structural subunits and assembly factors was an isolated RCC I deficiency in the investigated tissues of the patients, as defined by the referring centres. Furthermore, common mtDNA mutations should have been excluded within the routine molecular-genetic diagnostics by the referring clinical centres.

Towards the aim of finding genotype-phenotype correlations and general information about patients with RCC I deficiency, the phenotypes of investigated patients have been collected using a standardized questionnaire (2.3). An official research report about the results of the high-throughput mutation screen was sent to the referring centres, combined with a letter of enquiry for blood or DNA samples to confirm the findings and to get more details about the phenotype of patients. The majority of DNA samples has been referred from the 'Städtisches Klinikum Schwabing' Munich, where enzyme measurements of the RCC in biopsied patients are performed in cooperation with the Department of Paediatrics at the University of Salzburg in Austria (**Table. 12**). I reviewed the charts of these patients and clinical information has been collected by the standardized questionnaire. The clinical information of patients referred from Italy, the Czech Republic and Croatia was assessed by the referring doctors in form of the questionnaire.

A single patient from the Italian working group of Milano, harbouring a nDNA mutation, was added to the group of 150 patients analysed. The statistical analysis was performed in this cohort of 151 patients.

**Table. 12.** Overview of institutions which sent DNA samples from patients with RCC I deficiency

| Institution | Frequency |
|---|---|
| 'Städtisches Klinikum Schwabing', Munich | 73 |
| IRRCS Foundation Neurological Institute 'C.Besta', Milan | 50 |
| Department of Paediatrics, Paracelsus Medical University Salzburg | 15 |
| Department of Paediatrics, Charles University in Prague | 11 |
| Department of Paediatrics, Zagreb | 2 |
| **Total** | **151** |

## 2.3 Standardized questionnaire

For the ascertainment of clinical features of patients with RCC I deficiency, a standardized questionnaire has been developed. The questionnaire developed from the Working Group of Paediatric Metabolic Disorders [Freisinger et al. 2007] was used as template. Briefly, they collected signs and symptoms deemed to indicate a mitochondrial disorder and sent it to members of the guideline group and experts in paediatric neurology. Referring to the second evaluating step (members assessed items according to their experience), items were selected with statistical analysis and substantive vote of the members. For further information about questionnaire design methodology see Freisinger et al. [2007, p.33-34]. According to this template, we modified the questionnaire to have a suitable short overview about patients' 'clinical history', which could be completed both retrospective and relating to actual conditions of the patients. The questionnaire is divided into three parts. The first part presents the family history including basic information like name of the patient, birth, sex and family anamnesis beside main information about responsible doctors, referring institutions and date of assessment. The second part ('clinical symptoms and signs') encompasses clinical phenotypes which were classified according to different organs or organ systems (in total 68 phenotypes). They can be evaluated whether they are present, not present, have not been examined or no information is/was available. Two possibilities for additional symptoms are given as well. Five cardinal symptoms had to be named for summarizing the clinical phenotype of the patients. Information about cMRI and MRS, laboratory parameters in blood samples and CSF, information about tissue biopsies and the histochemical investigations beside the results of biochemical measurements of the RCC enzymes were documented in part three of the questionnaire ('imaging and laboratory parameters'). For complete questionnaire see **Appendix. 6**.

The questionnaire has been sent to the clinical institutions, which referred DNA samples or tissue biopsies of patients who were not seen in the 'Städtisches Klinikum Schwabing'. The main part of questionnaires was completed by retrospective chart research by me in the archive of the hospital, because the majority of DNA samples from patients that have been analysed within the mutation screen was referred from the 'Städtisches Klinikum Schwabing' (Department of clinical chemistry and molecular diagnostics). Nevertheless, without the kind cooperation work of other European clinical centres with interests in metabolic diseases this comprehensive collection of patients with isolated RCC I deficiency would not exist (see 2.2 for the name of participating institutions).

## 2.4 Database of collected patient information and statistical analysis

Data from 151 patients with isolated RCC I deficiency were collected retrospectively using a standardized questionnaire (2.3). The database was generated with Microsoft Office Access 2003, licensed for the Helmholtz Zentrum München (ID. 73931-640-0887165-57224). Statistical analysis was performed by assistance of the SPSS 16.0 version. For the database, including all patients with biochemical, clinical and molecular genetic information, a numerical code has been assessed. The five 'cardinal symptoms' were integrated for categorizing patients (see 3.2.5) and also assigned with code numbers for the analysis and comparability within the patient cohort.

Because of the database structure, descriptive statistics like frequency distribution and cross-classified tables have been predominantly used for describing the patient cohort. The chi-square-test ($x^2$-test) was used to compare proportions between the different categories of patients regarding severity of RCC I deficiency, age of onset and death, genotype and phenotype and others and to identify significant correlations between these groups. A significance of results was assumed when the p-value for a two-sided test was $\leq 0.05$. For evaluating the outcome of patients, Kaplan-Meier survival analysis was performed to study the survival rates within the different groups.

# 3 Results

## 3.1 High-throughput mutation screen of candidate genes

Former medical students (Florence Madignier, Rene Drost and Martin Freitag) performed a mutation screen of RCC I subunit and four assembly factor encoding genes in 150 RCC I deficiency patients and could establish a molecular-genetic diagnosis for 18% of patients. Assuming that other genes are essential for proper RCC I function candidate genes, namely C10ORF65, C3ORF60, C20ORF7 and C7ORF10, these genes were selected for investigations in the 150 DNA samples of patients with RCC I deficiency [Pagliarini et al. 2008]. XPNPEP3 has been newly associated with patients with RCC I deficiency during my work and was therefore additionally included in the screen [O'Toole et al. 2010].

In order to establish exon specific assays, intronic primers were designed using the ExonPrimer and LightScanner Primer Design program. PCR-protocols with three different annealing temperatures $T_A$ were tested for all exons. In some cases TouchDown and temperature gradient protocols, two additional DNA-polymerases and up to four different primer pairs were designed and tested. However, for seven exons out of 37 no PCR could be established. The final protocols are listed in **Appendix. 5**. In total, 30 coding exons have been screened with the high-resolution melting point analysis. Altogether, 170 samples displaying melting curves different from wild-type were analysed by Sanger sequencing. An example of the melting profile analysis performed by the Idaho Technology software is given in **Fig. 9.**

Not all exons of the candidate genes (meaning exon 1 of C7ORF10, C3ORF60, C10ORF65 and C20ORF7, exon 5 of C7ORF10, exon 6 of C10ORF65 and exon 2 and 4 of C3ORF60) could be analysed because no specific PCR product could be obtained. PCR of these exons have been repeated several times using different conditions (different primer concentrations or different reaction conditions), but failed to get satisfying results.

No sequence variants were identified in both C10ORF65 and C3ORF60 (see **Table. 13**). One patient with XPNPEP3 mutations has been part of the investigated patient cohort [O'Toole et al. 2010]. The pathogenic homozygous frame-shift mutation in XPNPEP3 was confirmed by the high-resolution melting profile screening procedure. Furthermore, three patients with a heterozygous sequence variant in XPNPEP3, two with a missense and one with a frame-shift mutation, were identified. Another heterozygous single nucleotide change in the intronic region next to exon 6 (position -2) was found. However, sequencing of all other exons did not discover additional mutations in these samples. Since an autosomal-recessive way of inheritance is assumed, these heterozygous changes are most likely not the cause of the RCC I defect.

Three heterozygous sequence variants were identified in the encoding regions of C20ORF7. Two patients harboured a heterozygous single nucleotide exchange resulting in the change of highly conserved amino acids. One patient was detected to have a heterozygous variant in an intronic region. Additionally, a new SNP has been identified in three patients (2% frequency). This variant is not annotated in the SNP database (NCBI; www.ncbi.nlm.nih.gov/projects//SNP/).

In C7ORF10, the largest analysed gene, encompassing 15 coding exons, only one heterozygous missense mutation was found.

In all patients with one mutation identified, the remaining exons were Sanger sequenced. Beside the confirmation of the homozygous frame-shift mutation in XPNPEP3, no other patient has been identified to harbour two mutations in one of the analysed genes.

**Table. 13.** Identified sequence variants of candidate genes in 150 patients with RCC I deficiency.

| Gene | Exon | Patient ID | Ranking | Sequence variant | Description |
|---|---|---|---|---|---|
| *C10ORF65: no variants identified* | | | | | |
| *C3ORF60: no variants identified* | | | | | |
| *XPNPEP3: 10 coding exons (all of them have been analysed)* | | | | | |
| XPNPEP3 | 6 | 33023 | 1 | c.[931_934delAACA]+ [931_934delAACA] p.[N311fs5]+[N311fs5] | frame-shift mutation, nephronophthisis and encephalomyopathy |
| XPNPEP3 | 2 | 33255 | 2 | c.[71_74delTGTT], p.[M24Sfs22] | frame shift mutation |
| XPNPEP3 | 6 | 33030 | 2 | c.[856-2A>G] | intronic region |
| XPNPEP3 | 8 | 35821 | 3 | c.[1070A>T], p.[Q357L] | medium conserved |
| XPNPEP3 | 9 | 33035 | 2 | c.[1244G>A], p.[R415Q] | highly conserved |
| *C20ORF7: 11 coding exons (exon 1 could not be analysed by DNA melting profile assay)* | | | | | |
| C20ORF7 | 2 | 33353 | 2 | c.[247G>C], p.[V83L] | highly conserved |
| C20ORF7 | 4 | 33334 | 3 | c.[327+16 A>G] | intronic region |
| C20ORF7 | 5 | 33485 | 2 | c.[449A>G], p.[N150S] | highly conserved |
| C20ORF7 | 7 | 35808 | - | c.[582C>T], p.[L194L] | novel SNP |
| C20ORF7 | 7 | 33325 | - | c.[582C>T], p.[L194L] | novel SNP |
| C20ORF7 | 7 | 33283 | - | c.[582C>T], p.[L194L] | novel SNP |
| *C7ORF10: 15 coding exons (exon 1 and 5 could not be analysed by DNA melting profile assay)* | | | | | |
| C7ORF10 | 15 | 35794 | 2 | c.[1214C>T], p.[T405M] | highly conserved |

Ranking: 1=pathogenic mutation, 2=possible pathogenic mutation, 3=pathogenic character unlikely
Using a ranking score, the pathogenicity of identified mutations has been assessed by their conservation across the individuals and the rate of heteroplasmy in mtDNA mutations. Additionally, information from literature or the mitomap database (www.mitomap.org, January 2009) and genpat database (http://genpat.uu.se/mtDB/, January 2009) were integrated to score the identified mutations.

## 3.2 Clinical data of the patients with isolated RCC I deficiency

A clinical questionnaire based on the 'Guidelines issued by the Working Group on Paediatric Metabolic Disorders' [Freisinger et al. 2007] has been developed in cooperation with paediatricians from the different sample referring clinics (see **2.2**). In total, the questionnaire encountered 125 items including different kinds of information (family history, clinical signs, imaging and laboratory investigation results) and collected information of patients has been analysed using SPSS statistical program. I searched for detailed information of all patients by chart research in the referring centres and contacted the responsible doctors directly. Some patients had been lost in follow-up. This collection of patients with RCC I deficiency is the largest study to date. Within the following chapters, I try to illustrate the most interesting findings, however the relatively low case numbers (especially when patients have been categorized by their clinical presentation) hamper statements about the significance of results.

Similar to previously reports of patients with mitochondrial disorders, the male/female ratio observed was 1 : 1.15 (68 male, 78 female patients) in the cohort of 151 patients with RCC I deficiency [Kirby et al. 1999; Rubio-Gozalbo et al. 2000; Skladal et al. 2003b].

### 3.2.1 Biochemical results of RCC I in the biopsies of patients

Measurements of RCC activities had been performed in fresh or frozen muscle tissue biopsies in 142 patients. Combined analysis in skeletal muscle and skin fibroblasts had been made in 41 out of these 142 patients (29%). Enzyme activities of the RCC were typically lower in the muscle biopsy than in skin fibroblasts. A liver biopsy had been carried out on four patients in addition to the muscle biopsy (3%). Investigations of heart tissues had only been performed in two patients (1%).

We analysed RCC I activity per citrate synthase (CS) first and in cases with missing values RCC I per non-collagen protein (NCP). The relative activities of RCC I are given in percentages of the lowest control values, which had been denoted from the corresponding institutes. The mean RCC I residual activity per CS in the 142 patients was 51% of the lowest control values (SD[10] ± 29%) with a range of 1-127%. The mean RCC I activity was higher and more variable when measured per NCP (58.1% with SD ± 38.3%; range of 7-190%). The distribution of RCC I residual activity is illustrated in **Fig. 14**.

Activities of RCC II-V and the pyruvate dehydrogenase (PDH) were within normal ranges.

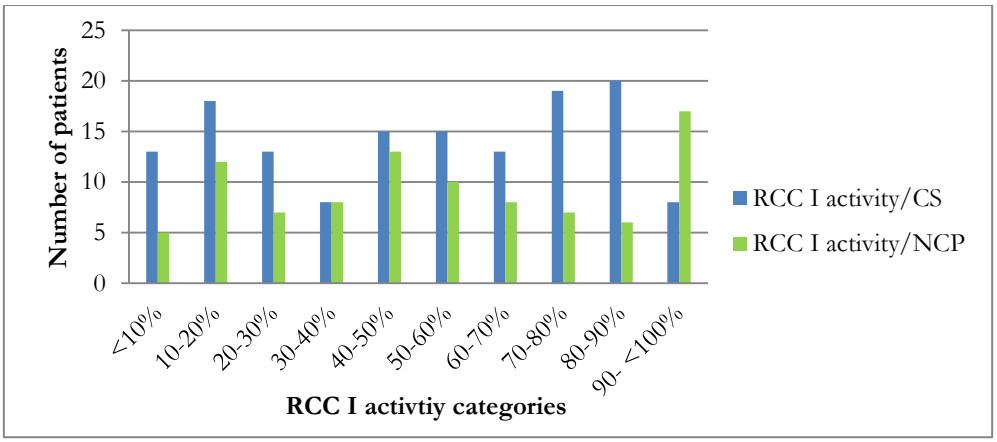



**Fig. 14.** RCC I activity distribution in the patient cohort (RCC I/CS n=142; RCC I/NCP n=93) all indicated as percentages of the lowest control value.

[10] SD= standard derivation

### 3.2.2 Morphological analysis of the biopsies

Information about histological investigations was available in 93 out of the 142 patients (66%). Forty-nine patients presented normal findings (53%) which is in agreement with findings from Rollins et al. [2001] and Patterson [2004] who found that 45-50% of the patients with mitochondrial disease had no abnormalities in microscopy investigations. Fourteen patients showed nonspecific changes (15%), whereas 30 presented abnormalities typical for a mitochondrial disorder (33%). Eleven of these 30 patients showed signs of muscular dystrophy (37%), 13 RRF (43%) and 20 had COX-negative fibers (67%) in histological examinations.

### 3.2.3 Age distribution and outcome

Most patients of the cohort with RCC I deficiency were paediatric cases, showing first symptoms at age of 0-10 years (80%) as illustrated in **Fig. 15**. The onset of first symptoms within the neonatal period is dominating (54%), followed by symptom presentation within the first six months of life in further 17% of the patients.

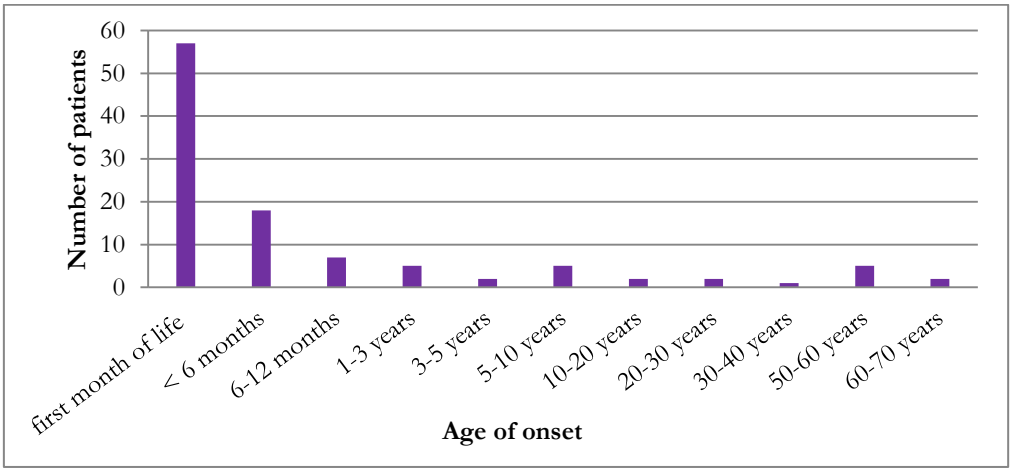



**Fig. 15.** Age of onset in the RCC I deficiency patient cohort (n=106).

Half of the patients presented first symptoms in the neonatal period (0-1 month), but only 9% of the patients underwent a diagnostic tissue biopsy within the first month of life (**Fig. 16**).
The patients were diagnosed with RCC I deficiency based on biochemical measurements of the RCC activities at a median age of two years, which is about one year after the occurrence of first symptoms. The large range up to 50 years illustrates the challenge and differences in diagnostic procedures leading to the diagnosis of a mitochondrial disorder. However, 37% of the patients underwent diagnostic biopsy between one and six months after first showing symptoms.

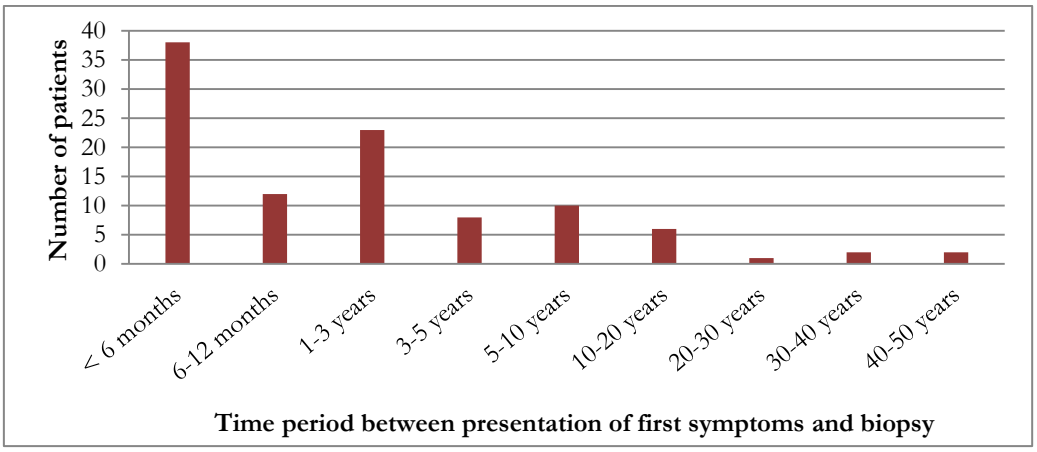



**Fig. 16.** High variance of the time period between presentation of first symptoms and tissue biopsy in 102 patients.

The mutation analysis project of RCC I started by DNA sample collection in 2006. During DNA collection and analysis, 37 of the patients died. Fifty-five were still alive at time of the analysis and from 59, no information about the actual clinical status could be evaluated.
Exact age of death was not examined in the first version of the questionnaire. Date of death was only available in 19 patients. This relatively small number of cases limits the power of the survival analysis. Twenty-one percent died within the first six months of life and additionally 36% did not reach the age of one year. Only 12% of patients died after the age of ten.
Fifty-two percent of the patients with onset <6 months died (32/62), whereas only 17% (4/24) of the patients died in the group with onset >6 months of life (p=0.003). This observation fits with the results from a previous report, where the age of onset has been described as an important predictive factor of the outcome of patients with mitochondrial disease [Skladal et al. 2003b; Debray et al. 2008].
No significant correlations were observed between RCC I residual activity and both the age of onset and death. This has been also described by Skladal et al. [2003b]. Low activity does not correlate with early onset or death, for example 13 patients with an onset of first symptoms within the first year of life had a RCC I residual activity of 80-90%. In contrast, two patients with activity less than 10% showed first symptoms after one year of life.

### 3.2.4 Identified mutations in the patient cohort

Thirteen patients (9%) carried pathogenic mutations in mtDNA and 15 patients in nDNA (10%). Detailed information about identified mutations is given in the **Appendix. 2** (mtDNA mutations) and **Appendix. 3** (nDNA mutations). Using a ranking score, the pathogenicity of identified mutations has been assessed by their conservation across the individuals and the rate of heteroplasmy in mtDNA mutations. Additionally, information from literature or the mitomap database (www.mitomap.org, January 2009) and genpat database (http://genpat.uu.se/mtDB/, January 2009) were integrated to score the identified mutations.
The following results regarding phenotypes and identified mutations do not deal with the different genes encoded by both mtDNA and nDNA, but rather present an overview of RCC I deficiency patients harbouring mutations in the different genomes and their biochemical, morphological, metabolic and clinical phenotypes compared with patients without identified mutations.

### 3.2.4.1 Correlation of RCC I activity and mutation status

A significant enrichment (p=0.026) of patients with mutations (in mt or nDNA) was observed in the group with less than 50% RCC I activity. Nineteen of the 67 patients (28%) harboured pathogenic sequence variants, whereas in the group with >50% RCC I activity only eight patients of 75 (11%) carried mutations. Detailed distribution of pathogenic mutations on RCC I activity groups is given in **Fig. 17**. No significant differences of median RCC I activities were observed between patients with mtDNA (38%; SD ± 27%; n=12) or nDNA mutations (36%; SD ± 24%; n=15). The group without mutations had a higher median residual activity (59%; SD ± 29.9%; n=115).

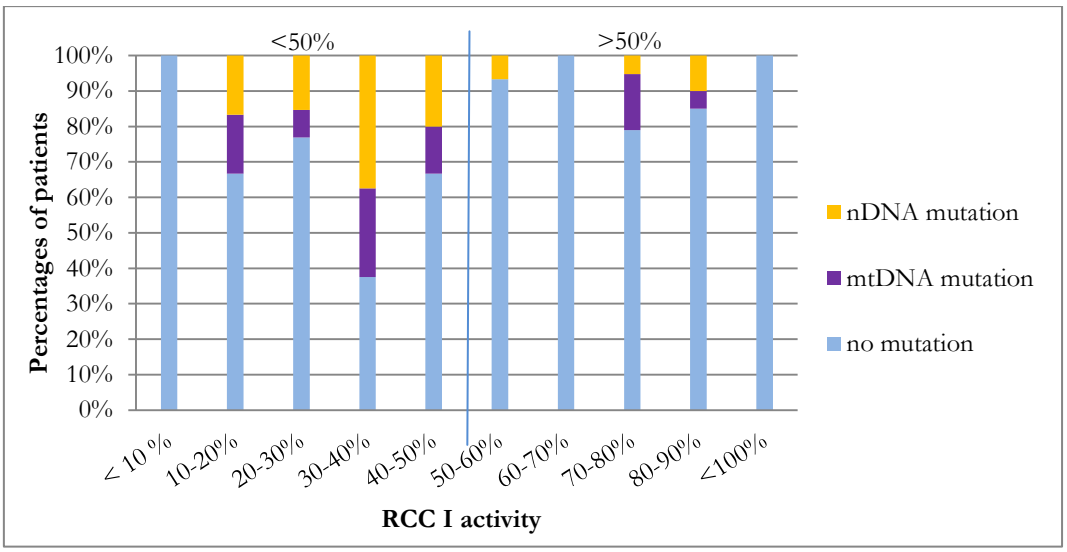



**Fig. 17.** Pathogenic mutations and their distribution among RCC I residual activity categories (n=142).

### 3.2.4.2 Histological findings and mutations

As mentioned in 3.2.2, 30 of the 93 patients with information about the histological investigations presented with findings seen typically in tissues affected by mitochondrial disease. Five of the 30 patients (17%) with pathological alterations in histological or histochemical biopsy investigations harboured pathogenic mtDNA mutations, whereas only one patient had a mutation in the nDNA (3%). RRF have been observed in 38% of the patients with mtDNA mutations (n=3) and were absent in patients harbouring nDNA mutations (n=9). This observation is in agreement with previous reports that RRF are more often associated with mtDNA mutations than nuclear mutations [Lamont et al. 1998; Patterson 2004; Malfatti et al. 2007; Koenig 2008].

### 3.2.4.3 Metabolic parameters and mutations

The most frequent recognized laboratory parameter in patients with mitochondrial disease is lactic acidosis [Koenig 2008]. In the analysed patient cohort, 58% had elevated blood lactate levels and 34% lactate level elevations in CSF. This finding is similar to the results from Jackson et al., who studied 51 patients with mitochondrial disorders and found 50% with lactic acidosis [Jackson et al. 1995]. Munnich et al. examined a cohort of 235 patients with mitochondrial disorders and found elevated venous blood lactate levels in only 30% [Munnich et al. 1996a].

The combination of both, elevated blood and CSF lactate levels was significantly higher (p=0.003) in patients with nDNA mutations (55%) and mtDNA mutations (40%) compared to patients without a pathogenic mutation (15%) as illustrated in **Fig. 18**. A similar correlation was observed for the single laboratory parameter CSF lactate, which seems to be more sensitive in diagnostic procedures of mitochondrial disease than blood lactate.

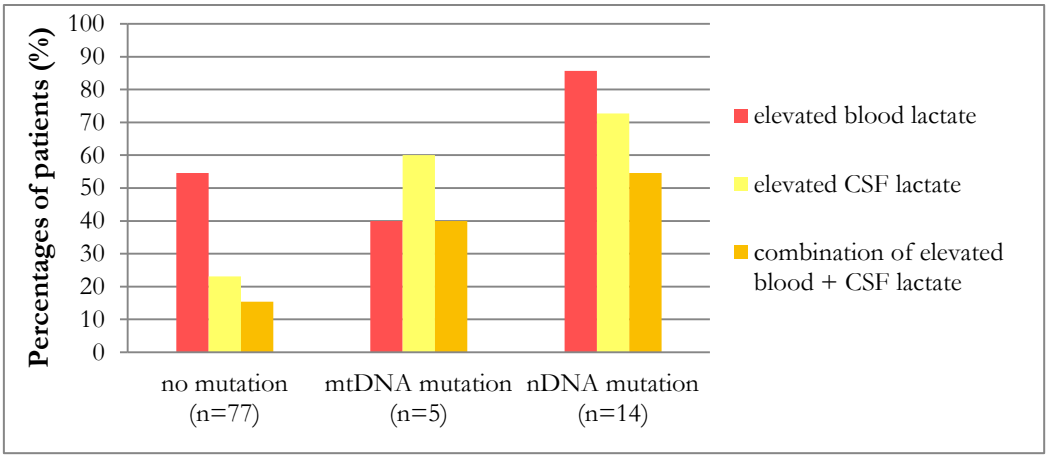



**Fig. 18.** Distribution of patients with elevated blood, CSF lactate levels and both in correlation with the genotype (n=96).

A significant enrichment (p=0.005) for patients with mtDNA mutations and elevated creatine kinase (CK) was found in contrast to those patients with no or nDNA mutations (**Fig. 19**).

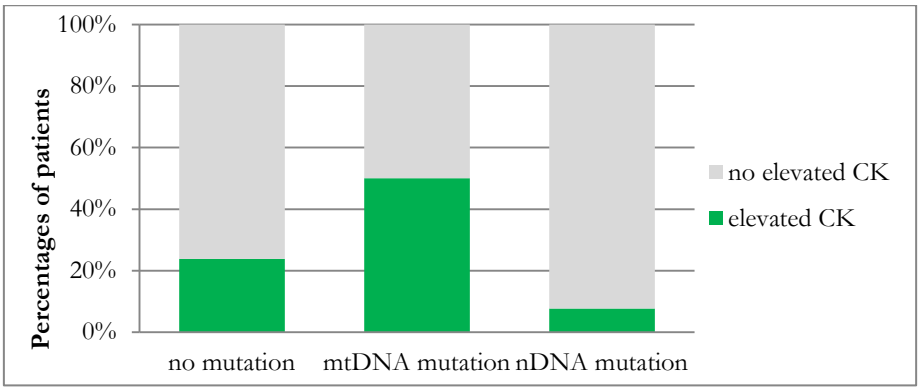



**Fig. 19.** Elevated CK in patients with RCC I deficiency (n=82).

For the remaining laboratory parameters, including acyl carnitines, organic acids and amino acids, no statistical correlations were found regarding the genotype of patients.

#### 3.2.4.4 Age of onset and mutation status

Age at first symptom occurrence was significant higher in patients with mtDNA mutations compared with those with mutations in nDNA (p=0.048). Patients with mtDNA mutations showed first symptoms at a median age of 18 months (range 0-66 years; n=7), whereas the patients with nDNA mutations had onset at median age of five months (range 0-2 years; n=13). **Fig. 20** shows the concentration of patients with nDNA in the groups of onset at an early age and the patients with mtDNA mutations, which are more distributed over the different age of onset categories. These data are consistent with previous suggestions that patients with mitochondrial disorders caused by mutations in mtDNA tend to become symptomatic at an older age [Rubio-Gozalbo et al. 2000; Skladal et al. 2003a]. However, two patients presenting with Leigh-like syndrome and one with severe nonspecific

encephalomyopathy harboured pathogenic mtDNA mutations and showed first symptoms at an early age, demonstrating that mtDNA mutations are also associated with severe phenotypes and fatal courses of disease in young patients.

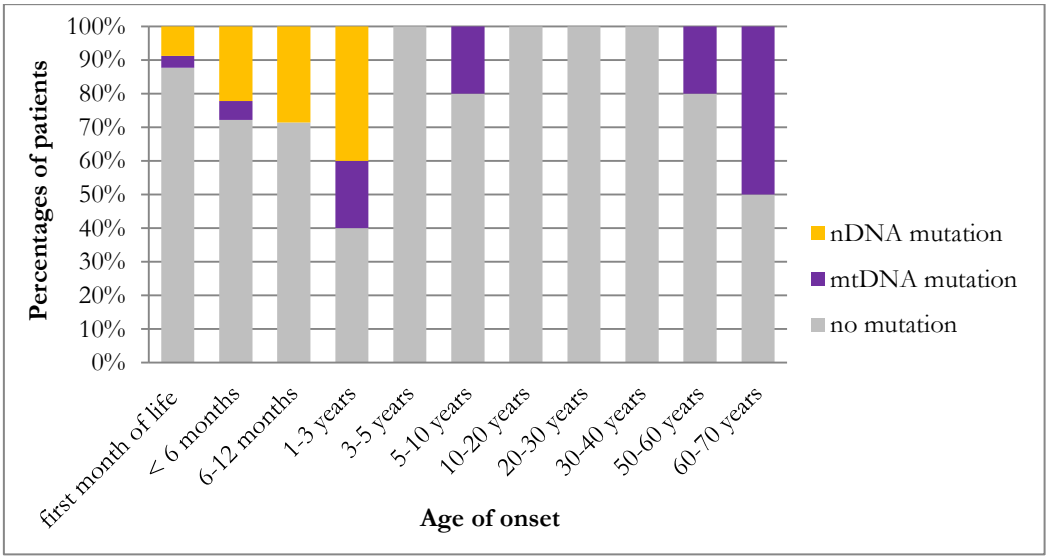



**Fig. 20.** Coloured percentages show part of patients with pathogenic nDNA or mtDNA mutations distributed over the age of onset categories (n=106).

### 3.2.5 Clinical phenotypes in the patient cohort

Information about clinical symptoms was available from 133 out of 151 patients (88%). The cardinal symptoms of the patients were assigned to eight different phenotype categories described in the literature for patients with mitochondrial disorders or isolated RCC I deficiency [Loeffen et al. 2000; Skladal et al. 2003b; Bugiani et al. 2004; Scaglia et al. 2004; Debray et al. 2007a]. The dominating symptoms were used to classify the patient cohort, but an overlap of symptoms is also possible.

**Fig. 21** illustrates the different phenotype categories that were present in our cohort of RCC I deficiency patients.

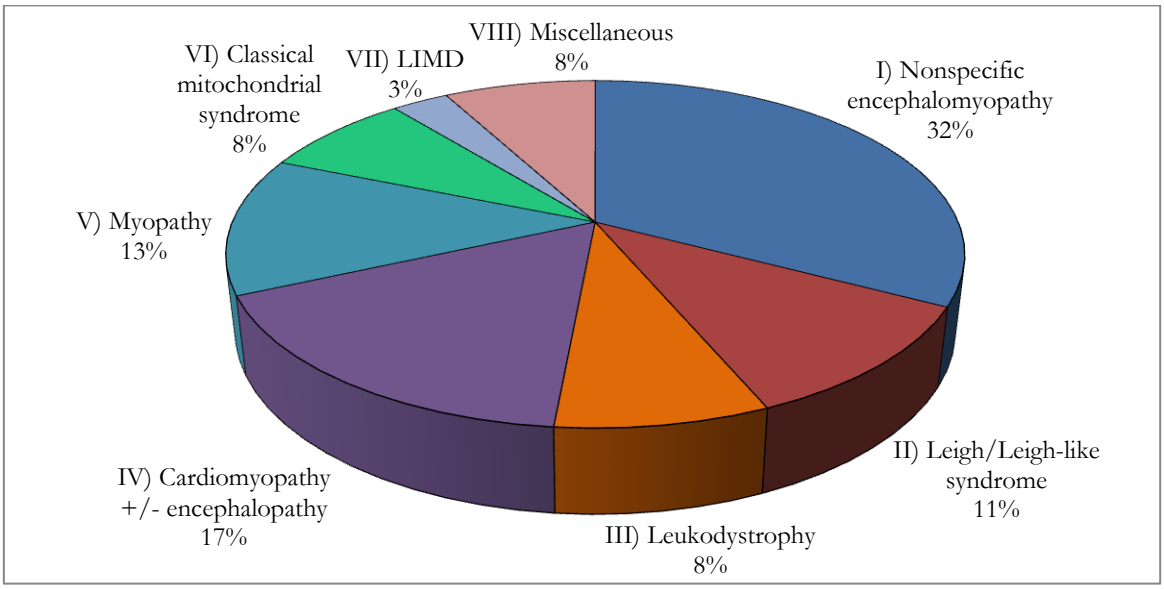



**Fig. 21.** Overview of clinical phenotypes in 133 patients with RCC I deficiency. Roman numbers indicate the phenotype categories which are used in the following chapters to describe the categories.

I - Nonspecific encephalomyopathy

Most frequently affected organ was the nervous system, which has been described previously [Munnich et al. 1996a; Koenig 2008]. One third of the patient cohort displayed nonspecific encephalomyopathy without the typical changes in brain imaging characterizing Leigh syndrome or leukodystrophy and variable neuromuscular symptoms (**Fig. 21**). Psychomotor development delay or retardation has been observed in 87% and 62% had decreased muscle tone. Seizures as a frequent symptom in this category were reported in 44% of the patients.

II - Leigh or Leigh-like syndrome

This phenotype was observed in 14 of 133 RCC I deficiency patients (11 %). We only had one patient with neuropathological findings in the autopsy, segregating with the characteristic alterations in Leigh's disease, which is primarily defined by pathological findings of gliosis, necrosis, spongiosis or capillary proliferation in the CNS. The remaining 13 patients displayed a pattern of signalling abnormalities (symmetrical lesions in the basal ganglia and/or brainstem) in the brain imaging investigations suggestive for a Leigh syndrome and were defined to have Leigh-like syndrome. Compared with published reports we had less patients with Leigh or Leigh-like syndrome [Loeffen et al. 2000; Rubio-Gozalbo et al. 2000,; Skladal et al. 2003b; Debray et al. 2007a]. Frequent symptoms observed in this group were psychomotor development delay or retardation (91%), decreased muscle tone (64%), dystonia (50%), spasticity and swallowing difficulties (both 40%).

III - Leukodystrophy

Eleven patients of the cohort suffered from progressive leukodystrophy, which was observed in the neuroimaging investigations (leukencephalopathy with large cavitations in subcortical white matter). Macrocephaly was not found in any of these patients similar to findings from Bugiani et al., but has been previously reported in patients with RCC I deficiency and leukodystrophy [Loeffen et al. 2000; Bugiani et al. 2004]. Ten of them had psychomotor development delay or regression (91%) and swallowing difficulties or poor sucking (36%). Other symptoms, without differences in frequency compared to categories I and II, were muscular hypotonia (46%), seizures (18%), ataxia and dystonia (30%).

IV - Cardiomyopathy +/- encephalomyopathy

Twenty-three patients presented with cardiomyopathy and 13 of them had signs of encephalomyopathy (56%). Two more patients with signs of cardiomyopathy were included into the group of patients with 'miscellaneous' presentations (see VIII), because of dominating additional symptoms. Hypertrophic cardiomyopathy was found in 74% patients (n=17). The remaining patients (n=6) had dilated cardiomyopathy, whereas this form is seen as long-term consequence of the hypertrophic cardiomyopathy [Scaglia et al. 2004]. Conduction impairments were reported in 17% (n=4). The frequency of cardiomyopathy in the cohort of patients (17%) fits to observations from Holmgren and colleagues who diagnosed 17 patients from 101 patients (16.8%) with mitochondrial disorder to have a cardiomyopathy [Holmgren et al. 2003]. Only one patient had the combination of cardiomyopathy and myopathy in agreement with results from Skladal et al. [2003b].

V - Myopathic phenotype

In 17 from 133 patients dominating myopathic symptoms have been observed (13 %). Frequently reported symptoms were myalgia (65%), exercise intolerance (42%), muscular weakness (62%) and rhabdomyolysis (36%).

VI - 'Classical mitochondrial syndromes'

Ten patients showed symptoms typical for mitochondrial syndromes. In particular two patients had chronic progressive external ophthalmoplegia (CPEO), two CPEO+[11], two MELAS, two LHON, one patient presented the clinical pattern of Kearns-Sayre syndrome (KSS) and one with MERRF. Symptoms observed in this group of patients are mainly characterized by the diagnosis of the mitochondrial syndrome (see **Appendix. 1**) and therefore not separately listed in here.

VII - LIMD

The phenotype of four patients was characterized by neonatal onset and highly elevated lactate levels in blood with fulminant course of disease (all died during the first month of life). Other symptoms present in all patients were muscular hypotonia, feeding difficulties, seizures and apnoeas or respiratory insufficiency, respectively. Respiratory abnormalities were seen in categories I, II, III and IV as well, but with a lower frequency (10-20%).

VIII - Miscellaneous symptoms

The combination of symptoms affecting different organ systems that could not be ascribed to any of the specific phenotypes was observed in ten patients. Four patients had dominating GIT symptoms like enteritis or cyclic vomiting. Only two of them were mildly retarded and one additionally had abnormal elevated liver enzymes in laboratory investigations. One patient suffered from liver insufficiency and diabetes mellitus. One girl was admitted due to lactic acidosis, hyperexcitability and feeding problems but was stable at time of observations without development delay or mental retardation. One patient with mutations in XPNPEP3 presented with renal insufficiency, psychomotor development delay, epilepsy and developed cardiomyopathy. He died at age of eleven years due to cardiac failure [O'Toole et al. 2010]. A girl harbouring a mutation in the X-linked NDUFA1 gene had mildly elevated blood lactate levels, mildly decreased muscle tone and high frequency deafness but normal mental and psychomotor development (see 3.3.5). Another patient had hyperreflexia, ataxia, optic-nerve atrophy and panzytopenia and one presented with hypertrophic cardiomyopathy, diabetes mellitus, anaemia and decreased muscle tone. No correlation studies have been performed within this group of patients because of the strikingly different and variable clinical presentation.

Male/female ratios were equally distributed between the genders in patients with encephalomyopathy, miscellaneous symptoms, myopathy, cardiomyopathy, Leigh or Leigh like syndrome and even with 'classical mitochondrial syndromes' with ratios from 0.8-1.2 : 1, whereas the LIMD and leukodystrophy patients were more often males with a ratio of 3 : 1 and 1.75 : 1, respectively. Skladal and colleagues observed similar patterns in distribution between the genders for LIMD and nonspecific encephalomyopathy [Skladal et al. 2003b].

[11] Patients with signs of CPEO and myopathic symptoms are designated as CPEO+ patients.

Regarding observed symptoms in all patients, the CNS was most frequently affected, followed by dysfunction of the skeletal muscle and ophthalmological tract (**Fig. 22**), thus confirming the observation that defects in energy generating processes mainly affect those cells with high-energy demand.

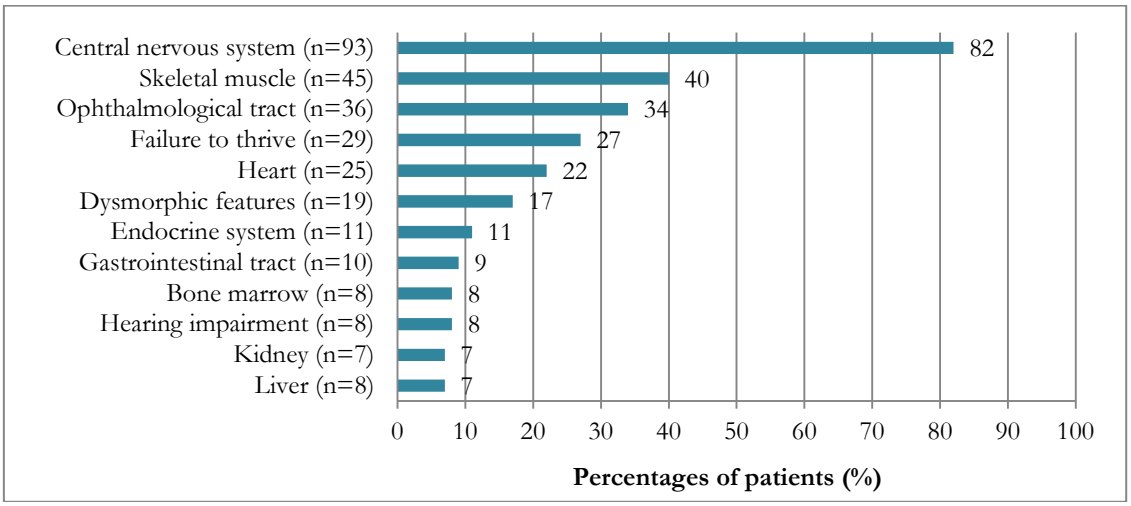



**Fig. 22.** Frequency of affected organ systems or general manifestations in the patient cohort (n=133).

### 3.2.5.1 RCC I activity among the different phenotypes

No significant correlation regarding the phenotype categories and severity of the RCC I defect was detected by statistical tests (**Fig. 23**). This approves previous observations that no correlations exist for the phenotype of patients and the residual enzyme activity [Korenke et al. 1990; Debray et al. 2008]. However, the observation that patients with severe phenotypes like Leigh syndrome or LIMD had clearly reduced RCC I activities (both median <25%) was remarkable. Patients with phenotypes III-VI showed nearly similar median RCC I residual activities (54-63%), although the clinical presentation and outcome were clearly different (see 3.2.5).

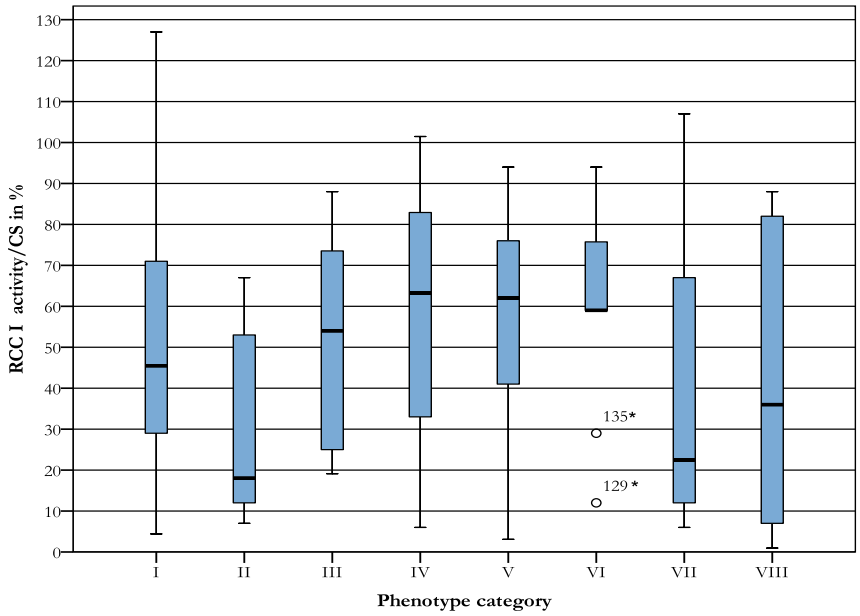



**Fig. 23.** RCC I residual activity of lowest control values per CS (n=127) and the distribution in the phenotype categories. *indicate case numbers from SPSS databank and their RCC I activity values that were remarkable different compared to remaining patients of the according phenotype category.

### 3.2.5.2 Histological findings and phenotypes

Most specific changes in the muscle biopsies have been observed in phenotype categories V and VI (**Fig. 24**), including RRF, COX-negative fibers and signs of muscular dystrophy. These observations are in accordance with numerous reports of patients with mitochondrial disease and their presentations [Loeffen et al. 2000; Rollins et al. 2001; Koenig 2008]. Fifty percent of the patients with 'classical mitochondrial syndrome' had RRF, whereas in the remaining groups, these findings were rare. This reflects the general opinion that it is an unusual finding in young children [Vogel et al. 2001; Scaglia et al. 2004].

In summary histological evaluation in children with unspecific phenotypes disease does not support achieving the diagnosis of a mitochondrial disorder as previously observed [Patterson 2004].

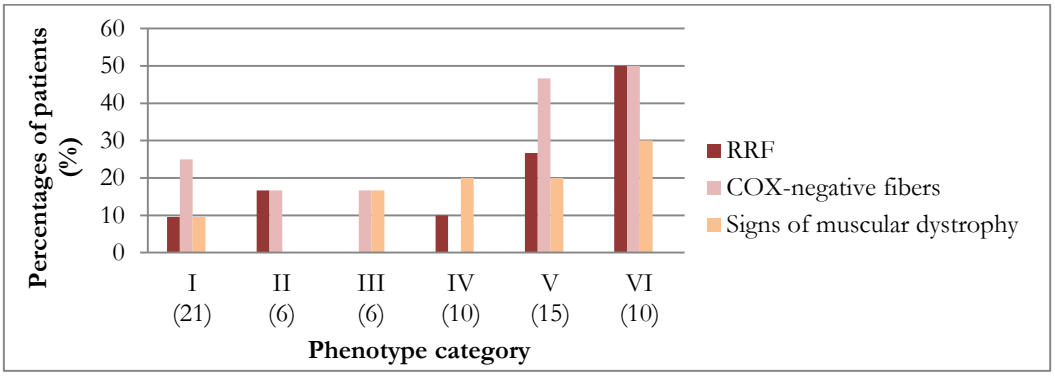



**Fig. 24.** Histological findings in muscle biopsies of patients with different phenotypes (n=68). Number of patients with information of each category indicated in brackets below roman number.

### 3.2.5.3 Metabolic parameters and phenotypes

Correlation analysis of metabolic parameters was only made for the elevation of lactate levels in blood and CSF. In patients with phenotypes of Leigh syndrome and LIMD all patients had abnormal lactate levels (**Fig. 25**), whereas over 50% of the patients of group II showed a combined elevation of blood and CSF lactate levels. No patient of the myopathic group (V) had abnormal results in lactate level investigations. Within the remaining phenotype groups the distribution of findings was equal. No significant correlations between abnormal lactate levels and the phenotype of patients have been detected supporting the results of previous reports [Debray et al. 2007a].

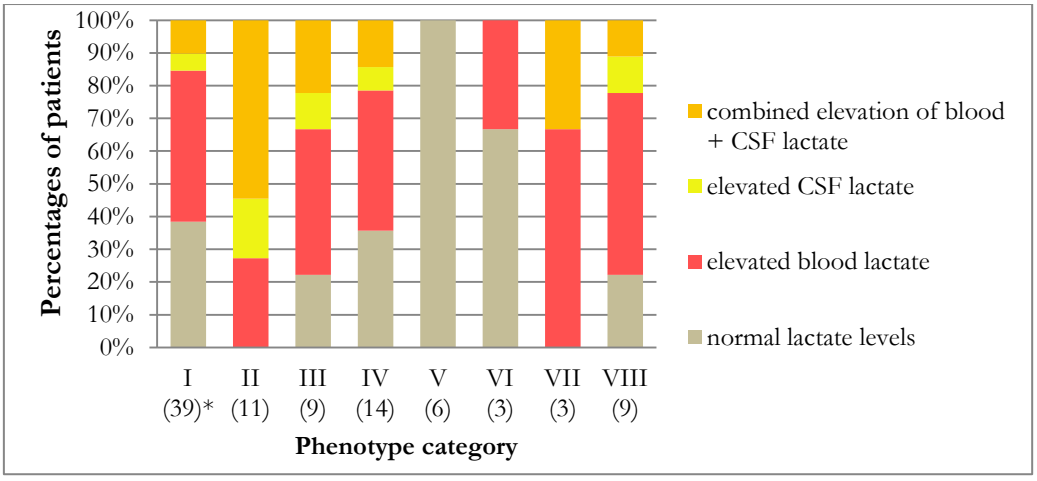



**Fig. 25.** Abnormalities in blood and CSF lactate levels in the different phenotype categories.
*n within brackets indicates number of patients of whom data about lactate parameters were available.

### 3.2.5.4 Outcome in phenotype categories

Regarding the different phenotype categories, distributions of age of onset are shown in **Fig. 26**. Median age of onset observed was significantly higher in patients with the phenotypes of myopathy (57 years) and mitochondrial syndromes (30 years). Within the other groups, median age of onset was within the first months of life (0-8 months). One exception in the group of patients with leukodystrophy has been observed. The patient developed dementia and spasticity at the age of 60 years (*81, **Fig. 26**).

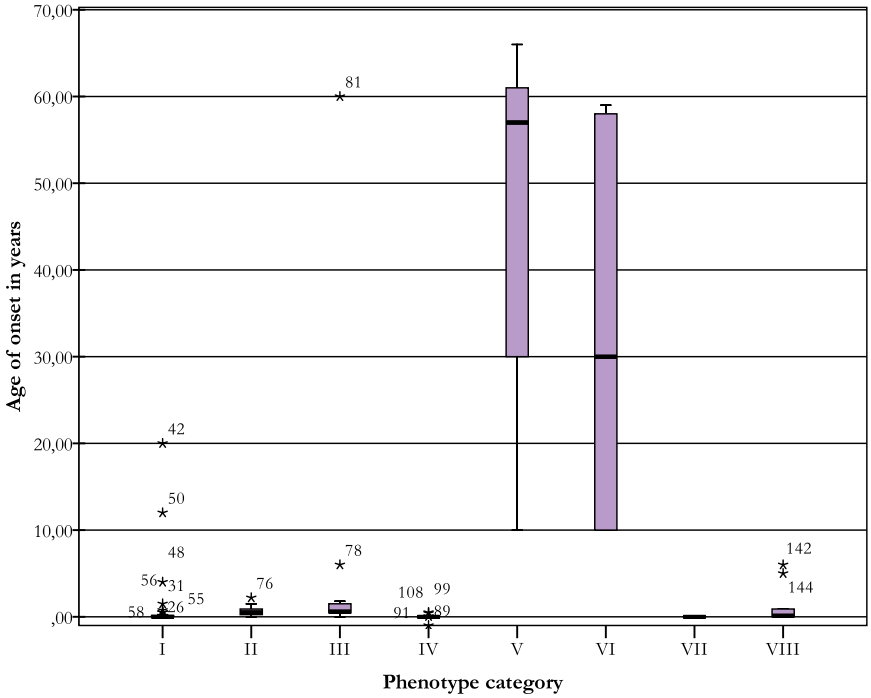



**Fig. 26.** Age of onset in the different phenotype categories (n=106). *indicate patient case numbers.

Within the questionnaire, specifications of age of death were primarily divided into three categories: death under one year of age, between one and ten years and after ten years of life. For these categories information from 87 of the 151 patients was available (58%). In categories I-IV 35-50% of patients expired at young age, but the poorest outcome showed patients with LIMD (VII). They all died during the first month of life. As expected the outcome in the myopathic group and group of patients with mitochondrial syndromes was remarkable different. Only one patient died in phenotype group VI at age 76 years.

### 3.2.5.5 Mutations of mtDNA and nDNA in phenotype categories

Regarding the genotype status (no mutation, mtDNA or nDNA mutation) different phenotype categories are associated with molecular-genetic basis of disease. Distribution of pathogenic mutations of single nuclear genes and mtDNA encoded genes (ND1-6, ND4L) among the different phenotypes is illustrated by **Fig. 27**. The figure also demonstrates that the majority of patients did not get a molecular-genetic diagnosis, although they have been included in a comprehensive mutation analysis for RCC I genes.

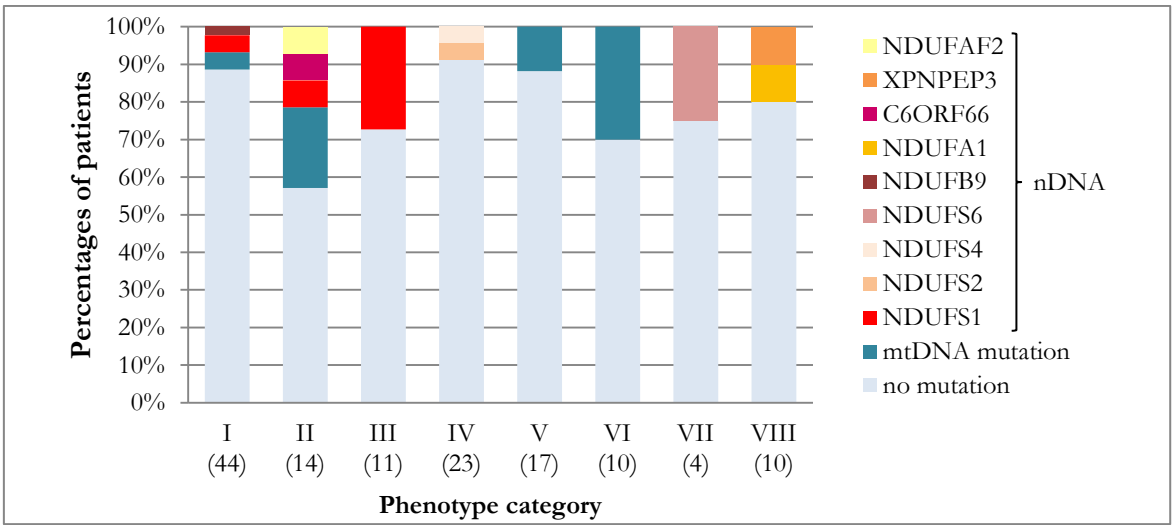



**Fig. 27.** Genotypes and their distribution among clinical phenotypes (n=133). In total, phenotypes of ten patients with mtDNA (three missing) and 14 with nDNA mutations (one missing) were known. Genes of nDNA encoded subunits are illustrated separately. Details of mutations are listed in **Appendix 2** and **3**.

mtDNA mutations were found in phenotypes I, II, V and VI (**Fig. 27**, turquoise bar). No patient with mtDNA mutation has had leukodystrophy, cardiomyopathy, LIMD or a miscellaneous phenotype. mtDNA and nDNA mutation rate in patients with Leigh syndrome was equal (both 21%) confirming that this phenotype is not specific for any kind of mutation [Rahman et al. 1996; Loeffen et al. 2000]. Mutations of nDNA encoded genes were found in nearly all phenotype categories, excluding the groups of patients with myopathic or mitochondrial syndrome. A significant enrichment of NDUFS1 mutations has been found in patients with leukodystrophy (III). For details about phenotype-genotype correlation analysis see 3.3.1. Mutations of NDUFS2 and NDUFS4 have been identified in patients with cardiomyopathy (IV), which fits to previous observations (details see 3.3.2 and 3.3.3). Mutations in NDUFS6 (see 3.3.4) were found in one patient with LIMD (VII). One female patient of the group with miscellaneous symptoms was found to have a heterozygous mutation in X-linked NDUFA1 (see 3.3.5 for further details).

#### 3.2.5.6 Brain imaging results in phenotype categories

In total, 72% of the RCC I deficiency patients showed abnormalities in the neuroimaging investigations (47/65 patients). The findings ranged from brain atrophy, leukodystrophy, lesions in basal ganglia or brainstem to cerebellar affection and others (e.g. agenesis of corpus callosum and grey matter lesions). Seventy percent of the patients of group I disclosed cMRI signs, including brain atrophy, of either basal ganglia or brainstem lesion (no combined defects) and cerebellar affection (16/23). All patients with information about the neuroimaging findings of group II and III presented abnormalities in CNS structures and were assigned to the categories because of these findings. Four out of twelve patients in category II with Leigh syndrome disclosed the combination of symmetrical lesions in basal ganglia and brainstem (33 %). In all other categories no patient was found to have this combination in brain imaging results. An example of these findings and the pathological correlate in macroscopic brain autopsy is shown in **Fig. 28**.

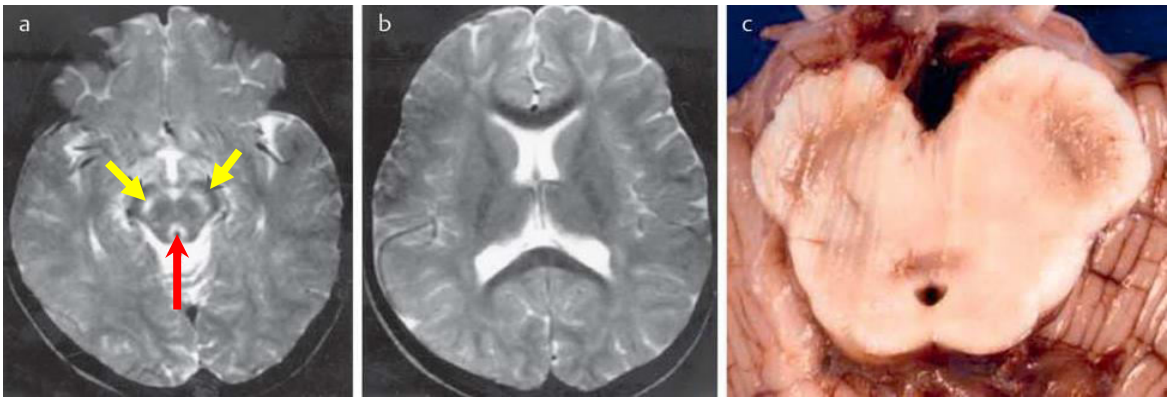



**Fig. 28.** cMRI ($T_2$-weighted images) shows increased signal in the midbrain in the substantia nigra (**a**: yellow arrow) and the periaqueductal region (**a**: red arrow), corpus callosum, internal capsule and basal nuclei are spared (**b**). Autopsy shows softening and brown discolouration of substantia nigra and subaqueductal area of the midbrain (**c**). [Herzer et al. 2010, p.31; see **Appendix. 7**]

The remaining eight patients had only one suspicious finding in either basal ganglia or brainstem. Within the group of patients with Leigh syndrome the feature of leukodystrophy has been additionally observed in 33%. Patients of group III only had lesions in white matter, without the affection of basal ganglia or brainstem despite other structures. As expected no patient of the myopathic group showed alterations in brain imaging investigations. In three patients (30%) with mitochondrial syndrome brain imaging investigations were performed. Stroke-like lesions were found in two of them and confirmed the diagnosis of MELAS. Investigated patients of group LIMD disclosed no abnormalities, which can be possible due to the early age at time of examinations [Spiegel et al. 2009]. In the miscellaneous group only one patient with ataxia showed signs of cerebellar affection.

## 3.3 Genotype – phenotype correlations

To date, no clear phenotype-genotype correlations have been described for patients with RCC I deficiency. One topic of this work was the analysis of the phenotypes presented by the patients with mutations in nDNA in comparison with phenotypes of patients reported in the literature. The analysis focused on mutations affecting nuclear encoded genes of RCC I. So far, about 50 cases with nDNA mutations and RCC I deficiency have been published. Within the mutation screen, 15 new patients with isolated RCC I deficiency were identified as carrying pathogenic mutations in nuclear encoded subunits and assembly factors of RCC I. This contributes to the understanding of the pathogenicity in RCC I defects caused by mutations in the nuclear genome. The relatively small number of identified patients with mtDNA mutations is no major contribution to previous knowledge about RCC I defects caused by mutations in mtDNA. Because of the high amount of previous reports about mtDNA mutations in RCC I deficiency patients and the small number of newly identified patients no correlation studies were performed within this present work.

For all genes discussed in the following no correlations between RCC I activity and the severity of the phenotype have been observed.

### 3.3.1 NDUFS1 – encephalomyopathy and Leigh syndrome

NDUFS1 is the largest conserved subunit of RCC I with a molecular weight of 75 kDa containing three Fe-S clusters [Ohnishi 1998; Papa et al. 2009]. So far, ten patients with mitochondrial encephalopathy have been found to harbour large scale deletions or point mutations in NDUFS1 [Bénit et al. 2001; Bugiani et al. 2004; Martin et al. 2005; Pagniez-Mammeri et al. 2009; Hoefs et al. 2010; Pagniez-Mammeri et al. 2010; Tuppen et al. 2010]. Twelve new mutations in six patients have been identified in our patient cohort. The clinical history of patients is shown in **Table. 15**. For patient 6 no details about the clinical phenotype were given [Pagniez-Mammeri et al. 2009].

**Fig. 29** illustrates that most patients with mutations in NDUFS1 have clearly reduced RCC I activity with a median of 36% (range 20-128%; n=13). The clinical phenotype did not correlate with the severity of the enzyme defect as demonstrated by the similar fatal course of disease in patient 1 and 8 (details see **Table. 15**).

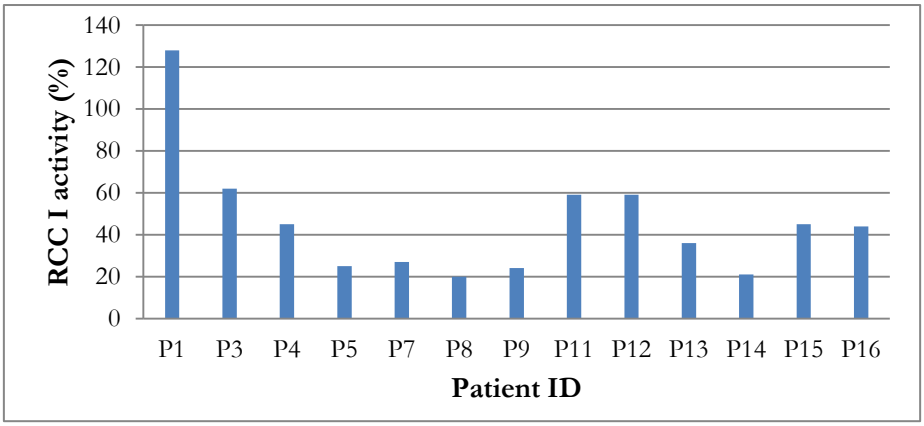



**Fig. 29.** Residual RCC I activity in percentages of lowest control values in patients with NDUFS1 mutations. Patient 2 is not shown because RCC I activity reported was not significantly reduced [Bénit et al. 2001, p.1345] and information for patient 6 and 10 were not given in the reports.

All patients carried at least one missense mutation[12] (see **Table. 14**).

**Table. 14.** Patients with NDUFS1 mutations and RCC I activities

| N° | Reference | Mutation | RCC I activity of lowest control values | Tissue |
|---|---|---|---|---|
| 1 | Bénit et al. 2001 | c.[664_666del; 755A>G]<br>p.[del222; D252G] | 128% (based on NCP) | Muscle homogenate |
| 2 | Bénit et al. 2001 | c.[721C>T; 1669C>T]<br>p.[R241W; R557X] | 226% (based on NCP) | Muscle mitochondria |
| 3 | Bénit et al. 2001 | c.[2219A>G], p.[M707V]<br>de novo deletion of paternal NDUFS1 allele | 62% (based on NCP) | Muscle mitochondria |
| 4 | Bugiani et al. 2004 | c.[1564C>A]+[1564C>A]<br>p.[Q522K]+[Q552K] | 45% (based on CS) | Muscle homogenate |
| 5 | Martín et al. 2005 | c.[691C>G]+[691C>G]<br>p.[L231V]+[L231V] | 25% (based on CS) | Muscle homogenate |
| 6 | Pagniez-Mammeri et al. 2009 | c.[683T>C; 755A>G]<br>p.[V228A; D252G] | N/A | N/A |
| 7 | Hoefs et al. 2010 | c.[1855G>A; 1669C>T]<br>p.[D619N; R557X] | 27% (based on COX) | Fibroblasts |
| 8 | Hoefs et al. 2010 | c.[1222C>T]+[1222C>T]<br>p.[R408C]+[R408C] | 20% (based on COX) | Fibroblasts |
| 9 | Pagniez-Mammeri et al. 2010 | c.[631_633delGAA]+[683T>C]<br>p.[211delE]+[V228A] | 24% (based on COX) | Fibroblasts |
| 10 | Tuppen et al. 2010 | c.[1222C>T]+[1222C>T]<br>p.[R408C]+[R408C] | N/A | N/A |
| 11 | IHG: 33255 | c.[497G>A; 683T>C]<br>p.[G166E; V228A] | 59% (based on CS) | Muscle homogenate |
| 12 | IHG: 33460 | c.[683T>C; 754A>G]<br>p.[V228A; D252G] | 59% (based on CS) | Muscle homogenate |
| 13 | IHG: 36179 | c.[212T>A;384T>A]<br>p.[V71D; C128X] | 36% (based on NCP) | Muscle homogenate |
| 14 | IHG: 35837 | c.[1669C>T; 1783A>G]<br>p.[R557X; T595A] | 21% (based on CS) | Muscle homogenate |
| 15 | IHG: 35822 | c.[2083T>C; 2084A>G]<br>p.[Y695H; Y695H] | 45% (based on CS) | Muscle homogenate |
| 16 | IHG: mt4258 | c.[1912delA; 2084A>G]<br>p.[T638fsX642; Y695C] | 44% (based on CS) | N/A |

N/A= no available information; IHG: Institute of Human Genetics TU Munich

Patients with NDUFS1 mutations showed first clinical symptoms between birth and 14 months of age (median age of five months; n=15). The age of death ranged from five to 46 months of age (median 18 months; n=12). Median lifetime from onset of first symptoms till death was twelve months (n=12).

First symptoms ranged from optic nerve atrophy or nystagmus to muscular hypotonia, failure to thrive and psychomotor development delay or regression. All patients shared pathological abnormalities in brain imaging investigations like leukodystrophy, signal abnormalities in basal ganglia and brainstem, brain atrophy or structural abnormalities in the cerebellum and mesencephalon.

Clinical hallmarks in patients with mutated NDUFS1 are symptoms due to the severe affected CNS correlating with the neuroimaging findings reported. All patients presented psychomotor development delay or regression. Another frequent symptom observed was failure to thrive in seven patients (47%). Most patients suffered from feeding problems, recurrent vomiting or dysphagia. For eleven patients muscular hypotonia has been reported (69%) and nine patients

[12] Missense mutations are point mutations in which a single nucleotide is changed, resulting in a codon that codes for a different amino acid.

(60%) developed spasticity or dystonia (signs of pyramidal syndrome[13]) during disease progression. The optical tract was less frequently affected with 47% of patients presenting with symptoms of optic nerve atrophy and nystagmus (n=7). Eleven patients displayed at least one time elevated lactate levels (73%). So far, in patients with NDUFS1 mutations no hepatic, cardiac or endocrine dysfunction has been described.

[13] A disorder characterized by dysfunction of the corticospinal (pyramidal) tracts of the CNS. Symptoms include an increase in the muscle tone in the lower extremities, spasticity, hyperreflexia, positive Babinski and a decrease in fine motor coordination.

**Table. 15.** Clinical features of patients with NDUFS1 mutations

| N° | Age of onset | First symptoms | Course | Brain imaging findings | Sex | Familiar |
|---|---|---|---|---|---|---|
| 1 | 4 months | Psychomotor retardation with hypotonia | 7 months: nystagmus, bilateral optic nerve atrophy, lactic acidosis (blood and CSF)<br>→ died from an acute episode of bradycardia at the age of 10 months | Leukodystrophy | m | + (a) |
| 2 | 2 months | Growth retardation, axial hypotonia, hepatomegaly, lactic acidosis | - developed macrocytic anaemia and dystonia<br>→ died at the age of 5 months | Hyperintensity of basal ganglia | m | + (a) |
| 3 | postnatal | Failure to thrive with muscular hypotonia, microcephaly, pyramidal syndrome | 5 months: lactic acidosis (blood and CSF), anaemia (necessitated blood transfusions) | Structural abnormalities of corona radiata, suggestive of Leigh syndrome | m | N/A |
| 4 | 6 months | Psychomotor regression evolving into spastic quadriparesis | - developed spastic quadriparesis with loss of postural control | Leukencephalopathy | m | + (a,b) |
| 5 | 8.5 months | Hospitalized with recurrent episodes of vomiting, floppiness and growth retardation, nystagmus, generalized muscular hypotonia | 13 months: deterioration with respiratory insufficiency, lactic acidosis and pyramidal signs<br>→ died 2 months later | Bilateral lesions affecting substantia nigra, midbrain | f | - |
| 7 | 8 months | Psychomotor regressions, abnormal crying | - spasticity, microcephaly, mental retardation and progressive neuropathy developed<br>→ died at the age of 12 years | Bilateral symmetrical atrophic, leukodystrophic and cystic lesions | f | - |
| 8 | 4 months | Episodic brainstem events | - abnormal breathing pattern, feeding problems, muscle dystrophy and muscular hypotonia<br>→ died at the age of 8 months | Leukencephalopathy | m | + (a,b) |
| 9 | 5 months | Nystagmus | 12 months: psychomotor and mental development stagnated, metabolic acidosis<br>15 months: loss of neurological abilities, failure to thrive, crying, eating difficulties, spasticity<br>→ died at the age of 2 years | Leukencephalopathy | f | - |
| 10 | 4 months | Axial muscular hypotonia, poor feeding, vomiting, failure to thrive | - developed intermittent nystagmus, no fixation<br>8 months: spasticity developed, dysphagia<br>→ at 10.5 months central hypopnoea, limb hypotonia and encephalopathy with death 24 h later; lactate levels elevated in blood and CSF | Extensive symmetrical abnormalities in cerebral peduncles, anterior pons, posterior limbs of internal capsule, changes in cerebellar white matter | f | + (a,b) |
| 11 | 5 months | Optic nerve atrophy | 9 months: psychomotor regression, spasticity, poor spontaneous movements<br>- nystagmus, dystonia, dysphagia, persistent lactic acidosis<br>→ died at the age of 3 years and 10 months | Leukodystrophy, brain atrophy, symmetrical lesions affecting pons and medulla oblongata, atrophic corpus callosum | N/A | - |

| | | | | | | |
|---|---|---|---|---|---|---|
| 12 | 6 months | Muscular hypotonia, psychomotor regression | - developed persistent lactic acidosis, neutropenia and dysphagia<br>→ died at the age of 19 months | Leukodystrophy, hyperintensity in medulla oblongata and periventricular areas, abnormalities of cerebellum | f | - |
| 13 | postnatal | Failure to thrive with muscular hypotonia, sucking weakness, seizures | - psychomotor development delay, myoclonus, lactic acidosis, optic nerve atrophy<br>→ died at the age of 5 months | Enlarged external cerebral fluid interspaces | m | - |
| 14 | 5 months | Psychomotor regression, nystagmus | - muscular hypotonia, spasticity, myoclonus, coma episodes with recurrent vomiting and persistent lactic acidosis<br>→ died at the age of 2.5 years | Leukodystrophy, signal abnormalities in cerebellum | f | - |
| 15 | 14 months | Psychomotor regression, pyramidal syndrome, muscular hypotonia | - dysphagia in course | Leukodystrophy | m | - |
| 16 | postnatal | Psychomotor regression, muscular hypotonia | - lactic acidosis and elevated CSF lactate values<br>→ died at the age of 9 months due to cardiorespiratory failure | Leukodystrophy, symmetrical lesions in basal ganglia, hyperintensity in cerebellum | f | - |

m: male; f: female; (a) one/more siblings affected or dead (b) related parents; N/A= no available information

### 3.3.2 NDUFS2 – encephalomyopathy and cardiomyopathy

NDUFS2 is one of the largest subunits of RCC I. It has been shown that disruption of the subunit results in complete absence of the peripheral arm of RCC I [Guenebaut et al. 1997]. So far, seven patients with different missense mutations in the NDUFS2 encoded subunit of RCC I have been reported with features of encephalopathy and cardiomyopathy by Loeffen et al. [2001] and Tuppen et al. [2010]. We identified one patient, harbouring two missense mutations, who showed signs of encephalopathy in cMRI and severe hypertrophic cardiomyopathy.

The median RCC I residual activity in patients with NDUFS2 mutations was 31.5% (range 12-68%; n=4). No correlation between the severity of enzyme defect and the presenting phenotype exists. For example patient 9 with clearly reduced RCC I activity had no progressive course and is actually stable, whereas the remaining patients died in young childhood. Detailed information about history and clinical features of the patients with NDUFS2 mutations are listed in **Table. 16**.

The patients had a median onset of first symptoms at seven months (range 0-34 months; n=8). Five of the eight patients presented with severe progressive course of disease and died at age of four days - 36 months (median 12 months; n=5). Two of the reported patients were still alive at time of publications and the newly identified patient is also actually well without neurological impairment and clinically stable [Baric 2009, personal communication]. Our patient showed initially severe hypertrophic cardiomyopathy, lactic acidosis and recurrent episodes of pseudo-obstruction soon after birth and had two affected sibs who died due to cardiac failure in the neonate period. Cardiomyopathy improved significantly under riboflavin therapy.

Cardiomyopathy has been reported to be a frequent symptom in patients harbouring NDUFS2 mutations. Here, only 50% of patients displayed signs of cardiac involvement, mainly hypertrophic cardiomyopathy [Loeffen et al. 2001]. Therefore, it should be noted that cardiac signs are seen in patients with NDUFS2 mutations, but are not as frequent as it was assumed.

Further common features are ophthalmological signs like nystagmus and optic nerve atrophy (56%; n=5). Other symptoms like muscular hypotonia, lactic acidosis, failure to thrive and dystonia were present as well. However, no remarkable frequency (e.g. for cardiomyopathy) or similar patterns of clinical symptoms and outcome has been observed in the patients.

**Table. 16.** Clinical features of patients with NDUFS2 mutations
m: male; f: female; (a) one/more siblings affected or dead (b) related parents. N/A: no information available;

| N° | Ref. | Mutation | Age of onset | First symptoms | Course | Brain imaging findings | Sex | Familiar |
|---|---|---|---|---|---|---|---|---|
| 1 | Loeffen et al. 2001 | c.[683G>A]+[683G>A]<br>p.[R228Q]+[R228Q] | 6 months | Neurological regression, nystagmus, bilateral optic nerve atrophy, axial muscular hypotonia, left ventricular hypertrophy, lactic acidosis | - neurological condition deteriorated, episodes of apnoea<br>→ died at age of 24 months | Bilateral hypodensities in basal ganglia | m | + (a, b) |
| 2 | | c.[686C>A]+[686C>A]<br>p.[P229Q]+[P229Q] | postnatal | Respiratory insufficiency, lactic acidosis, hypertrophic cardiomyopathy | → died at the age of 4 days due to cardiorespiratory failure | N/A | m | - |
| 3 | | c.[1237T>C]+[1237T>C]<br>p.[S413P]+[S413P] | 10 months | Cyclic vomiting, failure to thrive, nystagmus, muscular hypotonia, psychomotor development delay, lactic acidosis | 2.5 years: recurrent episodes of sleep-apnoea<br>→ died at age 3 years | Hypodensity of basal ganglia and midbrain | m | + (a, b) |
| 4 | Tuppen et al. 2010 | c.[353G>A; 875T>C]<br>p.[P20T; M929T] | postnatal | Poor feeding, vomiting and failure to thrive | 8 months: failure to thrive, delayed development, muscular hypotonia, nystagmus and metabolic acidosis<br>→ 22 months hypopnoea and death | Symmetrical abnormalities in cerebral peduncles, dorsal pons and upper medulla | f | - |
| 5 | | c. [875T>C; 1328T>A]<br>p. [M929T; M443K] | infancy | Gross motor skills delayed due to dystonia | - delayed speech additionally hampered by dysarthria, not able to walk until 3 years<br>- since age of 6 years seizures<br>- at time of the report 9 years old, attended mainstream school | Bilateral symmetrical lesions of basal ganglia | f | - |
| 6 | | c.[442G>A; 875T>C]<br>p.[E148K; M929T] | infancy | Psychomotor delay, learning difficulties, episodes of tonic upward eye deviation | - dystonia, optic nerve atrophy, dysarthria, dysphagia developed till 11 years | Bilateral low-density lesions in cerebral peduncles, high $T_2$-signal in thalami and front lobes (changes resolved at age 5 years, but new lesions in caudate and lentiform nuclei appeared) | f | - |
| 7 | | c.[413G>A; 1054C>G]<br>p.[R138Q; P352A] | postnatal | Metabolic acidosis | - feeding problems, vomiting, mild left ventricular hypertrophy<br>→ 3.5 months respiratory arrest and death | Normal | f | - |
| 8 | | c.[866+4A>G; 875T>C]<br>p.[5´splice-site intron6; M929T] | 34 months | Developmental delay, ataxia, nystagmus, optic nerve atrophy, mild persistent lactic acidosis | No further details about course of disease | Normal at age of 6 years | m | N/A |
| 9 | IHG: 33354 | c.[968G>A; 329A>T]<br>p.[ Q323R; D110V] | 3.5 months | Hypertrophic cardiomyopathy, recurrent episodes of pseudo-obstruction, lactic acidosis, signs of hepatopathy | - actually, 4 years old with normal neurological condition<br>- hypertrophic cardiomyopathy improved under riboflavin therapy | Generalized brain atrophy, diffuse increased signals in white matter (at age of 3.5 months) | f | + (a) |

### 3.3.3 NDUFS4 – severe encephalomyopathy and cardiomyopathy

First described in bovine heart mitochondria RCC I contains a nuclear encoded subunit which is phosphorylated by a cAMP[14]-dependent protein kinase [Sardanelli et al. 1995; Papa et al. 1996b]. This mature 18 kDa subunit of 175 amino acids is encoded by NDUFS4. The NDUFS4 subunit is highly conserved in mammals and the first patient described with a frame-shift mutation in this gene displayed clinics of a fatal neurological Leigh-like syndrome [Walker et al. 1992; van den Heuvel et al. 1998]. Papa et al. found that cAMP fails to promote phosphorylation of the NDUFS4 subunit in this patient and reasoned that cAMP-dependent phosphorylation of this subunit is necessary for RCC I activation [Papa et al. 2001]. Recently, a mouse model was developed by targeting the NDUFS4 subunit that exhibited several clinical features seen in RCC I deficiency patients mostly suggestive of progressive encephalopathy resembling Leigh syndrome in the analysed knock-out mouse which also had a risk of cardiomyopathy [Kruse et al. 2008; Quintana et al. 2010].

One known homozygous loss of function mutation in NDUFS4 has been identified in one patient during the mutation screen. So far, seven patients, all with loss of function mutations (frame-shift or stop mutations), have been described in the literature. The patients presented a phenotype suggestive for Leigh or Leigh-like syndrome with brainstem and basal ganglia involvement. Additionally, cardiomyopathy was noticed in three patients [van den Heuvel et al. 1998; Budde et al. 2000; Petruzella et al. 2001; Bénit et al. 2003b; Anderson et al. 2008; Leshinsky-Silver et al. 2009]. The clinical course of our patient was similar to the published patient with the same mutation [Budde et al. 2000]. Details of clinical course of the patients are listed in **Table. 17**.

**Fig. 30** shows the RCC I residual activities of the patients. The median RCC I activity was 48.5% (range 14-100%; n=8). Again, no correlations between the severity of clinical symptoms and the residual activity have been observed. For example, patient 7 had a RCC I residual activity of 54% and died at the age of 27 months (**Table. 17**) [Leshinsky-Silver et al. 2009]. In contrast, patient 6 had RCC I activity within normal ranges but had severe encephalomyopathy and died at the age of ten months [Anderson et al. 2008].

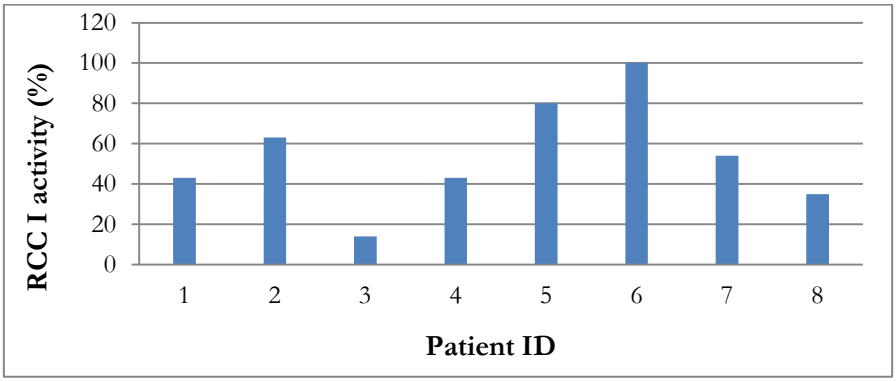



**Fig. 30.** RCC I activities in percentages of lowest control values in patients with NDUFS4 mutations.

The onset of first symptoms has been observed at a median age of two months (range 0-8 months; n=8). All patients had a severe progressive clinical course. The age of death ranged from three to 27 months (median 5.5 months; n=8). All patients presented psychomotor development delay or regression, pathological and inadequate reactions to their environment

[14] Cyclic adenosine-5'-monophosphate

and failure to thrive with symptoms like sucking weakness and recurrent vomiting. Further commonly observed features are seizures, lactic acidosis and muscular hypotonia. Respiratory insufficiency was frequent and in most cases the cause of death. Additionally, echocardiography disclosed hypertrophic cardiomyopathy in three patients (38%). Children with a multi-systemic metabolic disorder with heart involvement and RCC I deficiency should be screened for NDUFS4 and NDUFS2 (see 3.3.2) mutations. Compared with patients harbouring mutations in NDUFS2, the patients described with NDUFS4 mutations presented a more fulminant course of disease.

However, patients with NDUFS4 mutations showed the clinical picture of an acute and fulminant course of encephalomyopathy, which was often associated with changes in the CNS (typical seen in Leigh syndrome) in addition to failure to thrive and muscular hypotonia in all patients, no distinct pattern of symptoms has been observed.

**Table. 17.** Clinical features of patients with NDUFS4 mutations
m: male; f: female; (a) one/more siblings affected or dead (b) related parents. VEP: visual evoked potentials

| N° | Reference | Mutation | Age of onset | First symptoms | Course | Brain imaging findings | Sex | Familiar |
|---|---|---|---|---|---|---|---|---|
| 1 | van den Heuvel et al. 1998 | c.[466-470dupAAGTC]+[466-470dupAAGTC] p.[K158fs]+[K158fs] | 8 months | Admission due to cyclic vomiting, failure to thrive, muscular hypotonia | 13 months: motor development delay, seizures, cyanosis and bradypnoea<br>→ died at the age of 16 months due to cardiorespiratory failure | Generalized brain atrophy, symmetrical lesions in the basal ganglia | m | - |
| 2 | Budde et al. 2000 | c.[289-290del]+[289-290del] p.[W96X]+[W96X] | postnatal | Failure to thrive, muscular hypotonia, lethargy | 3 months: microcephaly, lactic acidosis, no detectable VEPs<br>→ died at the age of 3 months due to respiratory insufficiency | Symmetrical lesions in the basal ganglia | f | + (b) |
| 3 | Budde et al. 2000 | c.[316C>T]+[316C>T] p.[R106X]+[R106X] | 7 weeks | Muscular hypotonia, lack of visual and auditory attention | 3 months: admission due to respiratory insufficiency under pneumonia, facial dysmorphic features, hepatosplenomegaly, lactic acidosis<br>Echocardiogram: left ventricular hypertrophy<br>→ died at the age of 3 months due to cardiorespiratory failure | Symmetrical lesions in the basal ganglia | m | + (b) |
| 4 | Petruzella et al. 2001 | c.[44G>A]+[44G>A] p.[W14X]+[W14X] | 2 weeks | Tonic-clonic seizures, failure to thrive, vomiting | - severe progressive psychomotor development delay, loss of contact, muscular hypotonia, seizures<br>Echocardiogram: hypertrophic cardiomyopathy<br>→ comatose status (subsequent to apnoeic episode) until death at 7 months of age | Symmetrical lesions in the basal ganglia | f | - |
| 5 | Bénit et al. 2003b | 76bp deletion, resulted in complete skipping of exon 2 | 10 weeks | Sucking weakness, muscular hypotonia, poor movements and reactions | - lactic acidosis<br>→ died at 4 months of age of major swallowing difficulties, hypoventilation and severe brainstem involvement | Bilateral hypodensity of periventricular white matter, suggestive for Leigh syndrome | f | + (a,b) |
| 6 | Anderson et al. 2008 | c.[462delA]+[462delA] frame shift mutation | 3.5 months | Exotropia, ptosis, muscular hypotonia, head lag | 6 months: admission due to failure to thrive, respiratory insufficiency, decreased gut motility, tracheostomy, hypertrophic cardiomyopathy, developmental regression, became less responsive and more hypotonic<br>→ died at the age of 10 months | Bilateral abnormalities in cerebral peduncles, red nuclei, pons and medulla | m | + (a) |
| 7 | Leshinsky-Silver et al. 2009 | p.[D119H;K154fs] | 8 months | Myoclonus (during febrile illness) | 12 months: sudden onset exotropia, hyperreflexia<br>19 months: mildly delay of motor skills (wide-based unstable gait)<br>26 months: ophthalmoplegia, ataxia, tremor<br>→ died 6 weeks later due to respiratory insufficiency | Symmetrical lesions in medulla, midbrain and pons | m | - |
| 8 | IHG: 33253 | c.[316C>T]+[316C>T] p.[R106X]+[R106X] | postnatal | Muscular hypotonia, failure to thrive, dyskinetic movements | - microcephaly, sucking weakness, respiratory insufficiency, recurrent vomiting, nystagmus, lactic acidosis, cortical blindness<br>Echocardiogram: hypertrophic cardiomyopathy<br>Electrocardiogram: conduction impairments<br>→ died at 4 months of age due to cardiocirculatory failure | Brain atrophy, symmetrical lesions in the basal ganglia, brainstem and cerebellum | f | + (b) |

### 3.3.4 NDUFS6 – fatal infantile mitochondrial disease

NDUFS6 encodes a 13 kDa subunit of RCC I which is located in the Fe-S-fraction [Kirby et al. 2004b]. Kirby et al. assumed on the basis of their observations with complementation studies of fibroblasts from RCC I deficiency patients and the according phenotype, that mutations in NDUFS6 either prevent complete assembly or destabilize the peripheral arm of RCC I [Kirby et al. 2004b]. Patients harbouring NDUFS6 mutations presented with the clinical phenotype of LIMD [Kirby et al. 2004b; Spiegel et al. 2009]. The authors speculated that the severe phenotype is the result of impaired interaction of RCC I with RCC III [Spiegel et al. 2009]. The newly identified patient with a homozygous loss of function mutation displayed similar clinical features as reported patients. Details are listed in **Table. 18**. All patients showed signs of encephalopathy including muscular hypotonia, seizures, drowsiness, less or absent reactions on stimuli and severe lactic acidosis soon after birth. During first days of life all of them expired due to respiratory insufficiency and the persistent metabolic acidosis. Brain imaging results were normal in investigated patients, but neuropathological investigations disclosed congestion of the brain. Median RCC I residual activity was clearly reduced to 17% of the lowest control values (range 4-40%; n=4).

Clinical hallmark of patients with NDUFS6 mutations is the phenotype of LIMD and molecular-genetic analysis should be recommended for patients with RCC I deficiency having this phenotype.

**Table. 18.** Clinical features of patients with NDUFS6 mutations

| N° | Reference | Mutation | Age of onset | First symptoms | Course | Brain imaging findings | Sex | Familiar |
|---|---|---|---|---|---|---|---|---|
| 1 | Kirby et al. 2004b | 4.175 kb deletion exon 3+4 | postnatal | Muscular hypotonia, drowsiness, nystagmus, seizures, severe lactic acidosis, less spontaneous movements | - persistent lactic acidosis<br>- needed assisted ventilation due to hypoventilation<br>→ died on day 6 of life | Normal CT scan, but neuropathology disclosed congestion of basal ganglia, thalamus and periventricular region of brainstem suggestive for severe hypoxia | m | + (b) |
| 2 | Kirby et al. 2004b | c.[186+2T>A]+[186+2T>A] frame-shift mutation | postnatal | | → died few days later due to central hypoventilation | | f | + (a,b) |
| 3 | Spiegel et al. 2009 | c.[344G>A]+ [344G>A] p.[C115T]+ [C115T] | postnatal | Muscular hypotonia, drowsiness, severe lactic acidosis | - comatose and apnoeic<br>→ died within the first week of life owing to respiratory insufficiency | Normal (brain ultrasound only performed in one patient) | m/f: 1/3 | + (a) |
| 4 | IHG: 35797 | c.[352C>T]+[352C>T] p.[Q118X]+[Q118X] | postnatal | Muscular hypotonia, comatose, seizures, myoclonus | - persistent lactic acidosis<br>- needed assisted ventilation<br>→ died few days later due to respiratory insufficiency | Normal | m | - |

m: male; f: female; (a) one/more siblings affected or dead (b) related parents

### 3.3.5 NDUFA1 – unspecific encephalomyopathy and cytopathy

NDUFA1 has been mapped to the X-chromosome and encodes a small integral membrane protein of 70 amino acids [Gabaldon et al. 2005]. It has been shown to play an essential role in assembly process and function of RCC I in mammals and fungi [Au et al. 1999]. So far, only male patients with RCC I deficiency and mutations in NDUFA1 have been reported with the phenotype of neurodegenerative mitochondrial disease with variable outcome [Fernandez-Moreira et al. 2007; Potluri et al. 2009]. A female patient showed a mild form of mitochondrial cytopathy and has been identified to harbour a heterozygous missense mutation known for three male patients reported by Potluri et al. [2009]. Details of phenotype presentation are shown in **Table. 19.**

The median RCC I activity of NDUFA1 patients was 25% (range 6-36%; n=4). Again, no correlation between severity of symptoms and RCC I activity was found. Patient 1 with 20% residual activity presented the most progressive course and died early, but the patient with 6% residual activity is clinically stable. This patient suffered from neurodegenerative mitochondriopathy associated with psychomotor and mental retardation, gait disturbances, ataxia, epilepsy, eye involvement and psychiatric symptoms [Fernandez-Moreira et al. 2007; Potluri et al. 2009]. The girl newly identified had been observed as having three times acetone-like mouth breath, mildly muscular hypotonia and mildly elevated blood lactate levels at this time [Mayr and Sperl, unpublished, Department of Paediatrics, University Hospital Salzburg]. She has had no signs of psychomotor or mental development delay and other organ involvements. The positive family history, including two male miscarriages, pointed towards an X-linked inherited disorder. Although, she is only a heterozygous mutation carrier X-inactivation could explain the observed phenotype. Indeed Mayr et al. found a correlation of RCC I activity and the rate of inactivation of the mutated allele in three analysed tissues (blood, muscle and fibroblasts). Compared to the phenotypes of male patients, she had only mild symptoms.

Although patients with mutations in NDUFA1 had clearly reduced RCC I activity, the clinical presentation and outcome of patients was relatively 'mild' compared to patients with mutations in other nuclear genes (3.3.1-3.3.4). The median age of onset was 8 months (range 4-48 months; n=4).

Molecular-genetic investigations of NDUFA1 should be considered in patients with RCC I deficiency and delayed psychomotor development and signs of affected cerebellum.

**Table. 19.** Clinical features of patients with NDUFA1 mutations

| N° | Reference | Mutation | Age of onset | First symptoms | Course | Brain imaging findings | Sex | Familiar |
|---|---|---|---|---|---|---|---|---|
| 1 | Fernandez-Moreira et al. 2007 | c.[22G>C], p.[G8R] hemizygous | 4 months | Psychomotor development delay | 7 months: muscular hypotonia, nystagmus, choreoathetosis, lactic acidosis<br>13 months: deterioration leading to respiratory insufficiency<br>→ died at 14 months of age owing to cardiorespiratory arrest | Bilateral lesions of thalamus, cerebral peduncles and brainstem | m | - |
| 2 | Fernandez-Moreira et al. 2007 | c.[251G>C] p.[R37S] hemizygous | 6 months | Psychomotor development delay | 4 years: supported walk, delayed language skills, muscular hypotonia<br>9 years: developed myoclonic epilepsy, AEP disclosed defect in auditory response in pons and mesencephalon<br>→ at 10 years of age stable clinical evolution | Cerebellar atrophy | m | - |
| 3 | Potluri et al. 2009 | c.[94G>C], p.[G32R] hemizygous | 4 years | Psychomotor regression, aphasia | - epilepsy, dementia, mental retardation<br>- ataxia, gait imbalance<br>- retinitis pigmentosa, sensorineural hearing loss<br>- progressive behavior disorder | Cerebellar atrophy, increased signal intensity in anterior medulla | m | + (a) |
| 4 | IHG: 33545 | c.[94G>C], p.[G32R] heterozygous | 11 months | Acetonic mouth breath | - mildly elevated lactate levels, mildly decreased muscle tone<br>→ normal mental and psychomotor development | N/A | f | + (a) |

m: male; f: female; (a) one/more siblings affected. AEP: auditory evoked potentials; N/A: no information available

### 3.3.6 NDUFAF2 – encephalomyopathy and Leigh syndrome

NDUFAF2 is a paralogue of the NDUFA12L gene, also known as B17.2L, mimitin, MMTM or FLJ22398 [Gabaldon et al. 2005]. It encodes for a 20 kDa protein that was found to be associated to RCC I subunits ND1, NDUFS2, NDUFS3, NDUFS4, NDUFV1, NDUFV2 and NDUFA13. This suggests that NDUFAF2 is required in a late stage of RCC I assembly and stabilizes RCC I intermediates [Ogilvie et al. 2005; Fernandez-Vizarra et al. 2009; Lazarou et al. 2009]. So far, three patients with different mutations have been described in the literature, all harbouring mutations resulting in a truncated NDUFAF2 protein [Ogilvie et al. 2005; Barghuti et al. 2008; Hoefs et al. 2009]. Barghuti and colleagues identified the same mutation in two unrelated patients by homozygosity mapping who showed similar clinical symptoms and cMRI findings. All patients displayed an isolated RCC I deficiency with 31% median activity of lowest control values (range 12-55%; n=5).

Detailed information about reported patients and the newly identified patient are given in **Table. 20**. Patients with NDUFAF2 mutations presented first symptoms at a median age of twelve months (range 3-20 months; n=5), whereas the dominant symptoms observed were nystagmus and gait disturbances as signs of cerebellar affection and acute apnoeic episodes. Ogilvie and Barghuti reported three patients which shared similar imaging findings and showed normal or only mildly delayed psychomotor development, nystagmus or optic nerve atrophy interspersed by acute episodes of encephalopathy with brainstem involvement and the cardinal symptoms of respiratory insufficiency and loss of abilities [Ogilvie et al. 2005; Barghuti et al. 2008]. Patient 4 presented first symptoms at an earlier age and showed no acute episodes. She deteriorated gradually until her death due to respiratory arrest. Furthermore, she was the only patient with symmetrical lesions found in basal ganglia beside the other findings in cMRI typically suggestive for Leigh-like syndrome [Hoefs et al. 2009].

Newly identified patient 5 resembled a very similar phenotype compared with the reported patients, especially to the patients of Barghuti and colleagues. The boy was the first child of non-consanguineous Austrian parents and born after uneventful pregnancy. Early psychomotor development was mildly delayed (able to walk free at 22 months). Horizontal nystagmus was noticed at the age of 14 months. At the age of 26 months, due to an infectious episode, he was found apnoeic twice and transferred to the intensive ward owing to respiratory assistance. The CT scan at this time was normal. The patient regained consciousness and was removed at home. Three weeks later he was found apnoeic, cyanotic and comatose at home again. On admission the boy showed signs of foot drop position and bilateral positive Babinski[15]. At this time, cMRI disclosed symmetric lesions of the pons, crura cerebri, substantia nigra, medial longitudinal fasciculus and the cortico-spinal tracts, which is comparable to the findings of reported patients (lesions mainly in brainstem). Lactate measurements in blood and CSF were only elevated once, during an acute episode of respiratory insufficiency. Other clinical laboratory parameters were all within normal ranges [Herzer et al. 2010, see **Appendix. 7**]. Patients 2 and 3 repeatedly had normal lactate values and patient 1 presented only once with elevated CSF lactate. Patient 4 had lactate levels above normal values several times [Ogilvie et al. 2005; Barghuti et al. 2008; Hoefs et al. 2009]. For

[15] Babinski sign = hallux dorsal flexion and the other toes fan out, indicating damage of the CNS. Positive Babinski is physiological seen in newborn children because the cortico-spinal pathways that run from the brain down the spinal cord are not fully myelinated at this age, so the reflex is not inhibited by the cerebral cortex.

patients with NDUFAF2 mutations, lactate is no reliable indicator for the diagnosis. Problems with ventilation occurred and the patient died owing to cardiorespiratory failure at the age of 27 months. Post-mortem investigations revealed optic nerve atrophy, retinal pigment degeneration and signs of necrosis (status spongiosus and capillary proliferation) in brainstem without involvement of the basal ganglia, proving the diagnosis of Leigh syndrome (further description see **Appendix. 1**).

The patients with mutations in NDUFAF2 died at a median age of two years (range 1-13.5 years; n=5). All of them showed symptoms of severe encephalopathy with loss of abilities, especially respiratory problems due to the affected brainstem and coma episodes, which were partially observed to be reversible. Acute episodes of encephalopathy were common in these patients and cMRI findings were similar in all patients. Molecular-genetic analysis of assembly factor NDUFAF2 should be performed in patients with unspecific encephalomyopathy and RCC I deficiency.

**Table. 20.** Clinical features of patients with NDUFAF2 mutations

| N° | Ref. | Mutation | Age of onset | First symptoms | Course | Brain imaging findings | Sex | Familiar |
|---|---|---|---|---|---|---|---|---|
| 1 | Ogilvie et al. 2005 | c.[182C>T]+[182C>T]<br>p.[R45X]+[R45X] | 12 months | Nystagmus, wide-based gait | 3 years of age: (deterioration subsequent to infectious episode) ataxia, lethargy, deterioration of nystagmus, optic nerve atrophy<br>8 years of age: increasing muscular weakness, dysphagia and tracheostomy, coma for several months due to respiratory infection<br>→ immobile until death at the age of 13.5 years, no communication with environment, osteoporosis, sclerosis, severe muscle atrophy and polyneuropathy | Symmetrical lesions in mammilo-thalamic tracts, substantia nigra, medial lemniscus, medial longitudinal fasciculus, spino-thalamic tracts, hyperintensity in corpus medullare of the cerebellum (age of 3 years); extensive myelomalacia, leukodystrophy, cortical atrophy, vermian atrophy (age of 13 years) | f | - |
| 2 | Barghuti et al. 2008 | c.[1A>T]+[1A>T]<br>p.[M1L]+[M1L] | 8 months | Nystagmus, muscular hypotonia, optic nerve atrophy | 8 months: mild retardation of motor skills, nystagmus, optic nerve atrophy<br>18 months: apnoeic episodes, coma, respiratory insufficiency<br>→ died at the age of 21 months | Symmetrical lesions in mammilo-thalamic tracts, substantia nigra, medial lemniscus, medial longitudinal fasciculus, spino-thalamic tracts, periaqueductal gray matter, spared cortex and subcortical white matter | m | + (a,b) |
| 3 | | c.[1A>T]+[1A>T]<br>p.[M1L]+[M1L] | 20 months | Apnoeic episodes | normal psychomotor development till apnoeic episodes<br>- at admission: reanimation<br>- external ophthalmoplegia, respiratory insufficiency, coma with myoclonic seizures<br>- muscular hypotonia, nystagmus, ataxia<br>- stable in course, no deterioration<br>→ at 2 years of age again apnoeic episode but no effective reanimation | Symmetrical lesions in mammilo-thalamic tracts, substantia nigra, medial lemniscus, medial longitudinal fasciculus, spino-thalamic tracts, affected cerebellum, spared cortex and subcortical white matter | f | + (b) |
| 4 | Hoefs et al. 2009 | c.[114C>G]+[114C>G]<br>p.[Y38X]+[Y38X] | 3 months | Nystagmus | 7 months of age: admitted because of vomiting, failure to thrive (poor weight gain and feeding problems), renal tubular acidosis<br>- neurological: muscular hypotonia, developmental retardation, mainly affecting motor skills<br>- lactic acidosis, fumarate and malate in urine increased, development of dyskinetic movements, loss of abilities and respiratory alkalosis<br>→ died at the age of 1 year due to respiratory failure | Symmetrical hyperintensity in thalamus, cerebral peduncles, brainstem, spinal cord | f | - |
| 5 | IHG: 33462 | c.[9G>A]+[9G>A]<br>p.[W3X]+[W3X] | 14 months | Nystagmus, moderate gait disturbance | 26 months: two times apnoeic, cyanotic episodes<br>- tracheostomy 3 weeks later<br>→ died at the age of 27 months due to cardiorespiratory failure | Symmetrical lesions in substantia nigra, periaqueductal region, pontine tegmentum, spared corpus callosum and internal capsule, bilateral lesions in spinal cord, delayed myelination of cerebral white matter | m | - |

m: male; f: female; (a) one/more siblings affected or dead (b) related parents. VEP: visual evoked potentials

### 3.3.7 C6ORF66 – from LIMD to encephalomyopathy

The C6ORF66 (NDUFAF4) gene encodes for an RCC I assembly factor with a molecular weight of 20 kDa. So far, it is not clear how this assembly factor contributes to RCC I maturation. Saada has described five patients with a homozygous mutation (194T>C), which resulted in an exchange of leucine to proline at position 65 [Saada et al. 2009]. All patients presented immediately after birth with severe metabolic acidosis and three of them died during first week of life. The remaining patients suffered from recurrent exacerbations of the metabolic acidosis, developed a severe encephalopathy and one patient displayed a cardiomyopathy in echocardiography. The cMRI disclosed atrophy of both grey and white matter, pons, medulla and cerebellum. Only two patients were still alive at the time of the report (16 months and 7 years) showing severe encephalomyopathy including no reaction to external stimuli, spastic tone and contractures. The remaining four expired due to persistent lactic acidosis at 9-18 months of age [Saada et al. 2009]. One C6ORF66 patient, identified by the mutation screen (1.4.1), carried a homozygous missense mutation (23G>A) resulting in an amino acid exchange of a highly conserved glycine to aspartate at position eight. This patient was admitted soon after birth, due to poor sucking and failure to thrive beside a severe lactic acidosis (**Table. 21.**). He developed encephalopathy with seizures, muscular hypotonia and dyskinetic movements and an echocardiogram displayed signs of hypertrophic cardiomyopathy. Hypomyelination and symmetrical lesions in basal ganglia and brainstem have been observed. Like the other patients, he had severe progressive course of disease and died owing to cardiocirculatory failure at 10 months of age. All patients had a clearly reduced activity of RCC I in enzymatic measurements (range undetectable-21%; n=6).

The phenotype is variable in patients with mutations in assembly factor C6ORF66 ranging from LIMD to encephalopathy and cardiomyopathy. RCC I deficiency children presenting with severe metabolic acidosis, signs of encephalomyopathy and signs of cardiac involvement should be referred for molecular-genetic analysis of C6ORF66.

**Table. 21**. Clinical features of patients with mutations in C6ORF66

| Ref. | Age of onset | First symptoms | Course | Brain imaging findings | Sex | Familiar |
|---|---|---|---|---|---|---|
| Saada t al. 2008 | postnatal | Severe lactic acidosis | - 3 patients died within the first week of life (one with cardiomyopathy) due to LIMD<br>- remaining patients had recurrent episodes of lactic acidosis and severe encephalopathy, signs of an affected ophthalmological and auditory system | Severe brain atrophy of both grey and white matter, demyelination, atrophy of pons, medulla and cerebellum | m/f: 2/3 | + (a,b) |
| IHG: 33006 | 2 months | Poor sucking, failure to thrive, lactic acidosis | - persistent lactic acidosis<br>- muscular hypotonia, dyskinetic movements<br>- Echo: left ventricular hypertrophy<br>- cranial ultrasound: hyperintensity in basal ganglia, thalamus<br>- cerebral seizures<br>- psychomotor development delay<br>→ died at age of 10 months due to acute metabolic acidosis and subsequent cardiocirculatory failure | Hypomyelination, symmetrical lesions in basal ganglia and brainstem | m | - |

m: male; f: female; (a) one/more siblings affected or dead (b) related parents

# 4 Discussion

## 4.1 Mutation analysis of candidate genes in RCC I deficiency patients

In a previous mutation study, 150 samples of RCC I deficiency patients were analysed for sequence variants in all structural subunits, encoded by the mitochondrial or the nuclear genome, and in some assembly factors (NDUFAF1, NDUFAF2, Ecsit, C6ORF66, and C3ORF38). This has been so far the largest study of patients with isolated RCC I deficiency and in 18% of the patients pathogenic mutations have been identified [similar findings by Skladal et al. 2003a]. Numerous studies on RCC I deficiency patients and the molecular-genetic basis of the defect have been reported since the first mutation of nuclear encoded subunits was published in 1998 [Loeffen et al. 1998; van den Heuvel et al. 1998]. In our cohort, a nuclear genetic aetiology has been identified in 10% of the patients. Others reported frequencies of nDNA mutations ranging from 3.4% to 25% [Loeffen et al. 2000; Bénit et al. 2004a; Bugiani et al. 2004; Tuppen et al. 2010]. The variable range of the mutation detection rate is a result of different inclusion criteria and the methods used to detect mutations. The lower frequency of nuclear mutations observed in our patient cohort can be a result of unidentified mutations or probably reflect the more reliable mutation rate considering the cohort size. Common mtDNA mutations have been excluded previously in the cohort of analysed patients, which explains the low percentage of 8.6% of pathogenic mtDNA mutations identified. However, mtDNA mutations can account for a substantial fraction of RCC I deficiency patients and analysis of mtDNA should be recommended as an essential step in the diagnostic workout [Shoffner et al. 1996; Loeffen et al. 2000; Bugiani et al. 2004].

In contrast to the assumptions of Tuppen and colleagues, that the underlying genetic defect is only identified in about 50% of the patients as a result of missing high-throughput approaches in the routine diagnostic of RCC I deficiency patients, the high-throughput mutation screen performed at the IHG did not reveal the molecular basis of disease in the majority of patients [Tuppen et al. 2010]. New genes can be causative in some patients with RCC I defect, which have not been analysed or identified until now.

Within the present work, an analysis of five candidate genes previously associated with proper RCC I assembly and function was performed in the 150 patients with isolated RCC I deficiency [Pagliarini et al. 2008]. No mutation was identified in the C10ORF65, C3ORF10, C20ORF7 and C7ORF10 gene that could explain the defect in RCC I. In one patient with predominant neurological and nephritic manifestation, the screen has confirmed the previously described homozygous mutation in XPNPEP3 [O'Toole et al. 2010]. Despite heterozygous sequence variants no other patient has been identified to harbour two mutated XPNPEP3 alleles. Analysed exons and identified sequence variances are listed in **Table. 13**. In total, 81% (30/37) of the exons were analysed leaving the possibility of undetected mutations. In cases with one heterozygous mutation and possible pathogenic character (novel and highly conserved) all exons of the gene were sequenced, but in none of these cases further mutations could be detected.

In order to explain the RCC I defect, we assumed that the disorder is inherited in an autosomal-recessive way. However, other possibilities exist including autosomal-dominant hereditary or the deletion of one complete allele either maternal or paternal [Zeviani et al. 1990; Bénit et al. 2001] and therefore, it is possible that sequence variants we assumed not to

be pathogenic may cause RCC I deficiency. It is also possible that selected candidate genes are not essential for RCC I function and assembly.

One advantage of the Idaho LightScanner Technology is the parallel analysis of 192 DNA samples in duplicates, using the high standardization format of 384-well plates. Furthermore, it constitutes a time and cost effective method and allows recovery of samples for further analysis (e.g. Sanger sequencing). Many research and clinical examples of high-resolution melting analysis are published that show the use of melting curve analysis to obviate or complement traditional sequencing efforts [Aten et al. 2008; Audrezet et al. 2008; Meisinger et al. 2009; van der Stoep et al. 2009; Vossen et al. 2009]. The approach of analysing DNA melting curve profile is sensitive and both a time and cost effective method. Sensitivity is about 90% for heterozygous mutations and therefore it can be expected that not all sequence variants have been detected. The sensitivity is lower for homozygous mutations. Additionally, only point mutations or small deletions/duplications can be detected by this approach. Large-scale deletions like exon deletions or deletions of one allele are not detected by this method. Exon-deletion screen using real time quantitative PCR[16] should be performed to exclude the prevalence of such deletions. Furthermore, from the intronic regions only ± 10 bp have been analysed, but theoretically, mutations in introns can have pathogenic character effecting splicing [Lebon et al. 2007a].

To ensure complete analysis of affected patients, other methods should be considered. One alternative is the traditional Sanger sequencing. Regarding the high number of patients which should be analysed, this method is not suitable for high-throughput analysis, because it has a tenfold time and cost factor compared to melting curve analysis used in here. For future approaches more genes should be analysed. Routine whole genome sequencing of large numbers of individuals is still not feasible, partly due to the high costs and the time factor associated with the technique. At present, it is necessary to use an alternative approach, in which certain regions of the genome ('exome'[17]) are targeted, enriched and sequenced, which requires ~5% as much sequencing as a whole genome. In total there are about 180,000 exons found in the human genome. The sequencing of the exome is an efficient strategy to selectively sequence the coding regions of the human genome to identify novel genes associated with rare and common disorders. These are no suitable methods for high-throughput approaches, but they constitute good alternatives for complete analysis of single patients. The understanding of the molecular-genetic background for many inherited disorders including mitochondrial disorders and the susceptibility to acquired diseases is expected to improve in the future by the advance of the new screening methods.

[16] A technique based on the PCR, which is used to amplify and simultaneously quantify a targeted DNA molecule. It enables both detection and quantification (as absolute number of copies or relative amount when normalized to DNA input or additional normalizing genes) of one or more specific sequences in a DNA sample.

[17] The 'exome' represents all the exons in the human genome.

## 4.2 Analysed patient cohort with RCC I deficiency

Focus of previously performed work at the IHG was the establishment of the approach and identification of mutations in patients suffering from RCC I deficiency. Very poor clinical data were available at this time. The development of a standardized questionnaire to enable standardized assessment of history, clinical and examination parameters of the patients was the focus of my work. This should facilitate correlation analysis between identified genotypes and the phenotypes of patients with isolated RCC I deficiency. Previous reports described a maximum of 27 patients with isolated RCC I deficiency and compared to these, our collection of 151 patients build the largest cohort of studied patients with this special entity of mitochondrial dysfunction [Loeffen et al. 2000; Bugiani et al. 2004; Distelmaier et al. 2009]. The prevalence of symptoms should improve our understanding of patients suffering from mitochondrial disease and RCC I deficiency.

### 4.2.1 Methods for collection and evaluation of patient information

A questionnaire was developed with the goal of assessing important basic information of the clinical presentation of patients in a relatively simple, not time-intensive and reproducible way. Clinical symptoms and investigation results built the assortment chosen by evaluation of experienced clinicians (see 2.3). Most important information about the patients and their phenotypes could be collected using this questionnaire. Information about the phenotypes of patients has been mainly collected by me using chart research. In the remaining cases, doctors in charge completed the questionnaire and referred information. Most patients who had been referred from different clinical centres were seen some years ago in the clinical centres for the diagnostic workout and in some cases it was hard to find a responsible doctor. The generated lack of information did not allow the reconstruction of patient's history and the clinical course for each case. In total for 133/151 patients phenotype categories could be evaluated. This hampers complete analysis of clinical presentations within the patient cohort and lowers the power of the analysis. Additionally, the questionnaire included no items evaluating the clinical course of patients. Only for a small number of patients information about age of onset and age of death was obtained retrospectively. Therefore, no suitable analysis regarding the outcome of disease could be performed for the entire patient cohort. Parameters about birth and pregnancy were not recorded. The majority of patients described in the literature had uneventful pregnancy and birth parameters. Abnormal parameters have been reported in only few cases. This is consistent with the theory that the organism is especially well protected and supplied during the prenatal period and the demand for OXPHOS derived energy increases rapidly soon after birth [Bénit et al. 2003a; von Kleist-Retzow et al. 2003; Debray et al. 2007a; Distelmaier et al. 2009]. This aspect can also be used for explaining the frequently observed deterioration of the clinical status of patients during infectious episodes or fasting.

Not all symptoms and observations reported in patients with mitochondrial disease or especially RCC I defect could be evaluated by the questionnaire, which is a clear limitation of this study (especially missing information about course of disease by follow-up investigations and quantitative collection of metabolic parameters). The balance of having a suitable and equally comprehensive tool for data assessment of patients not going beyond the scope of practicable cooperation with the clinicians was an important fact. To overcome the limitations of this retrospective study it is an aim for future studies to assess patient information in a prospective way. The follow-up of each patient to analyse clinical course is mandatory. The

'mitoNET' (www.mitonet.org) is a German network of mitochondrial research centres. It includes numerous projects to improve awareness, clinical management, diagnosis and development of therapeutic strategies for patients with mitochondrial disorders. One project developed a web-based register for the evaluation of clinical data of patients ('mitoREGISTER') and is one great step forward to allow the performance of prospective multi-centric studies. It enables systematic annotation of phenotypes and documentation of follow-up investigations. The validated 'Newcastle mitochondrial disease adult scale (NMDAS)' [Schaefer et al. 2006] and 'Newcastle paediatric mitochondrial disease scale (NPMDS)' [Phoenix et al. 2006] served as templates for the 'mitoNET' in addition to an advanced version of the questionnaire used in here. Despite complete data assessment good cooperation and exchange of information between clinicians and research laboratories forms the basis for improved management of patients with mitochondrial disorders and should be an aim of every institution taking care of affected patients and their families.

### 4.2.2 RCC I residual activity distribution

RCC I activities have been referred from the clinical centres. In general, remaining RCC II-V activities have been referred to be in normal ranges. The biochemical diagnosis and dimension of the enzyme defect has not been confirmed at the Institute of Human Genetics. One limitation of measurements is the determination of activities under 'in vitro' conditions not corresponding to the physiological, cytosolic environment of mitochondria and the fragile RCC I composition. Unfortunately, no universally agreed standardization exists for the assays or assay conditions, and inter-laboratory variability in test results is common [Gellerich et al. 2004]. This makes comparisons of the test results difficult and misdiagnoses are possible [Rodenburg 2010]. Therefore, it is possible that some patients included in our study had no isolated RCC I defect which could reduce the power of analysis presented in here. Genes encoding other RCC subunits and assembly factors should be considered for molecular-genetic diagnosis following the confirmation of the enzyme defect in these cases.

The referred data of RCC I activities per NCP showed wider ranges than previously described. Ratios between mitochondrial enzymes (e.g. RCC activities expressed per CS) give a narrower range of normal values compared to activities expressed on the basis of protein content samples [Rustin et al. 1991; Rodenburg 2010]. Therefore, RCC I activities measured per CS have been used for our analysis. Only when activities per CS were not given, RCC I/NCP results were used for the statistics. This constitutes a non-conformal analysis and can introduce bias.

We could not find a significant correlation between the severity of enzyme deficiency and both the phenotype and genotype of patients which confirms numerous reports [Korenke et al. 1990; Debray et al. 2008; Rodenburg 2010]. However, we found a tendency that patients with severe phenotypes like Leigh syndrome or leukodystrophy had lower median residual RCC I activities (<25% of lowest control value).

### 4.2.3 Metabolic findings in the patient cohort

In contrast to the previously reported correlation, that lactic acidosis is more commonly found in patients with mtDNA mutations [Munnich et al. 1996b], in the investigated cohort 86% of the patients with nDNA mutation showed elevated lactate levels. Only 40% of patients harbouring mutations in mtDNA displayed abnormal lactate levels. The relatively small case number in our cohort may limit the conclusion. There were no comments on the prevalence

of mtDNA mutations in the population studied by Munnich, making a direct comparison difficult. Lactate levels can change during course of disease. Especially during infectious episodes, increased lactate levels have been observed [Loeffen et al. 2000]. In our questionnaire, we recorded only if lactate was abnormal once and had no information about the course of this metabolic parameter. Although lactate level measurements in both blood and CSF are very useful tests in diagnostic workout, several studies reported that normal levels does not exclude the evidence of a mitochondrial disorder [Touati et al. 1997; Loeffen et al. 2000; Debray et al. 2007a]. Patients with mutations can have normal lactate levels in repeated measurements, as found in patients with NDUFAF2 mutations [Herzer et al. 2010].

### 4.2.4 Neuroimaging findings in patients with RCC I deficiency

Neuroimaging studies in patients suffering from metabolic disease provide a very useful tool in diagnostics. Abnormalities seen in the investigations of cranial CT or MRI are often present in patients with inherited or acquired defects in energy metabolism. Changes are usually not specific for a distinct disorder or enzyme defect. However, investigations provide important information about the extension of disease in the CNS and disease progression beside therapeutic effects [Loeffen et al. 2000]. Within the patient cohort, a wide spectrum of abnormalities in brain structures was observed, ranging from lesions in basal ganglia, brainstem and the cerebellum to extensive white matter lesions (leukodystrophy) and general brain atrophy. This has been previously reported elsewhere [Scaglia et al. 2004]. For patients with Leigh or Leigh-like syndrome and leukodystrophy imaging investigations build one main stand for establishing the diagnosis. Not all patients presenting with neurological symptoms had abnormal findings in the brain imaging investigations, thus implicating that the absence of alterations in brain structures does not exclude the evidence of a mitochondrial disease. Furthermore, the severity of changes observed in neuroimaging investigations is often not reflected by the clinical presentation [Koenig 2008].

### 4.2.5 Phenotypes of RCC I deficiency patients

Only a few studies have been previously published dealing with RCC I deficiency patients [Loeffen et al. 2000; Smeitink et al. 2001; Bugiani et al. 2004; Distelmaier et al. 2009; Tuppen et al. 2010]. There are more reports on cohorts of patients with mitochondrial disease caused by different RCC defects [Munnich et al. 1996a; Rubio-Gozalbo et al. 2000; Skladal et al. 2003b; Scaglia et al. 2004; García-Carzola et al. 2005; Debray et al. 2007a; Kim et al. 2009]. Isolated RCC I deficiency has been reported to be the most frequently observed defect in RCC dysfunction and accounts for about one third of patients with mitochondrial disorders. An overview of the most frequent symptoms in patients can be established only in a large patient cohort, which was one aim of this work. The cohort of patients analysed within the present work is the largest study of patients with isolated RCC I deficiency. It mainly confirmed previous observed results of RCC I deficiency patients (references mentioned above).

Mean age of onset in the cohort was 5.8 years, whereas the median age of onset has been observed to be within one month of age (n=106). This bias in results can be a result of the great age dispersion within the analysed patient cohort (from postnatal up to 76 years). In the literature median age of onset reported in paediatric patients with isolated RCC I deficiency was four months [Loeffen et al. 2000; Distelmaier et al. 2009]. In the cohorts of patients with mitochondrial disease including all kinds of OXPHOS defects reported, the age of onset

ranged from 7-44 months [Skladal et al. 2003b; Scaglia et al. 2004; Debray et al. 2007a; Distelmaier et al. 2009]. The wide range of age of onset and the fact that also adults can have RCC I deficiency, demonstrates again the variability of clinical presentation. Comparable wide ranges and differences in outcome of patients have been observed for the age of death and the clinical status at this time.

Patients were categorized into eight groups according to previously reported phenotypes of RCC I deficiency patients and the manifestations observed in the patient population (see 3.2.5). Nonspecific encephalomyopathy was the phenotype most observed in our cohort and assigned to patients who had no findings suggestive for Leigh/Leigh-like syndrome or leukodystrophy in brain imaging or neuropathological investigations. Despite frequent symptoms of psychomotor and/or mental development delay and muscular hypotonia seizures have been observed in this group with the highest frequency compared to the other patient categories. However, no significant differences in the frequency of seizures was found between patients with nonspecific encephalomyopathy and patients with both Leigh-like syndrome or leukodystrophy as observed by Loeffen et al. [2000]. Compared to the other studies we identified the phenotype of Leigh or Leigh-like syndrome in lower frequency (11% versus 35-48%). This is probably caused by the inclusion of patients for the mutation screen after common mtDNA mutations have been excluded. mtDNA mutations have been described to account for major part of patients with Leigh syndrome [Loeffen et al. 2000; Bugiani et al. 2004]. The combination of leukodystrophy and macrocephaly has not been observed in our cohort, but the frequency of patients with leukodystrophy was similar to previous reports [Bugiani et al. 2004].

The frequency of patients with cardiomyopathy within the patient cohort (17%) was comparable to the findings of a large study on patients with mitochondrial disorders [Holmgren et al. 2003]. Nevertheless, frequencies of the studies dealing with isolated RCC I deficiency patients were quite different (11% of patients with cardiomyopathy versus 26%) [Loeffen et al. 2000; Bugiani et al. 2004].

The main part of patients was referred from paediatric clinical centres. Therefore, the phenotype of pure myopathy with symptoms like myalgia, muscular weakness and exercise intolerance was no frequent presentation in our cohort. However, this observation demonstrates the variable spectrum of RCC I deficiency. No comparisons to other studies regarding this result are possible, because they all described cohorts of paediatric patients [Munnich et al. 1996a; Loeffen et al. 2000; Rubio-Gozalbo et al. 2000; Skladal et al. 2003b; Bugiani et al. 2004; Scaglia et al. 2005; Debray et al. 2007a].

Comparable to other reports, the phenotype of a 'classical mitochondrial syndrome' was rare in the cohort, because it is more common in adult patients [Smeitink et van den Heuvel 1999; Skladal et al. 2003b]. However, patients can present with 'classical mitochondrial syndrome' predominantly in adulthood and for these patients a defect of RCC I should be considered as possible cause of disease.

One of the most serious phenotypes associated with OXPHOS disorders in general and RCC I deficiency in particular is LIMD [Loeffen et al. 2000]. We observed this phenotype in only 3% of the patients, which is different to previous reports where the frequency ranged from 11-13% [Loeffen et al. 2000; Bugiani et al. 2004].

The combination of different symptoms, affecting different organ systems, has been observed in ten patients, which have been assigned to the miscellaneous group. Because of the variable

phenotypes within this group, comparisons do not make sense. However, the presentation of mainly GIT-symptoms, signs of liver and renal dysfunction shows that RCC I deficiency can affect any organ system. Although, these visceral symptoms have not been reported in all reports of RCC I deficiency patients, they are common features observed in patients with mitochondrial disorders [Munnich et al. 1996a; Loeffen et al. 2000; Skladal et al. 2003b; Bugiani et al. 2004; García-Cazorla et al. 2005; Debray et al. 2007a; Distelmaier at al. 2009].
For future clinical management it has been shown that some signs and symptoms of the different phenotypes may help in dealing with RCC I deficiency patients. For example, abnormalities in metabolic investigations (mainly lactate levels in blood and CSF) have been observed more frequently in patients with severe phenotypes, including encephalomyopathy, Leigh syndrome, leukodystrophy and LIMD. But no significances of results have been observed for these parameters as reported elsewhere [Debray et al. 2007a]. Despite the great share for some gene mutations no predictive genotype-phenotype correlations were found, which segregates with the following results of the detailed analysis of RCC I deficiency patients with different nuclear gene mutations (4.3).

## 4.3 Phenotype-genotype correlations

For the diagnostic algorithm and the classification of patients with isolated RCC I deficiency, it is a desirable aim to establish phenotype-genotype correlations which can relieve and expedite this procedure, and furthermore, improve the clinical management for patients with the diagnosis of a mitochondrial disease. The variable presentations of clinical signs and symptoms make it very difficult to get even towards the hint that the diagnosis of a mitochondrial disorder should be considered. When diagnostic workout has started and results of investigated tissue biopsies give rise for a defect within the RCC, the next challenge is waiting. Numerous genes encoding subunits and assembly factors beside other proteins, which are essential for the inter-genomic communication, are involved to build proper functional RCC enzymes. To date, there are no efficient approaches, which can deal with the analysis of all possible disease-causing genes, to identify the pathogenic mutation. Furthermore, patients are seen in hospitals and not every institution has opportunities to perform high-throughput analysis of all questionable genes. Summarizing these thoughts, the cooperation of institutions, which take care and treat the affected patients, with research centres and highly equipped laboratories is mandatory to cope with the demand on finding the molecular-genetic basis of the RCC defect. It would be easier if the combinations of symptoms can lead to a selection of possible disease-causing genes. Therefore, phenotypes of patients with mutations reported and newly identified were analysed.

Although mitochondrial disorders are generally regarded as very heterogeneous, a more consistent clinical picture can be obtained for patients with isolated RCC I deficiency caused by nuclear mutations [Schuelke et al. 1999; Triepels et al. 1999; Loeffen et al. 2001; Budde et al. 2003; Visch et al. 2004; Koopman et al. 2005a; Visch et al. 2006a; Distelmaier et al. 2009]. Mutations of mtDNA encoded subunits form a widely heterogeneous phenotype spectrum, ranging from onset at any age (early childhood to late adolescence) and a broader variability in clinical symptoms, which has also been demonstrated by the analysis of our patient cohort. For example, within the group of patients with Leigh or Leigh-like syndrome, 21% of the patients had mutations in the mtDNA and only for patients with 'classical mitochondrial syndrome' the mtDNA mutation rate was higher with 30% (see 3.2.5.5). While no significant difference was observed in these two groups regarding the mutation rate, the age of onset and outcome of patients was quite different. Patients with Leigh syndrome presented first symptoms at median age of six months in contrast to those with a mitochondrial phenotype, who had onset at a median age of 30 years. These results support the general opinion that mutations affecting mtDNA encoded subunits generate a wider spectrum of symptom and organ manifestations. This can be seen as a result of the heteroplasmy between wild-type and mutant mtDNA, which is different for each tissue. Furthermore, the ratio varies during cell division known as mitotic segregation [Loeffen et al. 2000; Triepels et al. 2001; Bénit et al. 2004b; Bugiani et al. 2004; Janssen et al. 2006b; Distelmaier et al. 2009].

The genotype-phenotype analysis focused on patients with mutations in nuclear encoded genes. Patients with mutations in NDUFS1, NDUFS2, NDUFS4, NDUFS6, NDUFA1 and assembly factors NDUFAF2 and C6ORF66 have been compared to patients reported in the literature. Only for some gene mutations an overlap of clinical symptoms and course of disease has been observed.

The largest group is formed by patients with mutations in the nuclear encoded NDUFS1 subunit (15 with biochemical information). But in all patients with nuclear gene mutations

(irrespective from size of the groups) no correlation for the severity of the biochemical defect and the phenotype has been observed (**Fig. 31**), confirming previous reports [Korenke et al. 1990; Debray et al. 2008; Distelmaier et al. 2009; Rodenburg 2010].

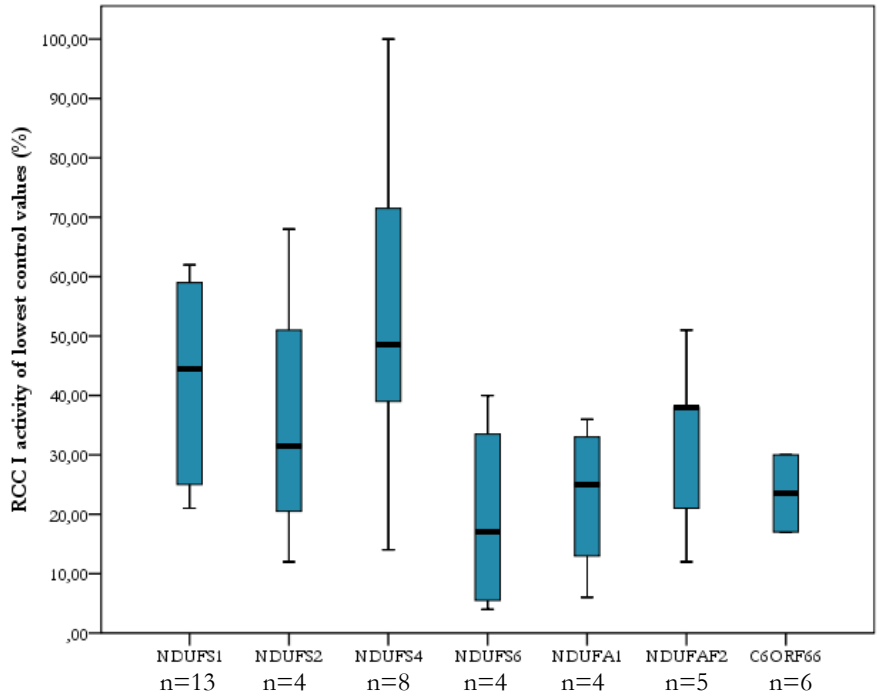



**Fig. 31.** Distribution of RCC I residual activities in patients with mutations in nuclear genes (n=44).

Comparing the age of onset and age of death between the patients with different nuclear gene mutations the severity of RCC I deficiency becomes obvious (**Fig. 32**). Most patients presented first symptoms at young age and expired several months after the onset of disease. Patients with mutations in NDUFS6 presented first symptoms soon after birth and disclosed the poorest outcome regarding the age of death (**Fig. 32**, pink pentagon). Patients with mutations in C6ORF66 showed nearly similar results regarding the age of first symptoms (median in both NDUFS6 and C6ORF66 postnatal), but compared to patients harbouring NDUFS6 mutations the age of death was more variable [Saada et al. 2009]. In summary, no significant correlations were found supporting several reports of the difficulties in finding genotype-phenotype correlations [Bénit et al. 2004b; Distelmaier et al. 2009]. Patients presenting at young age have been observed to be alive at time of reports and on the other hand patients who showed first signs after normal development died relatively soon after their first clinical presentation (e.g. patients with NDUFAF2 mutations, **Fig. 32**, darkblue points).

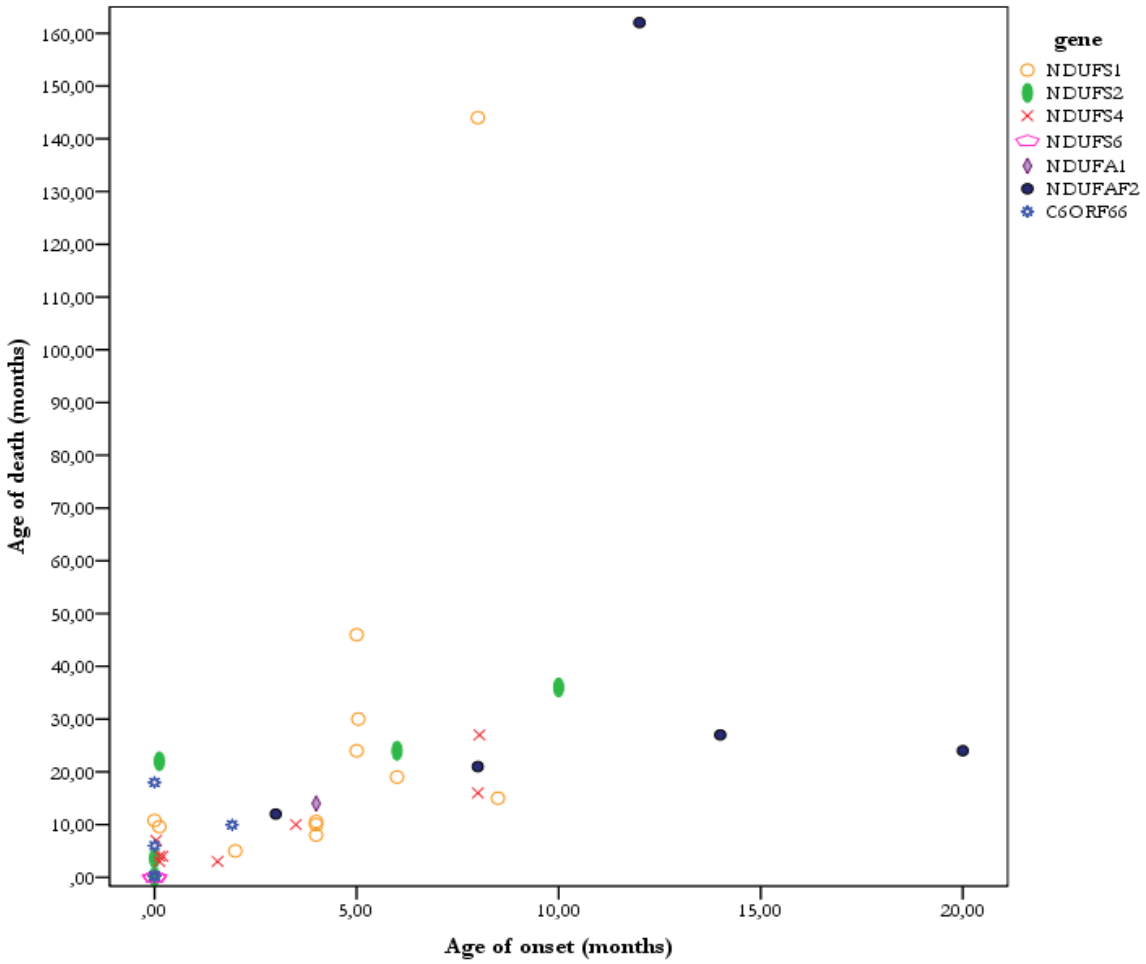



**Fig. 32.** Distribution of age of onset and death in patients with different nDNA mutations (n=40). Seven patients were still alive at time of the report/analysis and are not included in the graph. From four patients (three with NDUFS1 and one with NDUFS2 mutation) no information about course of disease was available.

Although some mutations have a great share in certain clinical features and constellations of those, obvious mutation-related phenotypes are not apparent (**Fig. 33**) confirming the observations previously reported [Budde et al. 2003; Distelmaier et al. 2009]. For example, the presentation of cardiomyopathy has been reported to be rather characteristic in patients with NDUFS2 mutations [Loeffen et al. 2001], but was also observed in patients harbouring mutations in NDUFS4 and C6ORF66 (**Fig. 33**). Moreover, cardiomyopathy has been reported in patients with mutations in NDUFV2 and NDUFA2 [Bénit et al. 2003a; Hoefs et al. 2008]. Another example presents the feature of leukodystrophy, frequently seen in patients with NDUFS1 mutations. It has been reported in patients with NDUFV1 mutations as well [Bénit et al. 2001; Schuelke et al. 1999]. On the other hand, not all patients with NDUFS1 mutations disclosed the finding of leukodystrophy in imaging investigations. They also presented lesions in basal ganglia and brainstem resembling the phenotype of Leigh syndrome.

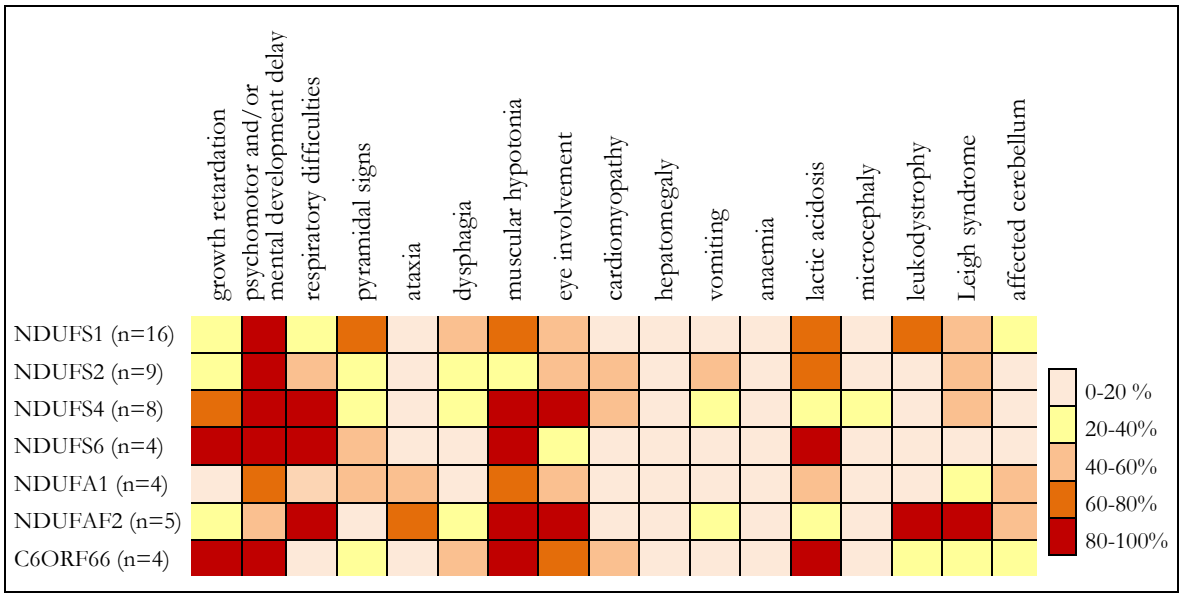



**Fig. 33.** Heat-map analysis demonstrates frequencies of phenotypes in 50 patients with different nuclear gene mutations.

In view of the summarized findings, it can be regarded as very difficult to establish genotype-phenotype correlations and sometimes even arbitrary. As the aetiopathology of the defect caused by nuclear mutations suggest every organ system can be affected and may display abnormalities to a variable extent, which is mainly a result of its specific demand on energy derived from the OXPHOS and the tissue-specific residual RCC I activity [Distelmaier et al. 2009].

Although no clear correlations were found, the comparison of the clinical symptoms of patients with suspected RCC I deficiency with patients previously described can lead towards a selection of possible disease causing genes. These genes should be considered for molecular-genetic analysis in the diagnostic workout of patients with suspected mitochondrial disease and isolated RCC I deficiency.

# 5 Literature


Anderson, S., Bankier, A. T., Barrell, B. G., de Bruijn, M. H., Coulson, A. R., Drouin, J., Eperon, I. C., Nierlich, D. P., Roe, B. A., Sanger, F., Schreier, P. H., Smith, A. J., Staden, R., Young, I. G. (1981). 'Sequence and organization of the human mitochondrial genome.' Nature **290**(5806): 457-65.

Anderson, S. L., Chung, W. K., Frezzo, J., Papp, J. C., Ekstein, J., Dimauro, S., Rubin, B. Y. (2008). 'A novel mutation in NDUFS4 causes Leigh syndrome in an Ashkenazi Jewish family.' J Inherit Metab Dis **31 Suppl 2**: 461-7.

Andersson, S. G., Karlberg, O., Canback, B., Kurland, C. G. (2003). 'On the origin of mitochondria: a genomics perspective.' Philos Trans R Soc Lond B Biol Sci **358**(1429): 165-77; discussion 177-9.

Andreu, A. L., Hanna, M. G., Reichmann, H., Bruno, C., Penn, A. S., 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**(14): 1037-44.

Aten E., White S.J., Kalf M.E., Vossen R.H., Thygesen H.H., Ruivenkamp C.A., Kriek M., Breuning M.H. and den Dunnen J.T. (2008). 'Methods to detect CNVs in the human genom.' Cytogenet Genome Res **123**: 313-321.

Au, H. C., Seo, B. B., Matsuno-Yagi, A., Yagi, T., Scheffler, I. E. (1999). 'The NDUFA1 gene product (MWFE protein) is essential for activity of complex I in mammalian mitochondria.' Proc Natl Acad Sci U S A **96**(8): 4354-9.

Audrezet M.P., Dabricot A., Le Marechal C. and Ferec C. (2008). 'Validation of high-resolution DNA melting analysis for mutation scanning of the cystic fibrosis transmembrane conductance regulator (CFTR) gene.' J Mol Diagn **10**: 424-434.

Barel, O., Shorer, Z., Flusser, H., Ofir, R., Narkis, G., Finer, G., Shalev, H., Nasasra, A., Saada, A., Birk, O. S. (2008). 'Mitochondrial complex III deficiency associated with a homozygous mutation in UQCRQ.' Am J Hum Genet **82**(5): 1211-6.

Barghuti, F., Elian, K., Gomori, J. M., Shaag, A., Edvardson, S., Saada, A., Elpeleg, O. (2008). 'The unique neuroradiology of complex I deficiency due to NDUFA12L defect.' Mol Genet Metab **94**(1): 78-82.

Barrientos, A., Barros, M. H., Valnot, I., Rotig, A., Rustin, P., Tzagoloff, A. (2002). 'Cytochrome oxidase in health and disease.' Gene **286**(1): 53-63.

Bauer, M. F., Gempel, K., Hofmann, S., Jaksch, M., Philbrook, C., Gerbitz, K. D. (1999). 'Mitochondrial disorders. A diagnostic challenge in clinical chemistry.' Clin Chem Lab Med **37**(9): 855-76.

Bender, A., Krishnan, K. J., Morris, C. M., Taylor, G. A., Reeve, A. K., Perry, R. H., Jaros, E., Hersheson, J. S., Betts, J., Klopstock, T., Taylor, R. W., Turnbull, D. M. (2006). 'High levels of mitochondrial DNA deletions in substantia nigra neurons in aging and Parkinson disease.' Nat Genet **38**(5): 515-7.

Bénit, P., Chretien, D., Kadhom, N., de Lonlay-Debeney, P., Cormier-Daire, V., Cabral, A., Peudenier, S., Rustin, P., Munnich, A., Rotig, 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**(6): 1344-52.

Bénit, P., Beugnot, R., Chretien, D., Giurgea, I., De Lonlay-Debeney, P., Issartel, J. P., Corral-Debrinski, M., Kerscher, S., Rustin, P., Rotig, A., Munnich, A. (2003a). 'Mutant NDUFV2 subunit of

mitochondrial complex I causes early onset hypertrophic cardiomyopathy and encephalopathy.' Hum Mutat **21**(6): 582-6.

Bénit, P., Steffann, J., Lebon, S., Chretien, D., Kadhom, N., de Lonlay, P., Goldenberg, A., Dumez, Y., Dommergues, M., Rustin, P., Munnich, A., Rotig, 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**(5-6): 563-6.

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

Bénit, Lebon S, Chol M, Giurgea I and Rötig A. (2004b). 'Mitochondrial NADH Oxidation Deficiency in Humans Current Genomics.' Current Genomics **5**: 137-146.

Berger, I., Hershkovitz, E., Shaag, A., Edvardson, S., Saada, A., Elpeleg, O. (2008). 'Mitochondrial complex I deficiency caused by a deleterious NDUFA11 mutation.' Ann Neurol **63**(3): 405-8.

Bernier, F. P., Boneh, A., Dennett, X., Chow, C. W., Cleary, M. A., Thorburn, D. R. (2002). 'Diagnostic criteria for respiratory chain disorders in adults and children.' Neurology **59**(9): 1406-11.

Bianchi M.C., Tosetti M., Battini R., Manca M.L., Mancuso M., Cioni G., Canapicchi R. and Siciliano G. (2003). 'Proton MR spectroscopy of mitochondrial diseases: analysis of brain metabolic abnormalities and their possible diagnostic relevance.' AJNR Am J Neuroradiol **24**: 1958-1966.

Bourgeois, J. M. and Tarnopolsky, M. A. (2004). 'Pathology of skeletal muscle in mitochondrial disorders.' Mitochondrion **4**(5-6): 441-52.

Bourges I., Ramus C., Mousson de Camaret B., Beugnot R., Remacle C., Cardol P., Hofhaus G. and Issartel J.P. (2004). 'Structural organization of mitochondrial human complex I: role of the ND4 and ND5 mitochondria-encoded subunits and interaction with prohibitin.' Biochem J **383**: 491-499.

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

Breningstall G.N., Shoffner J. and Patterson R.J. (2008). 'Siblings with leukoencephalopathy.' Semin Pediatr Neurol **15**: 212-215.

Brown M.D., Trounce I.A., Jun A.S., Allen J.C. and Wallace D.C. (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.

Brown M.D., Starikovskaya E., Derbeneva O., Hosseini S., Allen J.C., Mikhailovskaya I.E., Sukernik R.I. and Wallace D.C. (2002). 'The role of mtDNA background in disease expression: a new primary LHON mutation associated with Western Eurasian haplogroup J.' Hum Genet **110**: 130-138.

Budde, S. M., van den Heuvel, L. P., Janssen, A. J., Smeets, R. J., Buskens, C. A., DeMeirleir, L., Van Coster, R., Baethmann, M., Voit, T., Trijbels, J. M., Smeitink, J. A. (2000). 'Combined enzymatic complex I and III deficiency associated with mutations in the nuclear encoded NDUFS4 gene.' Biochem Biophys Res Commun **275**(1): 63-8.

Budde, S. M., van den Heuvel, L. P., Smeets, R. J., Skladal, D., Mayr, J. A., Boelen, C., Petruzzella, V., Papa, S., Smeitink, J. A. (2003). 'Clinical heterogeneity in patients with mutations in the NDUFS4 gene of mitochondrial complex I.' J Inherit Metab Dis **26**(8): 813-5.

Bugiani, M., Invernizzi, F., Alberio, S., Briem, E., Lamantea, E., Carrara, F., Moroni, I., Farina, L., Spada, M., Donati, M. A., Uziel, G., Zeviani, M. (2004). 'Clinical and molecular findings in children with complex I deficiency.' Biochim Biophys Acta **1659**(2-3): 136-47.

Carroll, J., Shannon, R. J., Fearnley, I. M., Walker, J. E., 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**(52): 50311-7.

Carroll, J., Fearnley, I. M., Shannon, R. J., Hirst, J., Walker, J. E. (2003). 'Analysis of the subunit composition of complex I from bovine heart mitochondria.' Mol Cell Proteomics **2**(2): 117-26.

Carroll, J., Fearnley, I. M., Skehel, J. M., Shannon, R. J., Hirst, J., Walker, J. E. (2006). 'Bovine complex I is a complex of 45 different subunits.' J Biol Chem **281**(43): 32724-7.

Chow, S. L., Rooney, Z. J., Cleary, M. A., Clayton, P. T., Leonard, J. V. (2005). 'The significance of elevated CSF lactate.' Arch Dis Child **90**(11): 1188-9.

Chretien D. and Rustin P. (2003). 'Mitochondrial oxidative phosphorylation: pitfalls and tips in measuring and interpreting enzyme activities.' J Inherit Metab Dis **26**: 189-198.

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

De Coo, I. F., Renier, W. O., Ruitenbeek, W., Ter Laak, H. J., Bakker, M., Schagger, H., Van Oost, B. A., Smeets, H. J. (1999). 'A 4-base pair deletion in the mitochondrial cytochrome b gene associated with parkinsonism/MELAS overlap syndrome.' Ann Neurol **45**(1): 130-3.

De Lonlay, P., Valnot, I., Barrientos, A., Gorbatyuk, M., Tzagoloff, A., Taanman, J. W., Benayoun, E., Chretien, D., Kadhom, N., Lombes, A., de Baulny, H. O., Niaudet, P., Munnich, A., Rustin, P., Rotig, A. (2001). 'A mutant mitochondrial respiratory chain assembly protein causes complex III deficiency in patients with tubulopathy, encephalopathy and liver failure.' Nat Genet **29**(1): 57-60.

De Meirleir, L., Seneca, S., Damis, E., Sepulchre, B., Hoorens, A., Gerlo, E., Garcia Silva, M. T., Hernandez, E. M., Lissens, W., Van Coster, R. (2003). 'Clinical and diagnostic characteristics of complex III deficiency due to mutations in the BCS1L gene.' Am J Med Genet A **121A**(2): 126-31.

De Meirleir L., Seneca S., Lissens W., De Clercq I., Eyskens F., Gerlo E., Smet J. and Van Coster R. (2004). 'Respiratory chain complex V deficiency due to a mutation in the assembly gene ATP12.' J Med Genet **41**: 120-124.

Debray, F. G., Lambert, M., Chevalier, I., Robitaille, Y., Decarie, J. C., Shoubridge, E. A., Robinson, B. H., Mitchell, G. A. (2007a). 'Long-term outcome and clinical spectrum of 73 paediatric patients with mitochondrial diseases.' Paediatrics **119**(4): 722-33.

Debray, F. G., Mitchell, G. A., Allard, P., Robinson, B. H., Hanley, J. A., Lambert, M. (2007b). 'Diagnostic accuracy of blood lactate-to-pyruvate molar ratio in the differential diagnosis of congenital lactic acidosis.' Clin Chem **53**(5): 916-21.

Debray, F. G., Lambert, M., Mitchell, G. A. (2008). 'Disorders of mitochondrial function.' Curr Opin Pediatr **20**(4): 471-82.

DiMauro, S., Andreu, A. L., De Vivo, D. C. (2002). 'Mitochondrial disorders.' J Child Neurol **17 Suppl 3**: 3S35-45; discussion 3S46-7.

DiMauro, S. and Davidzon, G. (2005). 'Mitochondrial DNA and disease.' Ann Med **37**(3): 222-32.

DiMauro, S. (2007). 'Mitochondrial DNA medicine.' Biosci Rep **27**(1-3): 5-9.

DiMauro, S. and Schon, E. A. (2008). 'Mitochondrial disorders in the nervous system.' Annu Rev Neurosci **31**: 91-123.

Dinopoulos A., Cecil K.M., Schapiro M.B., Papadimitriou A., Hadjigeorgiou G.M., Wong B., deGrauw T. and Egelhoff J.C. (2005). 'Brain MRI and proton MRS findings in infants and children with respiratory chain defects.' Neuropaediatrics **36**: 290-301.

Distelmaier, F., Koopman, W. J., van den Heuvel, L. P., Rodenburg, R. J., Mayatepek, E., Willems, P. H., Smeitink, J. A. (2009). 'Mitochondrial complex I deficiency: from organelle dysfunction to clinical disease.' Brain **132**(Pt 4): 833-42.

Dunning, C. J., McKenzie, M., Sugiana, C., Lazarou, M., Silke, J., Connelly, A., Fletcher, J. M., Kirby, D. M., Thorburn, D. R., Ryan, M. T. (2007). 'Human CIA30 is involved in the early assembly of mitochondrial complex I and mutations in its gene cause disease.' EMBO J **26**(13): 3227-37.

Efremov, R. G., R. Baradaran, Sazanov, L.A. (2010). 'The architecture of respiratory complex I.' Nature **465**(7297): 441-445.

Ernster, L., Ikkos, D., and Luft, R. (1959). 'Enzymic activities of human skeletal muscle mitochondria: a tool in clinical metabolic research.' Nature (Lond.) **184:** 1851-1854.

Fernandez-Moreira, D., Ugalde, C., Smeets, R., Rodenburg, R. J., Lopez-Laso, E., Ruiz-Falco, M. L., Briones, P., Martin, M. A., Smeitink, J. A., Arenas, J. (2007). 'X-linked NDUFA1 gene mutations associated with mitochondrial encephalomyopathy.' Ann Neurol **61**(1): 73-83.

Fernandez-Vizarra, E., Bugiani, M., Goffrini, P., Carrara, F., Farina, L., Procopio, E., Donati, A., Uziel, G., Ferrero, I., Zeviani, M. (2007). 'Impaired complex III assembly associated with BCS1L gene mutations in isolated mitochondrial encephalopathy.' Hum Mol Genet **16**(10): 1241-52.

Fernandez-Vizarra, E., Tiranti, V., Zeviani, M. (2009). 'Assembly of the oxidative phosphorylation system in humans: what we have learned by studying its defects.' Biochim Biophys Acta **1793**(1): 200-11.

Freisinger, P., Mayr, J.A., Burgard, P., Sperl, W. (2007) 'Diagnostic and Treatment: Approaches to Mitochondriopathies in Children and Adults', Guidelines issued by the Working Group on Paediatric Metabolic Disorders **Volume**, 45; www.aps-med.de/documents/MitoLL-22-12-2007.pdf

Friedrich, T. and Bottcher, B. (2004). 'The gross structure of the respiratory complex I: a Lego System.' Biochim Biophys Acta **1608**(1): 1-9.

Gabaldon, T., Rainey, D., Huynen, M. A. (2005). 'Tracing the evolution of a large protein complex in the eukaryotes, NADH:ubiquinone oxidoreductase (Complex I).' J Mol Biol **348**(4): 857-70.

Garcia-Cazorla A., De Lonlay P., Rustin P., Chretien D., Touati G., Rabier D., Slama A. and Saudubray J.M. (2006). 'Mitochondrial respiratory chain deficiencies expressing the enzymatic deficiency in the hepatic tissue: a study of 31 patients.' J Pediatr **149**: 401-405.

Gellerich F.N., Mayr J.A., Reuter S., Sperl W. and Zierz S. (2004). 'The problem of interlab variation in methods for mitochondrial disease diagnosis: enzymatic measurement of respiratory chain complexes.' Mitochondrion **4**: 427-439.

Gerards M., Sluiter W., van den Bosch B.J., de Wit L.E., Calis C.M., Frentzen M., Akbari H., Schoonderwoerd K., Scholte H.R., Jongbloed R.J., Hendrickx A.T., de Coo I.F. and Smeets H.J.

(2009). 'Defective complex I assembly due to C20orf7 mutations as a new cause of Leigh syndrome.' J Med Genet **47**: 507-512.

Ghezzi D., Goffrini P., Uziel G., Horvath R., Klopstock T., Lochmuller H., D'Adamo P., Gasparini P., Strom T.M., Prokisch H., Invernizzi F., Ferrero I. and Zeviani M. (2009). 'SDHAF1, encoding a LYR complex-II specific assembly factor, is mutated in SDH-defective infantile leukoencephalopathy.' Nat Genet **41**: 654-656.

Grigorieff, N. (1998). 'Three-dimensional structure of bovine NADH:ubiquinone oxidoreductase (complex I) at 22 A in ice.' J Mol Biol **277**(5): 1033-46.

Grossman, L. I. and Lomax, M. I. (1997). 'Nuclear genes for cytochrome c oxidase.' Biochim Biophys Acta **1352**(2): 174-92.

Guenebaut, V., Vincentelli R., Mills D., Weiss H. and Leonard K.R. (1997). 'Three-dimensional structure of NADH-dehydrogenase from Neurospora crassa by electron microscopy and conical tilt reconstruction.' J Mol Biol **265**(4): 409-18.

Haas R.H., Parikh S., Falk M.J., Saneto R.P., Wolf N.I., Darin N. and Cohen B.H. (2007). 'Mitochondrial disease: a practical approach for primary care physicians.' Paediatrics **120**: 1326-1333.

Haas, R. H., Parikh, S., Falk, M. J., Saneto, R. P., Wolf, N. I., Darin, N., Wong, L. J., Cohen, B. H., Naviaux, R. K. (2008). 'The in-depth evaluation of suspected mitochondrial disease.' Mol Genet Metab **94**(1): 16-37.

Hatefi, Y. (1985). 'The mitochondrial electron transport and oxidative phosphorylation system.' Ann. Rev. Biochem. **54**: 54.

Haut, S., Brivet M., Touati G., Rustin P., Lebon S., Garcia-Cazorla A., Saudubray J.M., Boutron A., Legrand A. and Slama A. (2003). 'A deletion in the human QP-C gene causes a complex III deficiency resulting in hypoglycaemia and lactic acidosis.' Hum Genet **113**(2): 118-22.

Henze, K. and Martin, W. (2003). 'Evolutionary biology: essence of mitochondria.' Nature **426**(6963): 127-8.

Herrmann, J. M. and Neupert, W. (2000). 'Protein transport into mitochondria.' Curr Opin Microbiol **3**(2): 210-4.

Herzer, M., Koch J., Prokisch H., Rodenburg R., Rauscher C., Radauer W., Forstner R., Pilz P., Rolinski B., Freisinger P., Mayr J.A. and Sperl W. (2010). 'Leigh disease with brainstem involvement in complex I deficiency due to assembly factor NDUFAF2 defect.' Neuropaediatrics **41**(1): 30-4.

Hirano, M. and Vu, T. H. (2000). 'Defects of intergenomic communication: where do we stand?' Brain Pathol **10**(3): 451-61.

Hirst, J., Carroll J., Fearnley I.M., Shannon R.J. and Walker J.E. (2003). 'The nuclear encoded subunits of complex I from bovine heart mitochondria.' Biochim Biophys Acta **1604**(3): 135-50.

Hoefs, S. J., Dieteren C.E., Distelmaier F., Janssen R.J., Epplen A., Swarts H.G., Forkink M., Rodenburg R.J., Nijtmans L.G., Willems P.H., Smeitink J.A. and van den Heuvel L.P. (2008). 'NDUFA2 complex I mutation leads to Leigh disease.' Am J Hum Genet 82(6): 1306-15.

Hoefs S.J., Dieteren C.E., Rodenburg R.J., Naess K., Bruhn H., Wibom R., Wagena E., Willems P.H., Smeitink J.A., Nijtmans L.G. and van den Heuvel L.P. (2009). 'Baculovirus complementation restores a novel NDUFAF2 mutation causing complex I deficiency.' Hum Mutat **30**: E728-736.

Hoefs, S. J., Skjeldal O.H., Rodenburg R.J., Nedregaard B., van Kaauwen E.P., Spiekerkotter U., von Kleist-Retzow J.C., Smeitink J.A., Nijtmans L.G. and van den Heuvel L.P. (2010). 'Novel mutations in the NDUFS1 gene cause low residual activities in human complex I deficiencies.' Mol Genet Metab **100**(3): 251-6.

Hofhaus G. and Attardi G. (1993). 'Lack of assembly of mitochondrial DNA-encoded subunits of respiratory NADH dehydrogenase and loss of enzyme activity in a human cell mutant lacking the mitochondrial ND4 gene product.' EMBO J **12**: 3043-3048.

Hofhaus G. and Attardi G. (1995). 'Efficient selection and characterization of mutants of a human cell line which are defective in mitochondrial DNA-encoded subunits of respiratory NADH dehydrogenase.' Mol Cell Biol **15**: 964-974.

Holmgren, D., Wahlander H., Eriksson B.O., Oldfors A., Holme E. and Tulinius M. (2003). 'Cardiomyopathy in children with mitochondrial disease; clinical course and cardiological findings.' Eur Heart J **24**(3): 280-8.

Holt, I. J., Cooper J.M., Morgan-Hughes J.A. and Harding A.E. (1988). 'Deletions of muscle mitochondrial DNA.' Lancet **1**(8600): 1462.

Honzik T., Wenchich L., Bohm M., Hansikova H., Pejznochova M., Zapadlo M., Plavka R. and Zeman J. (2008). 'Activities of respiratory chain complexes and pyruvate dehydrogenase in isolated muscle mitochondria in premature neonates.' Early Hum Dev **84**: 269-276.

Houstek J., Pickova A., Vojtiskova A., Mracek T., Pecina P. and Jesina P. (2006). 'Mitochondrial diseases and genetic defects of ATP synthase.' Biochim Biophys Acta **1757**: 1400-1405.

Horvath, R., Abicht A., Holinski-Feder E., Laner A., Gempel K., Prokisch H., Lochmuller H., Klopstock T. and Jaksch M. (2006). 'Leigh syndrome caused by mutations in the flavoprotein (Fp) subunit of succinate dehydrogenase (SDHA).' J Neurol Neurosurg Psychiatry **77**(1): 74-6.

Iuso, A., Scacco S., Piccoli C., Bellomo F., Petruzzella V., Trentadue R., Minuto M., Ripoli M., Capitanio N., Zeviani M. and Papa S. (2006). 'Dysfunctions of cellular oxidative metabolism in patients with mutations in the NDUFS1 and NDUFS4 genes of complex I.' J Biol Chem **281**(15): 10374-80.

Jackson, M., Schaefer J., Johnson M., Morris A., Turnbull D. and Bindoff L. (1995). 'Presentation and clinical investigation of mitochondrial respiratory chain disease: A study of 51 patients.' Brain **188**: 339-357.

Janssen A.J., Smeitink J.A. and van den Heuvel L.P. (2003). 'Some practical aspects of providing a diagnostic service for respiratory chain defects.' Ann Clin Biochem **40**: 3-8.

Janssen A.J., Trijbels F.J., Sengers R.C., Wintjes L.T., Ruitenbeek W., Smeitink J.A., Morava E., van Engelen B.G., van den Heuvel L.P. and Rodenburg R.J. (2006a). 'Measurement of the energy-generating capacity of human muscle mitochondria: diagnostic procedure and application to human pathology.' Clin Chem **52**: 860-871.

Janssen, R. J., Nijtmans L.G., van den Heuvel L.P. and Smeitink J.A. (2006b). 'Mitochondrial complex I: structure, function and pathology.' J Inherit Metab Dis **29**(4): 499-515.

Janssen A.J., Schuelke M., Smeitink J.A., Trijbels F.J., Sengers R.C., Lucke B., Wintjes L.T., Morava E., van Engelen B.G., Smits B.W., Hol F.A., Siers M.H., Ter Laak H., van der Knaap M.S., Van Spronsen F.J., Rodenburg R.J. and van den Heuvel L.P. (2008). 'Muscle 3243A-->G mutation load and capacity of the mitochondrial energy-generating system.' Ann Neurol **63**: 473-481.

Johns, D. R. and Neufeld, M. J. (1991). 'Cytochrome b mutations in Leber hereditary optic neuropathy.' Biochem Biophys Res Commun **181**(3): 1358-64.

Johns D.R. (1995). 'Seminars in medicine of the Beth Israel Hospital, Boston. Mitochondrial DNA and disease' N Engl J Med **333**: 638-644.

Jonckheere A.I., Hogeveen M., Nijtmans L.G., van den Brand M.A., Janssen A.J., Diepstra J.H., van den Brandt F.C., van den Heuvel L.P., Hol F.A., Hofste T.G., Kapusta L., Dillmann U., Shamdeen M.G., Smeitink J.A. and Rodenburg R.J. (2008). 'A novel mitochondrial ATP8 gene mutation in a patient with apical hypertrophic cardiomyopathy and neuropathy.' J Med Genet **45**: 129-133.

Kent WJ, Sugnet CW, Furey TS, Roskin KM, Pringle TH, Zahler AM, Haussler D. 'The human genome browser at UCSC.' Genome Res. 2002 Jun;12(6):996-1006.

Kim J.T., Lee Y.J., Lee Y.M., Kang H.C., Lee J.S. and Kim H.D. (2009). 'Clinical characteristics of patients with non-specific and non-categorized mitochondrial diseases' Acta Paediatr **98**: 1825-1829.

Kirby, D. M., Crawford M., Cleary M.A., Dahl H.H., Dennett X. and Thorburn D.R. (1999). 'Respiratory chain complex I deficiency: an underdiagnosed energy generation disorder.' Neurology **52**(6): 1255-64.

Kirby D.M., Kahler S.G., Freckmann M.L., Reddihough D. and Thorburn D.R. (2000). 'Leigh disease caused by the mitochondrial DNA G14459A mutation in unrelated families.' Ann Neurol **48**: 102-104.

Kirby D.M., Boneh A., Chow C.W., Ohtake A., Ryan M.T., Thyagarajan D. and Thorburn D.R. (2003). 'Low mutant load of mitochondrial DNA G13513A mutation can cause Leigh's disease.' Ann Neurol **54**: 473-478.

Kirby D.M., McFarland R., Ohtake A., Dunning C., Ryan M.T., Wilson C., Ketteridge D., Turnbull D.M., Thorburn D.R. and Taylor R.W. (2004a). 'Mutations of the mitochondrial ND1 gene as a cause of MELAS.' J Med Genet **41**: 784-789.

Kirby, D. M., Salemi R., Sugiana C., Ohtake A., Parry L., Bell K.M., Kirk E.P., Boneh A., Taylor R.W., Dahl H.H., Ryan M.T. and Thorburn D.R. (2004b). 'NDUFS6 mutations are a novel cause of lethal neonatal mitochondrial complex I deficiency.' J Clin Invest **114**(6): 837-45.

Kirby D.M., Thorburn D.R., Turnbull D.M. and Taylor R.W. (2007). 'Biochemical assays of respiratory chain complex activity.' Methods Cell Biol **80**: 93-119.

Kirkman M.A., Yu-Wai-Man P. and Chinnery P.F. (2008). 'The clinical spectrum of mitochondrial genetic disorders.' Clin Med **8**: 601-606.

Koenig, M. K. (2008). 'Presentation and diagnosis of mitochondrial disorders in children.' Pediatr Neurol **38**(5): 305-13.

Komaki H., Akanuma J., Iwata H., Takahashi T., Mashima Y., Nonaka I. and Goto Y. (2003). 'A novel mtDNA C11777A mutation in Leigh syndrome.' Mitochondrion **2**: 293-304.

Koopman W.J., Visch H.J., Verkaart S., van den Heuvel L.W., Smeitink J.A. and Willems P.H. (2005a). 'Mitochondrial network complexity and pathological decrease in complex I activity are tightly correlated in isolated human complex I deficiency.' Am J Physiol Cell Physiol **289**: C881-890.

Koopman W.J., Verkaart S., Visch H.J., van der Westhuizen F.H., Murphy M.P., van den Heuvel L.W., Smeitink J.A. and Willems P.H. (2005b). 'Inhibition of complex I of the electron transport chain causes O2-. -mediated mitochondrial outgrowth.' Am J Physiol Cell Physiol **288**: C1440-1450.

Korenke, G. C., Bentlage H.A., Ruitenbeek W., Sengers R.C., Sperl W., Trijbels J.M., Gabreels F.J., Wijburg F.A., Wiedermann V., Hanefeld F. (1990). 'Isolated and combined deficiencies of NADH dehydrogenase (complex I) in muscle tissue of children with mitochondrial myopathies.' Eur J Pediatr **150**(2): 104-8.

Kruse, S. E., Watt W.C., Marcinek D.J., Kapur R.P., Schenkman K.A. and Palmiter R.D. (2008). 'Mice with mitochondrial complex I deficiency develop a fatal encephalomyopathy.' Cell Metab **7**(4): 312-20.

Lamont P.J., Surtees R., Woodward C.E., Leonard J.V., Wood N.W. and Harding A.E. (1998). 'Clinical and laboratory findings in referrals for mitochondrial DNA analysis.' Arch Dis Child **79**: 22-27.

Laugel V., This-Bernd V., Cormier-Daire V., Speeg-Schatz C., de Saint-Martin A. and Fischbach M. (2007). 'Early-onset ophthalmoplegia in Leigh-like syndrome due to NDUFV1 mutations.' Pediatr Neurol **36**: 54-57.

Lazarou, M., McKenzie M., Ohtake A., Thorburn D.R. and Ryan M.T. (2007). 'Analysis of the assembly profiles for mitochondrial- and nuclear-DNA-encoded subunits into complex I.' Mol Cell Biol **27**(12): 4228-37.

Lazarou, M., Thorburn D.R., Ryan M.T. and McKenzie M. (2009). 'Assembly of mitochondrial complex I and defects in disease.' Biochim Biophys Acta **1793**(1): 78-88.

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

Lebon S., Minai L., Chretien D., Corcos J., Serre V., Kadhom N., Steffann J., Pauchard J.Y., Munnich A., Bonnefont J.P. and Rotig A. (2007a). 'A novel mutation of the NDUFS7 gene leads to activation of a cryptic exon and impaired assembly of mitochondrial complex I in a patient with Leigh syndrome.' Mol Genet Metab **92**: 104-108.

Lebon S., Rodriguez D., Bridoux D., Zerrad A., Rotig A., Munnich A., Legrand A. and Slama A. (2007b). 'A novel mutation in the human complex I NDUFS7 subunit associated with Leigh syndrome.' Mol Genet Metab **90**: 379-382.

Leigh, D. (1951). 'Subacute necrotizing encephalomyelopathy in an infant.' J Neurol Neurosurg Psychiatry **14**(3): 216-21.

Leshinsky-Silver, E., Lebre A.S., Minai L., Saada A., Steffann J., Cohen S., Rotig A., Munnich A., Lev D. and Lerman-Sagie T. (2009). 'NDUFS4 mutations cause Leigh syndrome with predominant brainstem involvement.' Mol Genet Metab **97**(3): 185-9.
Lin D.D., Crawford T.O. and Barker P.B. (2003). 'Proton MR spectroscopy in the diagnostic evaluation of suspected mitochondrial disease.' AJNR Am J Neuroradiol **24**: 33-41.

Lindal S., Lund I., Torbergsen T., Aasly J., Mellgren S.I., Borud O. and Monstad P. (1992). 'Mitochondrial diseases and myopathies: a series of muscle biopsy specimens with ultrastructural changes in the mitochondria.' Ultrastruct Pathol **16**: 263-275.

Loeffen, J., Smeitink J., Triepels R., Smeets R., Schuelke M., Sengers R., Trijbels F., Hamel B., Mullaart R. and van den Heuvel L. (1998). 'The first nuclear-encoded complex I mutation in a patient with Leigh syndrome.' Am J Hum Genet **63**(6): 1598-608.

Loeffen, J. L., Smeitink J.A., Trijbels J.M., Janssen A.J., Triepels R.H., Sengers R.C. and van den Heuvel L.P. (2000). 'Isolated complex I deficiency in children: clinical, biochemical and genetic aspects.' Hum Mutat **15**(2): 123-34.

Loeffen, J., Elpeleg O., Smeitink J., Smeets R., Stockler-Ipsiroglu S., Mandel H., Sengers R., Trijbels F. and van den Heuvel L. (2001). 'Mutations in the complex I NDUFS2 gene of patients with cardiomyopathy and encephalomyopathy.' Ann Neurol **49**(2): 195-201.

Malfatti, E., Bugiani M., Invernizzi F., de Souza C.F., Farina L., Carrara F., Lamantea E., Antozzi C., Confalonieri P., Sanseverino M.T., Giugliani R., Uziel G. and Zeviani M. (2007). 'Novel mutations of ND genes in complex I deficiency associated with mitochondrial encephalopathy.' Brain **130**(Pt 7): 1894-904.

Mancuso, M., Filosto M., Choub A., Tentorio M., Broglio L., Padovani A. and Siciliano G. (2007). 'Mitochondrial DNA-related disorders.' Biosci Rep **27**(1-3): 31-7.

Martin, M. A., Blazquez A., Gutierrez-Solana L.G., Fernandez-Moreira D., Briones P., Andreu A.L., Garesse R., Campos Y. and Arenas J. (2005). 'Leigh syndrome associated with mitochondrial complex I deficiency due to a novel mutation in the NDUFS1 gene.' Arch Neurol **62**(4): 659-61.

Massa, V., Fernandez-Vizarra E., Alshahwan S., Bakhsh E., Goffrini P., Ferrero I., Mereghetti P., D'Adamo P., Gasparini P. and Zeviani M. (2008). 'Severe infantile encephalomyopathy caused by a mutation in COX6B1, a nucleus-encoded subunit of cytochrome c oxidase.' Am J Hum Genet **82**(6): 1281-9.

Mayr, J. A., Paul J., Pecina P., Kurnik P., Forster H., Fotschl U., Sperl W. and Houstek J. (2004). 'Reduced respiratory control with ADP and changed pattern of respiratory chain enzymes as a result of selective deficiency of the mitochondrial ATP synthase.' Pediatr Res **55**(6): 988-94.

McBride, H. M., Neuspiel M. and Wasiak S. (2006). 'Mitochondria: more than just a powerhouse.' Curr Biol **16**(14): R551-60.

McFarland, R., Kirby D.M., Fowler K.J., Ohtake A., Ryan M.T., Amor D.J., Fletcher J.M., Dixon J.W., Collins F.A., Turnbull D.M., Taylor R.W. and Thorburn D.R. (2004). 'De novo mutations in the mitochondrial ND3 gene as a cause of infantile mitochondrial encephalopathy and complex I deficiency.' Ann Neurol **55**(1): 58-64.

Meisinger, C., Prokisch H., Gieger C., Soranzo N., Mehta D., Rosskopf D., Lichtner P., Klopp N., Stephens J., Watkins N.A., Deloukas P., Greinacher A., Koenig W., Nauck M., Rimmbach C., Volzke H., Peters A., Illig T., Ouwehand W.H., Meitinger T., Wichmann H.E. and Doring A. (2009). 'A genome-wide association study identifies three loci associated with mean platelet volume.' Am J Hum Genet **84**(1): 66-71.

Meyer LR, Zweig AS, Hinrichs AS, Karolchik D, Kuhn RM, Wong M, Sloan CA, Rosenbloom KR, Roe G, Rhead B, Raney BJ, Pohl A, Malladi VS, Li CH, Lee BT, Learned K, Kirkup V, Hsu F, Heitner S, Harte RA, Haeussler M, Guruvadoo L, Goldman M, Giardine BM, Fujita PA, Dreszer TR, Diekhans M, Cline MS, Clawson H, Barber GP, Haussler D, and Kent WJ. 'The UCSC Genome Browser database: extensions and updates 2010.' Nucleic Acids Res. 2010 Nov 15.

Munnich, A., Rotig A., Chretien D., Cormier V., Bourgeron T., Bonnefont J.P., Saudubray J.M. and Rustin P. (1996a). 'Clinical presentation of mitochondrial disorders in childhood.' J Inherit Metab Dis **19**(4): 521-7.

Munnich, A., Rotig A., Chretien D., Saudubray J.M., Cormier V. and Rustin P. (1996b). 'Clinical presentations and laboratory investigations in respiratory chain deficiency.' Eur J Pediatr **155**(4): 262-74.

Naini AB, Lu J, Kaufmann P, Bernstein RA, Mancuso M, Bonilla E, Hirano M, DiMauro S. Novel (2005). 'Mitochondrial DNA ND5 mutation in a patient with clinical features of MELAS and MERRF.' Arch Neurol **62**: 473-6

Ogilvie, I., Kennaway N.G. and Shoubridge E.A. (2005). 'A molecular chaperone for mitochondrial complex I assembly is mutated in a progressive encephalopathy.' J Clin Invest **115**(10): 2784-92.

Ohnishi, T. (1998). 'Iron-sulfur clusters/semiquinones in complex I.' Biochim Biophys Acta **1364**(2): 186-206.

O'Toole, J. F., Liu Y., Davis E.E., Westlake C.J., Attanasio M., Otto E.A., Seelow D., Nurnberg G., Becker C., Nuutinen M., Karppa M., Ignatius J., Uusimaa J., Pakanen S., Jaakkola E., van den Heuvel L.P., Fehrenbach H., Wiggins R., Goyal M., Zhou W., Wolf M.T., Wise E., Helou J., Allen S.J., Murga-Zamalloa C.A., Ashraf S., Chaki M., Heeringa S., Chernin G., Hoskins B.E., Chaib H., Gleeson J., Kusakabe T., Suzuki T., Isaac R.E., Quarmby L.M., Tennant B., Fujioka H., Tuominen H., Hassinen I., Lohi H., van Houten J.L., Rotig A., Sayer J.A., Rolinski B., Freisinger P., Madhavan S.M., Herzer M., Madignier F., Prokisch H., Nurnberg P., Jackson P., Khanna H., Katsanis N. and Hildebrandt F. (2010). 'Individuals with mutations in XPNPEP3, which encodes a mitochondrial protein, develop a nephronophthisis-like nephropathy.' J Clin Invest **120**(3): 791-802.

Pagliarini, D. J., Calvo S.E., Chang B., Sheth S.A., Vafai S.B., Ong S.E., Walford G.A., Sugiana C., Boneh A., Chen W.K., Hill D.E., Vidal M., Evans J.G., Thorburn D.R., Carr S.A. and Mootha V.K. (2008). 'A mitochondrial protein compendium elucidates complex I disease biology.' Cell **134**(1): 112-23.

Pagniez-Mammeri H., Lombes A., Brivet M., Ogier-de Baulny H., Landrieu P., Legrand A. and Slama A. (2009). 'Rapid screening for nuclear genes mutations in isolated respiratory chain complex I defects.' Mol Genet Metab **96**: 196-200.

Pagniez-Mammeri H., Landrieu P., Legrand A. and Slama A. (2010). 'Leukoencephalopathy with vanishing white matter caused by compound heterozygous mutations in mitochondrial complex I NDUFS1 subunit.' Mol Genet Metab **101**: 297-298.

Papa S. (1996). 'Mitochondrial oxidative phosphorylation changes in the life span. Molecular aspects and physiopathological implications.'' Biochim Biophys Acta **1276**: 87-105.

Papa, S., Sardanelli A.M., Cocco T., Speranza F., Scacco S.C. and Technikova-Dobrova Z. (1996). 'The nuclear-encoded 18 kDa (IP) AQDQ subunit of bovine heart complex I is phosphorylated by the mitochondrial cAMP-dependent protein kinase.' FEBS Lett **379**(3): 299-301.

Papa, S., Sardanelli A.M., Scacco S. and Technikova-Dobrova Z. (1999). 'cAMP-dependent protein kinase and phosphoproteins in mammalian mitochondria. An extension of the cAMP-mediated intracellular signal transduction.' FEBS Lett **444**(2-3): 245-9.

Papa, S., Scacco S., Sardanelli A.M., Vergari R., Papa F., Budde S., van den Heuvel L. and Smeitink J. (2001). 'Mutation in the NDUFS4 gene of complex I abolishes cAMP-dependent activation of the complex in a child with fatal neurological syndrome.' FEBS Lett **489**(2-3): 259-62.

Papa, S. (2002). 'The NDUFS4 nuclear gene of complex I of mitochondria and the cAMP cascade.' Biochim Biophys Acta **1555**(1-3): 147-53.

Papa S., Petruzzella V., Scacco S., Sardanelli A.M., Iuso A., Panelli D., Vitale R., Trentadue R., De Rasmo D., Capitanio N., Piccoli C., Papa F., Scivetti M., Bertini E., Rizza T. and De Michele G. (2009). 'Pathogenetic mechanisms in hereditary dysfunctions of complex I of the respiratory chain in neurological diseases.' Biochim Biophys Acta **1787**: 502-517.

Patterson K. (2004). 'Mitochondrial muscle pathology.' Pediatr Dev Pathol **7**: 629-632.

Pecina, P., Houstkova H., Hansikova H., Zeman J. and Houstek J. (2004). 'Genetic defects of cytochrome c oxidase assembly.' Physiol Res **53 Suppl 1**: S213-23.

Pequignot M.O., Dey R., Zeviani M., Tiranti V., Godinot C., Poyau A., Sue C., Di Mauro S., Abitbol M. and Marsac C. (2001). 'Mutations in the SURF1 gene associated with Leigh syndrome and cytochrome C oxidase deficiency.' Hum Mutat **17**: 374-381.

Petruzzella, V., Vergari R., Puzziferri I., Boffoli D., Lamantea E., Zeviani M. and 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**(5): 529-35.

Phoenix, C., Schaefer A. M., Elson J. L., Morava E., Bugiani M., Uziel G., Smeitink J. A., Turnbull D. M. and McFarland R. (2006). 'A scale to monitor progression and treatment of mitochondrial disease in children.' Neuromuscul Disord **16**(12): 814-20.

Pitkanen, S., Feigenbaum A., Laframboise R. and Robinson B.H. (1996). 'NADH-coenzyme Q reductase (complex I) deficiency: heterogeneity in phenotype and biochemical findings.' J Inherit Metab Dis **19**(5): 675-86.

Potluri P., Davila A., Ruiz-Pesini E., Mishmar D., O'Hearn S., Hancock S., Simon M., Scheffler I.E., Wallace D.C. and Procaccio V. (2009). 'A novel NDUFA1 mutation leads to a progressive mitochondrial complex I-specific neurodegenerative disease.' Mol Genet Metab **96**: 189-195.

Procaccio V. and Wallace D.C. (2004). 'Late-onset Leigh syndrome in a patient with mitochondrial complex I NDUFS8 mutations.' Neurology **62**: 1899-1901.

Quintana, A., Kruse S.E., Kapur R.P., Sanz E. and Palmiter R.D. (2010). 'Complex I deficiency due to loss of Ndufs4 in the brain results in progressive encephalopathy resembling Leigh syndrome.' Proc Natl Acad Sci U S A **107**(24): 10996-1001.

Rahman S., Blok R.B., Dahl H.H., Danks D.M., Kirby D.M., Chow C.W., Christodoulou J. and Thorburn D.R. (1996). 'Leigh syndrome: clinical features and biochemical and DNA abnormalities.' Ann Neurol **39**: 343-351.

Rodenburg, R. J. (2010). 'Biochemical diagnosis of mitochondrial disorders.' J Inherit Metab Dis: 1-10.

Rollins S., Prayson R.A., McMahon J.T. and Cohen B.H. (2001). 'Diagnostic yield muscle biopsy in patients with clinical evidence of mitochondrial cytopathy.' Am J Clin Pathol **116**: 326-330.

Rubio-Gozalbo, M. E., Dijkman K.P., van den Heuvel L.P., Sengers R.C., Wendel U. and Smeitink J.A. (2000). 'Clinical differences in patients with mitochondriocytopathies due to nuclear versus mitochondrial DNA mutations.' Hum Mutat **15**(6): 522-32.

Rustin P., Chretien D., Bourgeron T., Wucher A., Saudubray J.M., Rotig A. and Munnich A. (1991). 'Assessment of the mitochondrial respiratory chain." Lancet **338**: 60.

Rustin P., Chretien D., Bourgeron T., Gerard B., Rotig A., Saudubray J.M. and Munnich A. (1994). 'Biochemical and molecular investigations in respiratory chain deficiencies." Clin Chim Acta **228**: 35-51.

Rustin, P., Munnich A. and Rotig A. (2002). 'Succinate dehydrogenase and human diseases: new insights into a well-known enzyme.' Eur J Hum Genet **10**(5): 289-91.

Rustin, P. and Rotig A. (2002). 'Inborn errors of complex II--unusual human mitochondrial diseases.' Biochim Biophys Acta **1553**(1-2): 117-22.

Saada, A., Edvardson S., Rapoport M., Shaag A., Amry K., Miller C., Lorberboum-Galski H. and Elpeleg O. (2008). 'C6ORF66 is an assembly factor of mitochondrial complex I.' Am J Hum Genet **82**(1): 32-8.

Saada, A., Vogel R.O., Hoefs S.J., van den Brand M.A., Wessels H.J., Willems P.H., Venselaar H., Shaag A., Barghuti F., Reish O., Shohat M., Huynen M.A., Smeitink J.A., van den Heuvel L.P. and Nijtmans L.G. (2009). 'Mutations in NDUFAF3 (C3ORF60), encoding an NDUFAF4 (C6ORF66)-

interacting complex I assembly protein, cause fatal neonatal mitochondrial disease.' Am J Hum Genet **84**(6): 718-27.

Sardanelli, A. M., Technikova-Dobrova Z., Scacco S.C., Speranza F. and Papa S. (1995). 'Characterization of proteins phosphorylated by the cAMP-dependent protein kinase of bovine heart mitochondria.' FEBS Lett **377**(3): 470-4.

Sazanov, L. A. and Walker, J. E. (2000). 'Cryo-electron crystallography of two sub-complexes of bovine complex I reveals the relationship between the membrane and peripheral arms.' J Mol Biol **302**(2): 455-64.

Scaglia, F., Towbin J.A., Craigen W.J., Belmont J.W., Smith E.O., Neish S.R., Ware S.M., Hunter J.V., Fernbach S.D., Vladutiu G.D., Wong L.J. and Vogel H. (2004). 'Clinical spectrum, morbidity, and mortality in 113 paediatric patients with mitochondrial disease.' Paediatrics **114**(4): 925-31.

Schaefer, A. M., Taylor R.W., Turnbull D.M. and Chinnery P.F. (2004). 'The epidemiology of mitochondrial disorders--past, present and future.' Biochim Biophys Acta **1659**(2-3): 115-20.

Schaefer, A. M., Phoenix C., Elson J. L., McFarland R., Chinnery P. F. and Turnbull D. M. (2006). 'Mitochondrial disease in adults: a scale to monitor progression and treatment.' Neurology **66**(12): 1932-4.

Schapira, A. H., Gu M., Taanman J.W., Tabrizi S.J., Seaton T., Cleeter M. and Cooper J.M. (1998). 'Mitochondria in the etiology and pathogenesis of Parkinson's disease.' Ann Neurol **44**(3 Suppl 1): S89-98.

Schapira A.H. (1998). 'Inborn and induced defects of mitochondria.' Arch Neurol **55**: 1293-1296.

Schapira A.H. (2002a). 'The 'new' mitochondrial disorders.' J Neurol Neurosurg Psychiatry **72**: 144-149.

Schapira A.H. (2002b). 'Primary and secondary defects of the mitochondrial respiratory chain.' J Inherit Metab Dis **25**: 207-214.

Schatz, G. (1995). 'Mitochondria: beyond oxidative phosphorylation.' Biochim Biophys Acta **1271**(1): 123-6.

Schuelke, M., Smeitink J., Mariman E., Loeffen J., Plecko B., Trijbels F., Stockler-Ipsiroglu S. and van den Heuvel L. (1999). 'Mutant NDUFV1 subunit of mitochondrial complex I causes leukodystrophy and myoclonic epilepsy.' Nat Genet **21**(3): 260-1.

Schwartz, M. and Vissing, J. (2002). 'Paternal inheritance of mitochondrial DNA.' N Engl J Med **347**(8): 576-80.

Shoffner, J. M. (1996). 'Maternal inheritance and the evaluation of oxidative phosphorylation diseases.' Lancet **348**(9037): 1283-8.

Skladal, D., Halliday J. and Thorburn D.R. (2003a). 'Minimum birth prevalence of mitochondrial respiratory chain disorders in children.' Brain **126**(Pt 8): 1905-12.

Skladal, D., Sudmeier C., Konstantopoulou V., Stockler-Ipsiroglu S., Plecko-Startinig B., Bernert G., Zeman J. and Sperl W. (2003b). 'The clinical spectrum of mitochondrial disease in 75 paediatric patients.' Clin Pediatr (Phila) **42**(8): 703-10.

Smeitink, J. and L. van den Heuvel (1999). 'Human mitochondrial complex I in health and disease.' Am J Hum Genet **64**(6): 1505-10.

Smeitink, J., Sengers R., Trijbels, F. And van den Heuvel, L. (2001). "Human NADH:ubiquinone oxidoreductase." J Bioenerg Biomembr **33**(3): 259-66.

Sperl W., Sengers R.C., Trijbels J.M., Ruitenbeek W., Doesburg W.H., Smeitink J.A., Kollee L.A. and Boon J.M. (1992). 'Enzyme activities of the mitochondrial energy generating system in skeletal muscle tissue of preterm and fullterm neonates.' Ann Clin Biochem **29 (Pt 6)**: 638-645.

Spiegel R., Shaag A., Mandel H., Reich D., Penyakov M., Hujeirat Y., Saada A., Elpeleg O. and Shalev S.A. (2009). 'Mutated NDUFS6 is the cause of fatal neonatal lactic acidemia in Caucasus Jews.' Eur J Hum Genet **17**: 1200-1203.

Sue C.M., Hirano M., DiMauro S. and De Vivo D.C. (1999). 'Neonatal presentations of mitochondrial metabolic disorders.' Semin Perinatol **23**: 113-124.

Sugiana, C., Pagliarini D.J., McKenzie M., Kirby D.M., Salemi R., Abu-Amero K.K., Dahl H.H., Hutchison W.M., Vascotto K.A., Smith S.M., Newbold R.F., Christodoulou J., Calvo S., Mootha V.K., Ryan M.T. and Thorburn D.R. (2008). 'Mutation of C20orf7 disrupts complex I assembly and causes lethal neonatal mitochondrial disease.' Am J Hum Genet **83**(4): 468-78.

Taylor, R. W., Schaefer, A. M., Barron, M. J., McFarland, R., Turnbull, D. M. (2004). 'The diagnosis of mitochondrial muscle disease.' Neuromuscul Disord **14**(4): 237-45.

Taylor, R. W. and Turnbull, D. M. (2005). 'Mitochondrial DNA mutations in human disease.' Nat Rev Genet **6**(5): 389-402.

Thorburn, D. R. (2004). 'Mitochondrial disorders: prevalence, myths and advances.' J Inherit Metab Dis **27**(3): 349-62.

Thorburn, D. R., Sugiana C., Salemi R., Kirby D.M., Worgan L., Ohtake A. and Ryan M.T. (2004). 'Biochemical and molecular diagnosis of mitochondrial respiratory chain disorders.' Biochim Biophys Acta **1659**(2-3): 121-8.

Touati, G., Rigal O., Lombes A., Frachon P., Giraud M. and Ogier de Baulny H. (1997). 'In vivo functional investigations of lactic acid in patients with respiratory chain disorders.' Arch Dis Child **76**(1): 16-21.

Triepels R.H., van den Heuvel L.P., Loeffen J.L., Buskens C.A., Smeets R.J., Rubio Gozalbo M.E., Budde S.M., Mariman E.C., Wijburg F.A., Barth P.G., Trijbels J.M. and Smeitink J.A. (1999). 'Leigh syndrome associated with a mutation in the NDUFS7 (PSST) nuclear encoded subunit of complex.' Ann Neurol **45**: 787-790.

Triepels R.H., Van Den Heuvel L.P., Trijbels J.M. and Smeitink J.A. (2001). 'Respiratory chain complex I deficiency.' Am J Med Genet **106**: 37-45.

Tuppen, H. A., Hogan V.E., He L., Blakely E.L., Worgan L., Al-Dosary M., Saretzki G., Alston C.L., Morris A.A., Clarke M., Jones S., Devlin A.M., Mansour S., Chrzanowska-Lightowlers Z.M., Thorburn D.R., McFarland R. and Taylor R.W. (2010). 'The p.M292T NDUFS2 mutation causes complex I-deficient Leigh syndrome in multiple families.' Brain **133**(10): 2952-63.

Ugalde C., Triepels R.H., Coenen M.J., van den Heuvel L.P., Smeets R., Uusimaa J., Briones P., Campistol J., Majamaa K., Smeitink J.A. and Nijtmans L.G. (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 R.J., van den Heuvel L.P., Smeitink J.A. and Nijtmans L.G. (2004). '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. and Nijtmans L. (2004). 'Human mitochondrial complex I assembles through the combination of evolutionary conserved modules: a framework to interpret complex I deficiencies.' Hum Mol Genet **13**(20): 2461-72.

Ugalde C., Hinttala R., Timal S., Smeets R., Rodenburg R.J., Uusimaa J., van Heuvel L.P., Nijtmans L.G., Majamaa K. and Smeitink J.A. (2007). 'Mutated ND2 impairs mitochondrial complex I assembly and leads to Leigh syndrome.' Mol Genet Metab **90**: 10-14.

Valanne L., Ketonen L., Majander A., Suomalainen A. and Pihko H. (1998). 'Neuroradiologic findings in children with mitochondrial disorders.' AJNR Am J Neuroradiol **19**: 369-377.

Valentino M.L., Barboni P., Ghelli A., Bucchi L., Rengo C., Achilli A., Torroni A., Lugaresi A., Lodi R., Barbiroli B., Dotti M., Federico A., Baruzzi A. and 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.

van den Heuvel, L., Ruitenbeek W., Smeets R., Gelman-Kohan Z., Elpeleg O., Loeffen J., Trijbels F., Mariman E., de Bruijn D. and 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**(2): 262-8.

van der Knaap M.S., Jakobs C. and Valk J. (1996). 'Magnetic resonance imaging in lactic acidosis.' J Inherit Metab Dis **19**: 535-547.

van der Stoep N., van Paridon C.D., Janssens T., Krenkova P., Stambergova A., Macek M., Matthijs G. and Bakker E. (2009). 'Diagnostic guidelines for high-resolution melting curve (HRM) analysis: an interlaboratory validation of BRCA1 mutation scanning using the 96-well LightScanner.' Hum Mutat **30**: 899-909.

Victor, V. M., Apostolova N., Herance R., Hernandez-Mijares A. and Rocha M. (2009). 'Oxidative stress and mitochondrial dysfunction in atherosclerosis: mitochondria-targeted antioxidants as potential therapy.' Curr Med Chem **16**(35): 4654-67.

Visch H.J., Rutter G.A., Koopman W.J., Koenderink J.B., Verkaart S., de Groot T., Varadi A., Mitchell K.J., van den Heuvel L.P., Smeitink J.A. and Willems P.H. (2004). 'Inhibition of mitochondrial Na+-Ca2+ exchange restores agonist-induced ATP production and Ca2+ handling in human complex I deficiency.' J Biol Chem **279**: 40328-40336.

Visch H.J., Koopman W.J., Leusink A., van Emst-de Vries S.E., van den Heuvel L.W., Willems P.H. and Smeitink J.A. (2006a). 'Decreased agonist-stimulated mitochondrial ATP production caused by a pathological reduction in endoplasmic reticulum calcium content in human complex I deficiency.' Biochim Biophys Acta **1762**: 115-123.

Visch H.J., Koopman W.J., Zeegers D., van Emst-de Vries S.E., van Kuppeveld F.J., van den Heuvel L.W., Smeitink J.A. and Willems P.H. (2006b). 'Ca2+-mobilizing agonists increase mitochondrial ATP production to accelerate cytosolic Ca2+ removal: aberrations in human complex I deficiency.' Am J Physiol Cell Physiol **291**: C308-316.

Vogel H. (2001). 'Mitochondrial myopathies and the role of the pathologist in the molecular era.' J Neuropathol Exp Neurol **60**: 217-227.

Vogel, R. O., Janssen R.J., Ugalde C., Grovenstein M., Huijbens R.J., Visch H.J., van den Heuvel L.P., Willems P.H., Zeviani M., Smeitink J.A. and Nijtmans L.G. (2005). 'Human mitochondrial complex I assembly is mediated by NDUFAF1.' FEBS J **272**(20): 5317-26.

Vogel, R. O., Janssen R.J., van den Brand M.A., Dieteren C.E., Verkaart S., Koopman W.J., Willems P.H., Pluk W., van den Heuvel L.P., Smeitink J.A. and Nijtmans L.G. (2007). 'Cytosolic signaling

protein Ecsit also localizes to mitochondria where it interacts with chaperone NDUFAF1 and functions in complex I assembly.' Genes Dev **21**(5): 615-24.

von Ballmoos, C., Wiedenmann A. and Dimroth P. (2009). 'Essentials for ATP synthesis by F1F0 ATP synthases.' Annu Rev Biochem **78**: 649-72.

von Kleist-Retzow J.C., Cormier-Daire V., de Lonlay P., Parfait B., Chretien D., Rustin P., Feingold J., Rotig A. and Munnich A. (1998). 'A high rate (20%-30%) of parental consanguinity in cytochrome-oxidase deficiency.' Am J Hum Genet **63**: 428-435.

von Kleist-Retzow, J. C., Cormier-Daire V., Viot G., Goldenberg A., Mardach B., Amiel J., Saada P., Dumez Y., Brunelle F., Saudubray J.M., Chretien D., Rotig A., Rustin P., Munnich A. and De Lonlay P. (2003). 'Antenatal manifestations of mitochondrial respiratory chain deficiency.' J Pediatr **143**(2): 208-12.

Vossen R.H., Aten E., Roos A. and den Dunnen J.T. (2009). 'High-resolution melting analysis (HRMA): more than just sequence variant screening.' Hum Mutat **30**: 860-866.

Walker, J. E. (1992). 'The NADH:ubiquinone oxidoreductase (complex I) of respiratory chains.' Q Rev Biophys **25**(3): 253-324.

Walker, J. E., Arizmendi J.M., Dupuis A., Fearnley I.M., Finel M., Medd S.M., Pilkington S.J., Runswick M.J. and Skehel J.M. (1992). 'Sequences of 20 subunits of NADH:ubiquinone oxidoreductase from bovine heart mitochondria. Application of a novel strategy for sequencing proteins using the polymerase chain reaction.' J Mol Biol **226**(4): 1051-72.

Walker, J. E., Skehel J.M. and Buchanan S.K. (1995). 'Structural analysis of NADH: ubiquinone oxidoreductase from bovine heart mitochondria.' Methods Enzymol **260**: 14-34.

Walker, C., Byrne (1996). 'Respiratory chain encephalomyopathies: A diagnostic classification.' Eur Neurol **36**: 260-267.
Wallace, D. C., Singh G., Lott M.T., Hodge J.A., Schurr T.G., Lezza A.M., Elsas L.J., 2nd and Nikoskelainen E.K. (1988). 'Mitochondrial DNA mutation associated with Leber's hereditary optic neuropathy.' Science **242**(4884): 1427-30.

Wallace, K. B., Eells J.T., Madeira V.M., Cortopassi G. and Jones D.P. (1997). 'Mitochondria-mediated cell injury. Symposium overview.' Fundam Appl Toxicol **38**(1): 23-37.

Wang, H. and Oster G. (1998). 'Energy transduction in the F1 motor of ATP synthase.' Nature **396**(6708): 279-82.

Zafeiriou, D. I., Rodenburg, R. J., Scheffer, H., van den Heuvel, L. P., Pouwels, P. J., Ververi, A., Athanasiadou-Piperopoulou, F., van der Knaap, M. S. (2008). 'MR spectroscopy and serial magnetic resonance imaging in a patient with mitochondrial cystic leukoencephalopathy due to complex I deficiency and NDUFV1 mutations and mild clinical course.' Neuropaediatrics **39**(3): 172-5.

Zeviani M., Bresolin N., Gellera C., Bordoni A., Pannacci M., Amati P., Moggio M., Servidei S., Scarlato G. and DiDonato S. (1990). 'Nucleus-driven multiple large-scale deletions of the human mitochondrial genome: a new autosomal dominant disease.' Am J Hum Genet **47**: 904-914.

Zeviani, M., Bertagnolio B. and Uziel G. (1996). 'Neurological presentations of mitochondrial diseases.' J Inherit Metab Dis **19**(4): 504-20.

Zeviani, M. (2004). 'Mitochondrial disorders.' Suppl Clin Neurophysiol **57**: 304-12.

## Websites


Apweiler, R. (2002); UniProt Consortium
(http://www.uniprot.org/)
Status: 26.05.2009

Bethesda National Center for Biotechnology Information (2006): 'SNP database'
(http://www.ncbi.nlm.nih.gov/SNP/)
Status: 06.03.2009

Department of Chemistry, University of Maine, Orono, ME 04469 (Last revised: January 8th, 2015)
(http://chemistry.umeche.maine.edu/CHY431/Code4.html)
Status: 10.01.2015

Director: Bryan S. (2010); National Center for Biotechnology Information:
'The NCBI Structure Group'
(http://www.ncbi.nlm.nih.gov/Structure)
Status: 19.10.2010

Director: Mattaj, I. (2009): European Bioinformatic Institute: Tools>Sequence>Analysis>
ClustalW2 for alignments
(http://www.ebi.ac.uk/Tools/clustalw2/index.html)
Status: 01.11.2010

Geeknet, Inc. Sourgeforge (2010): 'Software sequence analysis Staden package'
(http://staden.sourceforge.net/staden_home.html)
Status: 25.02.2009

GenBankAccession (Last revised: January 29, 2010)
(http://www.ncbi.nlm.nih.gov/genbank/)
Status: 05.05.2009

Hamosh A. (1985); National Center for Biotechnology Information (NCBI), McKusick-Nathans Institute of Genetic Medicine and Johns Hopkins University School of Medicine:
'OMIM-Online Mendelian Inheritance in Man'
(www.ncbi.nlm.nih.gov/Omim/)
Status: 07.07.2009

Idaho Technology (2001-2010): 'LightScanner System'
(http://www.idahotech.com/LightScanner/index.html)
Status: 04.03.2009

Ingman, M. & Gyllensten, U. (2006) 'MtDB: Human Mitochondrial Genome Database, a resource for population genetics and medical sciences'
(http://www.genpat.uu.se/mtDB/)
Status: 10.05.2009

Kent J. (2002), UCSC In-Silico PCR
(http://genome.ucsc.edu/cgi-bin/hgPcr)
Status: 31.12.2008

Lott, M. (2010): 'A human mitochondrial genome database: MITOMAP Web'
(http://www.mitomap.org/MITOMAP)
Status: 11.02.2009

Mansfield, B.K. (2003); U.S. Department of Energy Genome Program's Biological and Environmental Research Information System (BERIS)
(http://www.ornl.gov/sci/techresources/Human_Genome/home.shtml)
Status: 16.06.2009

Rosenbloom, KR (2002); Genome Bioinformatics Group of UC Santa Cruz:
'UCSC Human (*Homo sapiens*) Genome Browser Gateway'
(http://genome.ucsc.edu/cgi-bin/hgGateway)
Status: 30.05.2009

Weizmann Institute of Science (1996): 'The GeneCards Human Gene Database'
(http://www.genecards.org)
Status: 12.04.2009

# 6 Appendix

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| Appendix 1 | Overview of 'classical mitochondrial syndromes' |
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**Appendix. 1.** Overview of most common mitochondrial syndromes and their mode of inheritance, clinical features and cardinal findings in clinical diagnostics.

| Denotation | Description | OMIM | Clinical symptoms | Findings | Inheritance |
|---|---|---|---|---|---|
| Alpers syndrome | Also known as Alpers Huttenlocher disease; progressive infantile poliodystrophy | 203700 | Infantile onset of progressive neurodegenerative disease (mental retardation, epilepsy) | cMRI: cortical atrophy | Autosomal-recessive |
| | | | Muscular hypotonia | typical neuropathological findings post-mortem | |
| | | | Spasticity (often leading to quadriplegia) | | |
| | | | Frequent: liver impairment, optic nerve atrophy, hearing loss | | |
| CPEO | Chronic progredient external ophthalmoplegia | 157640<br>258450<br>609283<br>609286<br>610131 | Ptosis (in general first symptom) | Ptosis | Sporadic, autosomal-dominant, autosomal-recessive |
| | | | Progressive external ophthalmoplegia | | |
| KSS | Kearns-Sayre syndrome | 530000 | Ptosis, CPEO, retinal degeneration | Retinitis pigmentosa | Sporadic |
| | | | Cardiac conduction impairments | ECG: conduction disturbances | |
| | | | Ataxia | cMRI: usually diffuse leukencepalopathy | |
| | | | Frequent: short stature, hearing loss, dysphagia | | |
| | | | Onset <20 years of age | | |
| Leigh syndrome[2] | Also known as Subacute Necrotizing EncephaloMyopathy (SNEM) | 256000<br>308930 | Neurodegenerative disease starting during first year of life | cMRI: symmetrical lesions in basal ganglia and brainstem | Autosomal-recessive, maternal, X-linked[1] |
| | | | Psychomotor retardation and regression | Typical neuropathological findings post-mortem | |
| | | | Frequent: muscular hypotonia, ataxia, seizures, neuropathy, optic nerve atrophy | | |
| | | | | Lactic acidosis | |
| LHON | Leber's hereditary optic nerve atrophy | 535000 | Vision loss in young adults | Optic nerve atrophy | Maternal, sporadic |
| | | | Possible: cardiac involvement, encephalopathic features | | |
| MELAS | Mitochondrial encephalomyopathy with lactic acidosis and stroke-like episodes | 540000 | Stroke-like episodes generally <40 years of age (often resulting in hemiparesis, migraine-like headaches, focal or generalized seizures, dementia) | cMRI: stroke-like lesions in vessel autonomous regions | Maternal |
| | | | Recurrent vomiting | Lactic acidosis | |
| | | | Myopathy (muscle weakness, myalgia) often observed as early symptoms | Ragged red fibers[3] | |
| | | | possible deafness or diabetes development at later stage | | |
| MERRF | Myoclonic epilepsy with ragged red fibers | 545000 | Progressive myoclonic epilepsy | Ragged red fibers[3] | Maternal |
| | | | Ataxia | | |
| | | | Myopathy | | |
| | | | Frequent: hearing loss, neuropathy, short stature | | |
| MNGIE | Mitochondrial neurogastrointestinal encephalomyopathy | 603041 | Myopathy | cMRI: diffuse leukencephalopathy | Autosomal-recessive |
| | | | Episodes of gastrointestinal dysmotility | | |
| | | | Frequent: neuropathy, ptosis, CPEO | | |

**Continuation of Appendix. 1.** Overview of most common mitochondrial syndromes and their mode of inheritance, clinical features and cardinal findings in clinical diagnostics.

| | | | | | |
|---|---|---|---|---|---|
| NARP | Neuropathy, ataxia and retinitis pigmentosa | 551500 | Neuropathy (in general sensory)<br>Ataxia<br>Vision loss | Retinitis pigmentosa | Maternal |
| Pearson syndrome | Pearson-Marrow-Pancreas syndrome | 557000 | Anaemia (transfusion-dependent)<br>Malabsorption<br>Furthermore frequent liver, renal and endocrine dysfunction, failure to thrive<br>Death often during infancy or early childhood, survivors developed KSS | Sideroblastic anaemia<br>Exocrine pancreas insufficiency | Sporadic |

[1]X-linked is a mode of inheritance in which the mutated gene is localized on the X chromosome and causes therefore the expression of the phenotype in males (who are hemizygous) and in females who inherited two mutated alleles.
[2]Striktely speaking per definition, Leigh syndrome is a neuropathological defined disease [Leigh 1951]. If the clinical presentation and suspicious findings in cMRI match together and are suggestive for a Leigh syndrome, it is characterized as Leigh-like syndrome [Loeffen et al. 2000, p.124].
[3]Ragged red fibers (RRF) are histological findings in electron microscopy and pathological correlates of accumulated faulty mitochondria seen in the Gomori-trichrome stain of muscle fibers.
Abbreviations: cMRI=cranial magnetic resonance imaging; ECHO=echocardiogram; ECG=electrocardiogram;

**Appendix. 2**. Results of detected sequence variants in mtDNA from the complex mutation analysis of RCC I genes in 150 patients with RCC I deficiency performed at the Institute of Human Genetics. Ranking system of mutations (1=confirmed mutation; 2=possible mutation; 3=unlikely) and description for the classification encompasses enquiry of frequency of mtDNA mutations (http://www.mitomap.org/) and level of conservation across the individuals.

| Gene | Patient ID | Sequence variants | Heteroplasmy rate | Ranking | Description |
|---|---|---|---|---|---|
| MT-ND1 | 35805 | c.3764C>T, p.T153M | >95% | 3 | <0.1% mitomap; not conserved |
| MT-ND2 | 32999 | c.4512G>A, p.A15T | >95% | 2 | new, mitomap 0 of 2704 |
| MT-ND2 | 33016 | c.5041T>C, p.M191T | >95% | 2 | new, mitomap 0 of 2704 |
| MT-ND3 | 33343[1] | c.10197G>A, p.A47T | ~80% | 1* | <0.1% mitomap, confirmed Leigh/dystonia/stroke? (according to Bandelt, Salas et al. 2009 unclear) |
| MT-ND3 | 33456 | c.10197G>A, p.A47T | ~80% | 1* | <0.1% mitomap, confirmed Leigh/dystonia/stroke? (according to Bandelt, Salas et al. 2009 unclear) |
| MT-ND3 | 33464 | c.10158T>C, p.S43P | >95% | 1* | mitomap confirmed mutation: encephalopathy, Leigh-like |
| MT-ND3 | 35841 | c.10191T>C, p.S45P | >95% | 1* | mitomap confirmed mutation: ESOC[2], Leigh-like [McFarland et al. 2004, p.58-59] |
| MT-ND3 | 35841 | c.10398 G>A, p.A114T | >95% | 2 | new, mitomap 0 of 2704 |
| MT-ND4 | 33346 | c.11360A>G, p.M201V | >95% | 3 | new, not conserved, 1 of 2704 |
| MT-ND5 | 33343[1] | c.13969A>T, p.S545C | >95% | 3 | new, not conserved, 1 of 2704 |
| MT-ND5 | 33328 | c.13042G>A, p.A236T | ~80% | 1* | reported in mitomap: MELAS [Naini et al. 2005] |
| MT-ND6 | 33328 | c.14470T>C, p.G68E | >95% | 3 | new, not conserved |
| MT-ND6 | 33346 | c.14487T>C, p.M63V | >95% | 1* | known Mutation (Leigh syndrome/dystonia) |
| MT-ND6 | 44732 | c.14459G>A, p.A72V | >95% | 1* | mitomap confirmed mutation: Leigh-like, LHON |
| MT-TL1 | 33041 | 3250T>C | | 1* | reported in mitomap: CPEO, myopathy |
| MT-TV | 33267, 33466 | 1607T>C | | 2 | new, mitomap 0 of 2704 (highly conserved) |
| MT-TV | 33354 | 1659insTT | | 2 | new, highly conserved |
| MT-TA | 35803 | 7570A>G | | 2 | new, mitomap 0 of 2704 (highly conserved) |
| MT-TY | 33344 | 5839C>T | | 2 | 3/2704, highly conserved |

*Indicates mutations which have been associated with special phenotypes in the mitomap and are declared as pathogenic.

[1]A homozygous mutation in the nuclear gene NDUFB8 has also been identified in this patient, whereas the pathogenic character of this sequence variant is not clear (see **Appendix. 3**).

[2]ESOC=epilepsy, stroke-like episodes, optic nerve atrophy and cognitive decline

**Comment**

Sequence variants in the mitochondrial genome are often difficult to interpret regarding their pathogenic character for the disease. The various numbers of SNPs situated in the mtDNA and the challenge to evaluate the pathogenicity of newly identified sequence variants are problems in the interpretation of mitochondrial gene mutations [DiMauro et al. 2005, p.227-29; Taylor et al. 2005, p.393]. Although, some special mutations of mtDNA encoded subunits of RCC I are associated with variable phenotypes like LHON, Leigh syndrome, MELAS and other mitochondrial syndromes (MITOMAP; www.mitomap.org), the variability of mtDNA (see 1.1.2 for further details) makes it a difficult issue to interpret newly identified sequence variants [Bénit et al. 2004, p.139-41].

**Appendix. 3.** Sequence variants which have been found in nuclear genes encoding for RCC I in the 150 patients analysed. Thirteen pathogenic mutations (compound heterozygous or homozygous) have been identified which can explain the molecular basis of mitochondrial disease in these patients and their phenotype (indicated with ranking score 1).

| Gene_exon | Gene locus | Patient ID | Sequence variants | Ranking | Conservation/ Reference |
|---|---|---|---|---|---|
| C6ORF66_1 | 6q16.1 | 33006 | c.[23G>A]+[23G>A], p.[G8D]+[G8D] | 1 | Prokisch et al., unpublished |
| NDUFA1_1 | Xq24 | 33545 | c.[94 G>C], p.[G32R] | 1 | all G (4 times) |
| NDUFA12L_1 | 5q12.1 | 33462 | c.[9G>A]+[9G>A] p.[W3X]+[W3X] | 1 | Herzer et al. 2010 |
| NDUFB9_2 | 8q13.3 | 35838 | c.[191T>C]+[192T>C], p.[L64P]+[L64P] | 1 | all L (5 times) |
| NDUFS1_7 | 2q33-q34 | 33255 | c.[497G>A(+)683T>C], p.[G166E(+)V228A] | 1 | all G, all V (6 times) |
| NDUFS1_8 | 2q33-q34 | 33460 | c.[683T>C(+)754A>G], p.[V228A(+)D252G] | 1 | all V, all D (6 times) |
| NDUFS1_15 | 2q33-q34 | 35837 | c.[1669C>T(+)1783A>G], p.[R557X(+)T595A] | 1 | HsMmDmCeNcAt (TTTTTT) |
| NDUFS1_15 | 2q33-q34 | 35847 | c.[1564C>A]+[1564C>A], p.[Q522K]+[Q522K] | 1 | HsMmDmCeNcAt (QQQQRQ) |
| NDUFS1_17 | 2q33-q34 | 55555 | c.[1912delA]+[ c.2084A>G], p.[T638fsX642]+[Y695C] | 1 | Frame shift |
| NDUFS1_18 | 2q33-q34 | 35822 | c.[2083T>C(+)2084A>G], p.[Y695H(+)Y695C] | 1 | HsMmDmCeNcAt (YYFYYY) |
| NDUFS1_4 | 2q33-q34 | 36179 | c.[212T>A(+)384T>A], p.[V71D(+)C128X] | 1 | HsMmDmCeNcAt (VVVIII), all C (6 times) |
| NDUFS2_10 | 1q23 | 33354 | c.[968G>A(+)329A>T], p.[Q323R(+)D110V] | 1 | HsMmCeNcAt (RRKNQ), HsMmCeNcAt (DDIDE)] |
| NDUFS4_3 | 5q11.1 | 33253 | c.[316C>T]+[316C>T], p.[R106X]+[R106X] | 1 | Stop codon |
| NDUFS6_4 | 5pter-p15.33 | 35797 | c.[352C>T]+[352C>T], p.[Q118X]+[Q118X] | 1 | Stop codon |
| XPNPEP3 | 22q13.2 | 33023 | c.[931_934delAACA]+[931_934delAACA] p.[N311LfsX5]+[N311LfsX5] | 1 | O'Toole et al. 2010 |
| NDUFA10_8 | 2q37.3 | 35831 | c.[849G>A], p.[W283X] | 2 | HsMmDmCe (WWAW) |
| NDUFA3_3 | 19q13.42 | 35827 | c.[146C>T], p.[T49M] | 2 | HsMmDr (TTV) |
| NDUFA6_3 | 22q13.1 | 33041 | c.[384T>A], p.[H128Q] | 2 | all H (6 times) |
| NDUFA7_3 | 19p13.2 | 35833 | c.[142A>C], p.[K48Q] | 2 | HsMmDmCe (KKLK) |
| NDUFA9_4 | 12p | 35844 | c.[373G>A], p.[V125M] | 2 | all V (6 times) |
| NDUFB9_2 | 8q13.3 | 33027 | [NDUFB9:c.140G>T]+[NDUFA5:c.-6G>A], [NDUFB9:p.R47L]+[NDUFA5:p.?] | 2 | HsMmDmCeAt (RRRRK) |
| NDUFS1_7 | 2q33-q34 | 35842 | c.[529A>G], p.[I177V] | 2 | all I (6 times) |
| NDUFS2_7 | 1q23 | 35788 | c.[671C>T], p.[A224V] | 2 | HsMmCeNcAt (AAAAH) |
| NDUFS7_3 | 19p13 | 35824 | c.[100G>A], p.[V34M] | 2 | HsMmNcAtDm (VVIAQ) |
| NDUFS8_6 | 11q13 | 33284 | c.[476C>A], p.[A159D] | 2 | HsMmDmCeAtNc (AAAAAS) |
| NDUFV3_4 | 21q22.3 | 33007 | c.[1289C>T], p.[P430L] | 2 | all P (3 times) |
| ECSIT_5_6 | 19p13.2 | 33026 | c.[832C>T], p.[R278C] | 3 | not conserved |
| ECSIT_5_6 | 19p13.2 | 33332 | c.[832C>T], p.[R278C] | 3 | not conserved |
| ECSIT | 19p13.2 | 33334 | [ECSIT:c.344G>A]+[AFG3L2:c.1895G>G/A], [ECSIT:p.R115H]+[AFG3L2:p.R632Q] | 3 | not conserved |
| ECSIT_3_2 | 19p13.2 | 33459 | c.[425G>A], p.[R142Q] | 3 | not conserved |

| | | | | | |
|---|---|---|---|---|---|
| NDUFA10_3 | 2q37.3 | 35830 | c.[296G>A]+[296G>A] p.[G99E]+[G99E] | 3 | not conserved |
| NDUFA10_3 | 2q37.3 | 36242 | c.[296G>A]+[296G>A] p.[G99E]+[G99E] | 3 | not conserved |
| NDUFA13_2 | 19p13.2-p13.1 | 33281 | c.[374C>A], p.[T125N] | 3 | not conserved |
| NDUFA6_3 | 22q13.1 | 33033 | c.[400C>T], p.[H134Y] | 3 | not conserved |
| NDUFA6_3 | 22q13.1 | 35815 | c.[400C>T], p.[H134Y] | 3 | not conserved |
| NDUFA6_3 | 22q13.1 | 35820 | c.[400C>T], p.[H134Y] | 3 | not conserved |
| NDUFAF1_3 | 15q13.3 | 33326 | c.[708G>A], p.[M236I] | 3 | not conserved |
| NDUFB8_4 | 10q23.2-q23.33 | 33331 | c.[364A>G], p.[T122A] | 3 | not conserved |
| NDUFB8_5 | 10q23.2-q23.33 | 35841[1] | c.[524C>T]+[524C>T], p.[S175F]+[S175F] | 3 | not conserved |
| NDUFS1_13 | 2q33-q34 | 33017 | c.[1291C>G], p.[L431V] | 3 | not conserved |
| NDUFS1_13 | 2q33-q34 | 37796 | c.[1291C>G], p.[L431V] | 3 | not conserved |
| NDUFS3_2 | 11p11.11 | 33023 | c.[94C>G], p.[L32V] | 3 | not conserved |
| NUBPL_5 | 14q12 | 33025 | c.[413G>A], p.[G138D] | 3 | |
| NUBPL_2 | 14q12 | 33277 | c.[166G>A], p.[G56R] | 3 | |
| NUBPL_7 | 14q12 | 33358 | c.[545T>C], p.[V182A] | 3 | |
| NUBPL_7 | 14q12 | 36230 | c.[593A>C], p.[N198T] | 3 | |

*Ranking score: 1=pathogenic mutation; 2=possible mutation; 3=unlikely

[1]This patient harbours also a pathogenic homoplasmic mtDNA mutation in MT-ND3 and another homoplasmic mutation with unspecified pathogenic relevance in MT-ND5 (see **Appendix. 2**).

**Appendix. 4.** Detailed information about analysed candidate genes

*C20ORF7*

During the present work and the genetic analysis of C20ORF7 (**Fig. 34**) in the patient cohort, the first pathogenic mutation has been identified by homozygosity mapping and subsequent sequencing of the candidate gene [Sugiana et al. 2008, p.470-72]. Green fluorescent protein (GFP) tagging and microscopy disclosed the localization of the protein within the inner mitochondrial membrane. Furthermore, they hypothesized that C20ORF7 is involved in methylation processes of conserved histidine residues because of its predicted S-adenosylmethionine(SAM)-dependent methyltransferase fold (http://www.ncbi.nlm.nih.gov/Structure) and therefore can serve as an essential requisite in RCC I assembly process [Sugiana et al. 2008, p.474-78]. Shortly after, another mutation affecting the highly conserved SAM-dependent methyltransferase domain of the protein has been associated with the phenotype of Leigh syndrome in a family with three affected children [Gerards et al. 2009, p.507]. Beside the similarities in molecular and biochemical characteristics of mutated assembly factor C20ORF7, the clinical presentation reported in the patients was quite different comparing both reports. The three patients from Gerards presented at three years of age with spasticity and developed a severe extrapyramidal movement disorder with dystonic posturing, spastic tetraplegia and dysarthria. Results from cMRI revealed lesions mainly affecting the basal ganglia beside discrete bifrontal global atrophy. Remarkable was, that disease progression seemed to slow down after puberty was reached [Gerards et al. 2009, p.511-13]. In contrast the patient reported from Sugiana presented with severe progressive neonatal mitochondrial disease with lactic acidosis and died at one month of age [Sugiana et al. 2008, p.470-72]. These two, particular different, reports of patients with mutations in the C20ORF7 assembly factor encoding gene suggest that there are no correlations between the phenotype and genotype. Furthermore, no pinpoint can be obtained from the phenotype of the patients to guide molecular-genetic diagnostics in the direction of analysing C20ORF7 firstly.

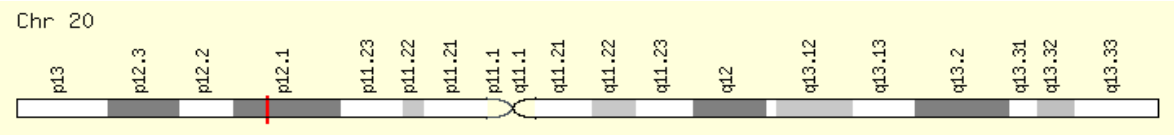


**Fig. 34**. Localization of C20ORF7 on chromosome 20p (start: 13,713,682 base-pair (bp), end: 13,745,874 bp) is labelled red. The gene encompasses 32,193 bases and two isoforms of the human protein are produced by alternative splicing (for mutation analysis isoform 1 with eleven coding exons was examined). http://www.genecards.org

*C7ORF10*

The protein encoded by C7ORF10 (**Fig. 35**) is affiliated to CoA-transferase family III. CoA-transferases are found in all organisms from all lines of descent and most of them, which are members of family I and II, are well known enzymes. But recent work revealed a third family of CoA-transferases, which are poorly characterized (http://www.ncbi.nlm.nih.gov/Structure). Within the research work for a mitochondrial compendium C7ORF10 was predicted to be important for RCC I function because of its high phylogenetic conservation [Pagliarini et al. 2008, p.114-17].

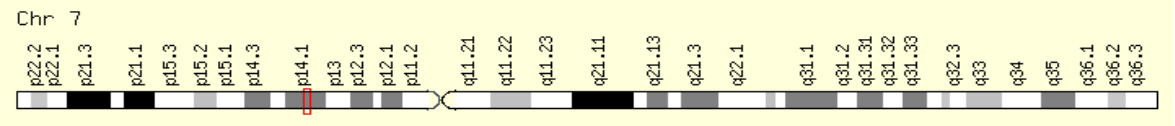


**Fig. 35.** Localization of C7ORF10 on chr. 7p (start: 40,141,100 bp, end: 40,866,882 bp) is labelled red. The gene encompasses 725,783 bases and 15 coding exons. http://www.genecards.org

*C10ORF65*

This gene (**Fig. 36**) was also mapped to be localized in mitochondria by the mitochondrial compendium and is affiliated to the dihydrodipicolinate synthase family (DHDPS-like protein isoform 1) which are members of the class I aldolases (http://www.ncbi.nlm.nih.gov/Structure). Phylogenetic analysis predicted this gene to be involved in functional stabilization of RCC I.

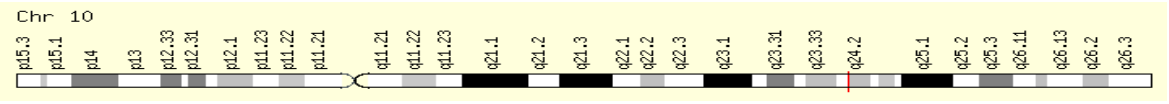


**Fig. 36.** Localization of C10ORF65 on chr. 10q (start: 99,334,070 bp, end: 99,362,549 bp) is labelled red. The gene encompasses 28,480 bases and two isoforms of the human protein are produced by alternative splicing (for mutation analysis isoform 1 with seven coding exons was examined). http://www.genecards.org

*C3ORF60*

Also known as NDUFAF3, this gene (**Fig. 37**) encodes a nuclear protein of unknown function. The similar rat nuclear protein is predominantly expressed in testis. Only structural data are available for this protein (Mth938_2P1-like, http://www.ncbi.nlm.nih.gov/Structure). Recently, patients with fatal neonatal mitochondrial disease have been shown to harbour mutations in C3ORF60 [Saada et al. 2009, p.718]. The patients presented with lactic acidosis soon after birth and all children died before the age of six months. However, they had different clinical features and outcome [Saada et al. 2009, p.719]. Using a baculoviral complementation approach it has been shown that the RCC I amount and activity could be restored to normal levels in fibroblasts of the patients. Additionally, Saada et al. showed that NDUFAF3 plays a role in RCC I assembly process in combination with NDUFAF4 (C6ORF66) and that the protein is localized to the mitochondrial membrane [Saada et al. 2009, p.721-26].

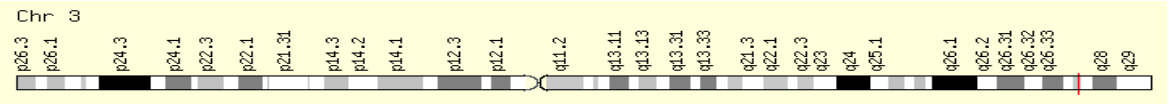


**Fig. 37**. Localization of C3ORF60 on chr. 3q (start: 186,913,774 bp, end: 186,918,649 bp) is labelled red. The gene encompasses 4,876 bases and two isoforms of the human protein are produced by alternative splicing (for mutation analysis isoform 1 with five coding exons was examined). http://www.genecards.org

*XPNPEP3*

XPNPEP3 encodes the x-prolyl aminopeptidase 3 (**Fig. 38**) and has been associated to patients with RCC I deficiency and nephronophthisis (NPHP) [O'Toole et al. 2010, p.791]. They identified a new locus on 22q13.2 containing 101 putative candidate genes performing whole genome wide linkage in 116 consanguineous families with NPHP or NPHP-like phenotype. By exon sequencing of 29 of these candidate genes, they found likely pathogenic sequence variants in XPNPEP3. The new gene product was localized in mitochondria - specifically expressed in distal convoluted tubule and the cortical collecting duct - and accompanied with RCC I deficiencies. One family with identified mutations had two affected children who presented with the phenotype of a mitochondrial disorder and decreased RCC I activity in muscle biopsies. They harboured a homozygous mutation in exon 6 (c.929.932delCAAA) resulting in a frame shift and premature stop codon (N311fsX315 homozygous). Both children had hypertrophic dilated cardiomyopathy, mental retardation, seizures, gastrointestinal involvement (chronic pancreatitis and hepatopathy) and required renal replacement therapy at the age of nine and eight years, respectively (died at the age of eleven and nine years) [O'Toole et al. 2010, p.795].

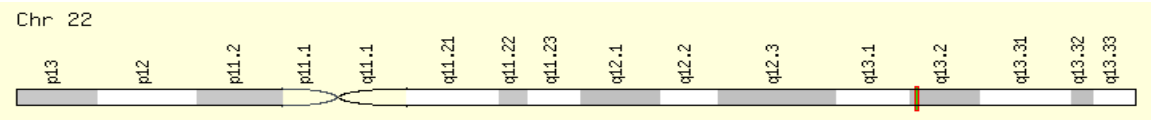


**Fig. 38.** Localization of XPNPEP3 on chr. 22q (start: 41,253,081 bp; end: 41,368,585 bp) is labeled red. The gene encompasses 115,505 bases and ten coding exons. http://www.genecards.org

**Appendix. 5.** Genes which were analysed on sequence variants with the according locus, primer sequences for amplification and PCR strings ($T_A$ = annealing temperature).

| Chr. | Gene | N° | Primer sequences | Product size | $T_A$ | PCR settings | Gene locus |
|---|---|---|---|---|---|---|---|
| | | | Primer forward: F/ Primer reverse: R | | | | |
| 22 | XPNPEP3_1 | 11667 | GGGCATGACGTCACAAC/GAGGAGACCGTGGGAGAA | 394 bp | 68°C | Standard | 22q13.2 |
| 22 | XPNPEP3_2-1 | 11668 | TGAATATTGAAGGAGAGAAGGAAG/ AATGGCCATGTGAGGTTAGTG | 387 bp | 65°C | Standard | 22q13.2 |
| 22 | XPNPEP3_2-2 | 11669 | CACATTGTAGAAAAAGCAATCAGG/ TTAAGGCCTGGAAAATGCAG | 475 bp | 65°C | Standard | 22q13.2 |
| 22 | XPNPEP3_3-1 | 11663 | TGCAGAAATGTAAAGCATCCC/ATGGTAATTGTTTGCCAGGG | 295 bp | 65°C | Standard | 22q13.2 |
| 22 | XPNPEP3_3-2 | 11664 | CCCTATACTTTCCACCAAGACAAC/ AGAAAGGTTTTAATCCCAATCTG | 312 bp | 65°C | Standard | 22q13.2 |
| 22 | XPNPEP3_4 | 11666 | GTTTAGGGGCAGAAAAGCTG/ GTATACGACCTCAAGAGCTTACG | 369 bp | 68°C | Standard | 22q13.2 |
| 22 | XPNPEP3_5 | 11672 | AACTTCAGGCTGACGAAATTG/TCCTCTCCAATGACATGCTG | 304 bp | 68°C | Standard | 22q13.2 |
| 22 | XPNPEP3_6 | 11673 | GGGGTGAGAATAAAAGAAATGG/ GAGGCATGTTAATAATTCCTCCTAAC | 500 bp | 68°C | Standard | 22q13.2 |
| 22 | XPNPEP3_7 | 11674 | GAGGCATGTTAATAATTCCTCCTAAC/ TTGCAACCTAACTTGACCAGG | 330 bp | 68°C | Standard | 22q13.2 |
| 22 | XPNPEP3_8 | 11675 | AAACATCAACAGTGCCCCAG/AAACATCAACAGTGCCCCAG | 445 bp | 68°C | Standard | 22q13.2 |
| 22 | XPNPEP3_9 | 11676 | TTAAACTGAAGTGGCAGAACC/GAATTGCTGAACCTGGGG | 391 bp | 68°C | Standard | 22q13.2 |
| 22 | XPNPEP3_10 | 11677 | ACTAGGTAGGTACCATAAAGATCCAG/ TCACACAGCTGCTATGCTCC | 427 bp | 68°C | Standard | 22q13.2 |
| 20 | C20ORF7_1 | 14737 | AGAAGATCTTTGGAGTAGACACA/GAGGAAAACAAGCCTCCG | 359 bp | 70°C | Q-solution; TouchDown | 20p12.1 |
| 20 | C20ORF7_2 | 14367 | AAATTGAAAAGGGGATAAAGGG/CTTCCAGGTTCTGCCATTTC | 261 bp | 65°C | Standard | 20p12.1 |
| 20 | C20ORF7_3 | 14368 | TAAGGTGAGGCTGAAAAGGC/ AAAACCACCATTGATTCTACCAC | 363 bp | 65°C | Standard | 20p12.1 |
| 20 | C20ORF7_4 | 14369 | TCAGCAATTGTTTGTGACTGG/ TCAGAAAAGAAAGCTAGTTTGAAGG | 205 bp | 65°C | Standard | 20p12.1 |
| 20 | C20ORF7_5 | 14370 | CATTGACTTCAATTTAACAAACAGTG/ CCTCATAGCAAAACTTTATGGC | 264 bp | 65°C | Standard | 20p12.1 |
| 20 | C20ORF7_6 | 14371 | TGGGTTATCATTAACCTGGTG/TCACCGATTTCTTTCCCAAC | 246 bp | 65°C | Standard | 20p12.1 |
| 20 | C20ORF7_7 | 14372 | TTTGGCAGAATAAAAGTGGTTG/TGCCATGAAACCATGAACAC | 414 bp | 65°C | Standard | 20p12.1 |
| 20 | C20ORF7_8 | 14373 | TGGGAGATTGGTCTGGGTAG/ TCTCAAAATGTTTCTCTCAAATGG | 251 bp | 65°C | Standard | 20p12.1 |
| 20 | C20ORF7_9_10 | 14374 | TGAGCTGTTCAGGGTGTTTG/ CCCCAAGTTAGTAAACAATAATCTG | 620 bp | 65°C | Standard | 20p12.1 |
| 20 | C20ORF7_11 | 14375 | GGCTTTAATTGGGGCTGTG/ TTAGATGTTAAAGCTATCCATTTCTG | 220 bp | 65°C | Standard | 20p12.1 |

**Continuation of Appendix. 5.** Genes which were analysed on sequence variants with the according locus, primer sequences for amplification and PCR strings ($T_A$ = annealing temperature).

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| 3 | C3ORF60_1 | 14714 | CCAACCCGGGGACTAAC/TAGGGGGCTGGATGGTC | 170 bp | 70°C | Q-solution; TouchDown | 3p21.31 |
| 3 | C3ORF60_2 | 14715 | CCCTGACCCTTTCCCTC/GGCCTCAGTTTCCACAC | 249 bp | 70°C | Q-solution; TouchDown | 3p21.31 |
| 3 | C3ORF60_3 | 14536 | AGTGGATGGAGATGGGGAG/CTGTTGAGAGGCTGCAGTGG | 206 bp | 70°C | Q-solution; TouchDown | 3p21.31 |
| 3 | C3ORF60_4 | 14716 | CTCAACAGAACTGTAGACTAGC/AGCATCAGCCTGTCTAGC | 205 bp | - | No effective primer strings found | 3p21.31 |
| 3 | C3ORF60_5 | 14538 | AAGTGCAGGACACGGTGAG/GAGTGAAAGCATTGGGAAGC | 267 bp | 63°C | Standard | 3p21.31 |
| 10 | C10ORF65_1 | 14717 | TCACTCTGGGACATAGACCAA/GTCCCACAGAGGACAGC | 282 bp | 65°C | Standard | 10q24.1 |
| 10 | C10ORF65_2_3 | 14458 | CCAATGTCCTAGTTGTTCGG/CCCTTATCTCCTCTCCCTGG | 558 bp | 65°C | Standard | 10q24.1 |
| 10 | C10ORF65_4_5 | 14459 | AGTCTCTGGCTCTTGGGACC/AGGTACCTGGGTATCTCTTGG | 607 bp | 65°C | Standard | 10q24.1 |
| 10 | C10ORF65_6 | 14460 | GATGCCTGGAGGGGAGAG/CAATGAGGACACAGACTGCC | 247 bp | 69°C | No effective primer strings found | 10q24.1 |
| 10 | C10ORF65_7 | 14461 | CCGAGTTCCAGATATGGGTG/CAAGTGCAAGGCAGGAGG | 271 bp | 65°C | Standard | 10q24.1 |
| 7 | C7ORF10_1 | 14736 | AGGCGACTAGTGCTCAG/CCACCCAGACCGAATCTC | 260 bp | 65°C | Qiagen | 7p14 |
| 7 | C7ORF10_2 | 14481 | GTGGTCTGTGGACTCCCTTG/ TCGTGAAAGATTAGAGGATGATTAAG | 561 bp | 65°C | Standard | 7p14 |
| 7 | C7ORF10_3 | 14482 | GTGTCTGTGTTGACGGGTTG/ TTCACAGGAAAACAAGAAATGG | 320 bp | 64°C | Standard | 7p14 |
| 7 | C7ORF10_5 | 14539 | GGGATTGGGCTTCTTGTG/ TGATATTCCAAACCCTAATTATAACC | 178 bp | 63°C | Standard | 7p14 |
| 7 | C7ORF10_6 | 14484 | TGGAAAACTATGTCCCTGGC/AGTTCAGCAACCTAAACGGC | 241 bp | 65°C | Standard | 7p14 |
| 7 | C7ORF10_7 | 14485 | AGTGGTTTTAGCACACCCTG/TGCAGAGTAAAATCCTTTTGG | 215 bp | 63°C | Standard | 7p14 |
| 7 | C7ORF10_8 | 14486 | GGGGAGAGAAATGCACTGAG/ GCAACAAGAGCAAAATTCCATC | 552 bp | 65°C | Standard | 7p14 |
| 7 | C7ORF10_9 | 14487 | AATGCCGTTCTTCATTTGTG/AAAACAGCTGAAGGATTTGTG | 379 bp | 65°C | Standard | 7p14 |
| 7 | C7ORF10_10 | 14488 | TTTAGAAATTAAACTTTTGTGGTCTTG/ TGGGCAAAACTGAACTTTCC | 209 bp | 65°C | Standard | 7p14 |
| 7 | C7ORF10_11 | 14489 | TGAACACTAGCACCCACAGG/ CAAATTGATCTCAAATAAGCCAAG | 273 bp | 65°C | Standard | 7p14 |
| 7 | C7ORF10_12 | 14490 | GGCCACATGATAGAAGAGGC/TGACTTAAGCTTGCGATCAATG | 264 bp | 65°C | Standard | 7p14 |
| 7 | C7ORF10_13 | 14491 | AGAATTCAAATCCTTGTGGTTG/GATTATCCTTTCCTGGCTGC | 197 bp | 65°C | Standard | 7p14 |
| 7 | C7ORF10_14 | 14492 | ATGGCTGTCCATGCCTTG/ AAACAGCATATGGGACAATTAATAAAG | 201 bp | 65°C | Standard | 7p14 |
| 7 | C7ORF10_15 | 14493 | AACACCCCAGGCTGCTTAG/TTGCCTTTGCCAGTGTATTC | 298 bp | 65°C | Standard | 7p14 |

**Appendix. 6.** Standardized questionnaire for patients with mitochondrial disorders 'general information and family history'.

| | **General Information** | **YES** | **NO** | **No info** | **Comment** |
|---|---|---|---|---|---|
| 0 | Date: | | | | |
| 1 | Date of Biopsy: | | | | |
| 2 | Institution: | | | | |
| 3 | Doctor in charge of the patient: | | | | |
| 4 | Other responsible doctors: | | | | |
| | **Index patient:** | | | | |
| 5 | Surname: | | | | |
| 6 | First name: | | | | |
| 7 | Date of birth: | | | | |
| 8 | Sex: | | | | |
| | **Family** | | | | |
| 9 | Ethnic background of the family: | | | | |
| 10 | Blood relationship of the parents | | | | |
| 11 | Degree of relationship: | | | | |
| 12 | Do or did family members have similar diseases? | | | | |
| 13 | If Yes, in respect of whom? | | | | |
| 14 | Which? | | | | |
| 15 | When did they arise? | | | | |
| 16 | Course? | | | | |
| 17 | Are there or were there neurological diseases in the family? | | | | |
| | Enquire specifically about: | | | | |
| 18 | Muscle diseases | | | | |
| 19 | Movement impairments | | | | |
| 20 | Epilepsy | | | | |
| 21 | Disabilities | | | | |
| 22 | Stroke | | | | |
| 23 | Migraine | | | | |
| 24 | Hearing impairment (sensorineural) in the family? | | | | |
| 25 | Visual impairment in the family? | | | | |
| 26 | Frequent miscarriages | | | | |

**Continuation of Appendix. 6.** Standardized questionnaire for patients with mitochondrial disorders 'clinical signs and symptoms'.

| | | YES | NO | Not examined | No info | Comment |
|---|---|---|---|---|---|---|
| | **Neurology:** | | | | | |
| 27 | Psychomotoric development delay | | | | | |
| 28 | Loss of abilities | | | | | |
| 29 | Episodes of unexplained coma | | | | | |
| 30 | Dementia | | | | | |
| 31 | Muscular hypotonia | | | | | |
| 32 | Muscular hypertonia | | | | | |
| 33 | Hyperreflexia | | | | | |
| 34 | Ataxia | | | | | |
| 35 | Dystonia | | | | | |
| 36 | Spasticity | | | | | |
| 37 | Swallowing difficulties | | | | | |
| 38 | Myoclonus | | | | | |
| 39 | Epilepsy | | | | | |
| 40 | Migraine-like headaches | | | | | |
| 41 | Stroke-like episodes | | | | | |
| 42 | Microcephaly | | | | | |
| 43 | Peripheral Neuropathy | | | | | |
| | **Muscles:** | | | | | |
| 44 | Myalgia | | | | | |
| 45 | Stress intolerance | | | | | |
| 46 | Weakness (decreased muscular force) | | | | | |
| 47 | Muscular atrophy | | | | | |
| 48 | Artificial ventilation | | | | | |
| 49 | Rhabdomyolysis | | | | | |
| | **Heart:** | | | | | |
| 50 | Cardiomyopathy hypertrophic | | | | | |
| 51 | Cardiomyopathy dilated | | | | | |
| 52 | Conduction impairments | | | | | |
| 53 | Pre-excitation syndrome WPW | | | | | |
| | **Eyes:** | | | | | |
| 54 | Ophthalmoplegia (CPEO) | | | | | |
| 55 | Ptosis | | | | | |
| 56 | Nystagmus | | | | | |
| 57 | Retinal pigment degeneration | | | | | |
| 58 | Optic nerve atrophy | | | | | |
| 59 | Cataract | | | | | |
| 60 | Reduction in visual acuity | | | | | |
| 61 | Field of vision losses | | | | | |
| | **Growth:** | | | | | |
| 62 | Intrauterine growth retardation | | | | | |
| 63 | Failure to thrive | | | | | |
| 64 | Short stature ( < 3th percentile) | | | | | |
| | **Gastrointestinal tract:** | | | | | |
| 65 | Pseudo-obstruction | | | | | |
| 66 | Cyclic vomiting | | | | | |
| 67 | Chronic-recurrent diarrhoea > 3 weeks | | | | | |
| 68 | Exocrine pancreas insufficiency | | | | | |

**Continuation of Appendix. 6.** Standardized questionnaire for patients with mitochondrial disorders 'clinical signs and symptoms'.

| | | | | | | |
|---|---|---|---|---|---|---|
| | **Liver:** | | | | | |
| 69 | Acute liver failure | | | | | |
| 70 | Chronic liver insufficiency (elevation of liver enzymes) | | | | | |
| 71 | Valproate-induced liver failure | | | | | |
| | **Endocrine system:** | | | | | |
| 72 | Pubertas tarda | | | | | |
| 73 | Hypothyroidism | | | | | |
| 74 | Hypoparathyroidism | | | | | |
| 75 | Diabetes mellitus | | | | | |
| 76 | Others | | | | | |
| | **Hearing:** | | | | | |
| 77 | Sensorineural hearing loss | | | | | |
| 78 | Ototoxicity of specific medications | | | | | |
| | **Skin:** | | | | | |
| 79 | Symmetrical lipomatosis | | | | | |
| 80 | Hypertrichosis | | | | | |
| 81 | Hair growth disorders | | | | | |
| | **Facial dysmorphia signs:** | | | | | |
| 82 | Dysmorphic features | | | | | |
| | **Kidneys:** | | | | | |
| 83 | Renal tubular acidosis | | | | | |
| 84 | Nephrotic syndrome | | | | | |
| 85 | Renal insufficiency | | | | | |
| | **Haematopoiesis system:** | | | | | |
| 86 | Panzytopenia | | | | | |
| 87 | Anaemia, hyporegenerative | | | | | |
| 88 | Neutropenia | | | | | |
| | **Natural Course:** | | | | | |
| 89 | Age of onset | | | | | |
| 90 | Progressive course | | | | | |
| 91 | Incremental occurrence of ≥ 2 neurological symptoms | | | | | |
| 92 | No death | | | | | |
| 93 | death < 1 y | | | | | |
| 94 | death 1 y – 10y | | | | | |
| 95 | death > 10y | | | | | |
| | **General:** | | | | | |
| 96 | Involvement of different organ systems | | | | | |
| 97 | Deterioration by infectious episodes | | | | | |
| | **Enter additional symptoms here:** | | | | | |
| 98 | Additional symptom 1 | | | | | |
| 99 | Additional symptom 2 | | | | | |
| | **Which are the cardinal symptoms (max. 5)?** | | | | | |
| 100 | Cardinal symptom 1 | | | | | |
| 101 | Cardinal symptom 2 | | | | | |
| 102 | Cardinal symptom 3 | | | | | |
| 103 | Cardinal symptom 4 | | | | | |
| 104 | Cardinal symptom 5 | | | | | |

**Continuation of Appendix. 6.** Standardized questionnaire for patients with mitochondrial disorders 'imaging and laboratory parameters'.

| | | YES | NO | not examined | Values | No info | Comment |
|---|---|---|---|---|---|---|---|
| | **MRI** | | | | | | |
| 105 | Leukodystrophy | | | | | | |
| 106 | Brain atrophy | | | | | | |
| 107 | Symmetrical basal ganglia lesions | | | | | | |
| 108 | Symmetrical brainstem lesions | | | | | | |
| 109 | Cerebellar affection | | | | | | |
| 110 | Others | | | | | | |
| | **MR-Spectroscopy** | | | | | | |
| 111 | Lactate elevated | | | | | | |
| 112 | Other pathologic metabolites | | | | | | |
| | **Body fluids** | | | | | | |
| 113 | Elevated blood lactate | | | | | | |
| 114 | Elevated CSF lactate | | | | | | |
| 115 | Elevated creatine kinase | | | | | | |
| 116 | Acyl carnitines abnormal | | | | | | |
| 117 | Organic acids abnormal | | | | | | |
| 118 | Amino acids abnormal | | | | | | |
| | **Tissue biopsies** | | | | | | |
| 119 | Muscle | | | | | | |
| 120 | Skin | | | | | | |
| 121 | Liver | | | | | | |
| 122 | Heart | | | | | | |
| | **Histology** | | | | | | |
| 123 | RRF | | | | | | |
| 124 | COX-negative fibers | | | | | | |
| 125 | Signs of muscular dystrophy | | | | | | |
| 126 | Others | | | | | | |
| 127 | Electron Microscopy | | | | | | |
| | **Enzyme activities** | **activity /NCP** | **Reference values** | **activity/CS** | **Reference values** | | |
| 128 | Complex I | | | | | | |
| 129 | Complex II/III | | | | | | |
| 130 | Complex II | | | | | | |
| 131 | Complex III | | | | | | |
| 132 | Complex IV | | | | | | |
| 133 | Complex V | | | | | | |
| 134 | PDHc | | | | | | |
| 135 | Citrate synthase | | | | | | |

**Appendix 7.** Neuropaediatrics paper by Herzer et al. 2010



# Leigh Disease with Brainstem Involvement in Complex I Deficiency due to Assembly Factor NDUFAF2 Defect

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Key words
- NDUFAF2
- complex I deficiency
- Leigh disease
- assembly factor
- brainstem
- involvement
- mitochondrial encephalomyopathy

## Abstract

Mitochondrial NADH: ubiquinone oxidoreductase (complex I) deficiency accounts for most defects in mitochondrial oxidative phosphorylation. Pathogenic mutations have been described in all 7 mitochondrial and 12 of the 38 nuclear encoded subunits as well as in assembly factors by interfering with the building of the mature enzyme complex within the inner mitochondrial membrane. We now describe a male patient with a novel homozygous stop mutation in the *NDUFAF2* gene. The boy presented with severe apnoea and nystagmus. MRI showed brainstem lesions without involvement of basal ganglia and thalamus, plasma lactate was normal or close to normal. He died after a fulminate course within 2 months after the first crisis. Neuropathology verified Leigh disease. We give a synopsis with other reported patients. Within the clinical spectrum of Leigh disease, patients with mutations in *NDUFAF2* present with a distinct clinical pattern with predominantly brainstem involvement on MRI. The diagnosis should not be missed in spite of the normal lactate and lack of thalamus and basal ganglia changes on brain MRI.

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Bibliography
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## Introduction

With a minimum birth prevalence of at least 1:5 000 [16] disorders of the mitochondrial respiratory chain account for a main part of inherited metabolic diseases. Embedded within the inner mitochondrial membrane, the respiratory chain is made up of 5 enzyme complexes which produce ATP by oxidative phosphorylation. Especially high energy-dependent organs like brain, heart and skeletal muscle are vulnerable to defects of the aerobic energy metabolism.

Isolated complex I deficiency encompasses about one third of these hereditary defects in the mitochondrial energy generating system [2, 9]. Being the largest complex of the respiratory chain, complex I contains 45 subunits, which are under genetic control of both mitochondrial and nuclear genes [4]. Pathogenic mutations causing complex I defects have been described in all 7 mitochondrial encoded subunits and 12 nuclear genes, namely NDUFS1, NDUFS2, NDUFS3, NDUFS4, NDUFS6, NDUFS7, NDUFS8, NDUFV1, NDUFV2, NDUFA1, NDUFA2 and NDUFA11, which are highly conserved and account for a large number of different phenotypes in the affected patients [2, 3, 5]. However, these mutations explain pathogenicity only for a small part of the entire complex I deficiency patients. For more than 50% of the patients molecular diagnostics fail to detect the cause of the disease. In previous reports assembly factor mutations have been shown to cause complex I deficiency.

Ogilvie and colleagues showed first that the NDUFAF2 gene, which shares similarities in amino acid sequence with subunit NDUFA12, serves as complex I assembly factor [12], as well as NDUFAF1, C6ORF66, C8ORF38, C20ORF7 and NDUFAF3, for which mutations have been described [1, 6, 7, 12–15, 17]. Herein, we describe a patient with complex I deficiency in both muscle and fibroblasts harbouring a new mutation in the NDUFAF2 gene. We compare the clinical picture with previously published cases and summarize the unique neuropathological pattern of the known patients with NDUFAF2 mutations.

## Case Report

The patient was the first child of non-consanguineous Austrian parents and was born after an uneventful pregnancy. Early psychomotor development was moderately delayed. He was able to

walk at the age of 22 months. Horizontal nystagmus was noticed at the age of 14 months and improved with orthoptist therapy. At the age of 26 months, during pneumonia, he was found to be apnoeic and needed respiratory assistance. The CT scan of the brain was normal, cerebrospinal fluid (CSF) showed no signs of central nervous system infection. He recovered promptly and was discharged from hospital after 8 days. Metabolic screening, virological investigations and lactate measurements in plasma and CSF were normal.

3 weeks later, with respiratory infection, he was found to be apnoeic, cyanotic and comatose. On admission he was mechanically ventilated and presented with foot drop position and bilateral positive Babinski signs. Neuroimaging at the age of 27 month (**Fig. 1**) revealed on $T_2$-weighted images lesions in the upper cervical spinal cord, the medulla oblongata, in the pontine tegmentum, in the midbrain in the substantia nigra and the periaqueductal region (**Fig. 1a**). In the cerebral white matter patchy and not completely confluent lesions, mainly located in the frontal and parietal deep white matter, are seen. The corpus callosum, internal capsule and basal ganglia and thalami are spared (**Fig. 1b**).

On admission lactate was elevated (serum 4.2 mmol/L, reference value <2.2 mmol/L; CSF 6 mmol/L, reference value <2.1 mmol/L), subsequently lactate levels were within the normal range. CSF protein was normal. Fundoscopy showed pale optic discs. Auditory evoked potentials disclosed signs of lesions in the rostral pontine auditory pathway. 3 weeks later the patient died under mechanical ventilation of cardiac arrest at the age of 27 months.

## Histological findings

Autopsy and biopsies of skin, muscle and liver were performed. Neuropathological examination of CNS revealed softening and brown discolouration of the substantia nigra and the tegmentum of the brainstem (**Fig. 1c**) and typical signs of Leigh disease with necrosis, spongy state and capillary proliferation (**Fig. 1d**) predominantly in the brainstem (substantia nigra, tegmentum of mesencephalon, pons and medulla oblongata), the optic nerves and chiasma. Some less pronounced changes were found in nucleus dentatus cerebelli and the uppermost spinal cord, and mild involvement of the medial thalamus and hypothalamus. The mammillary bodies were not affected. Dorsal spinal tracts, spinocerebral tracts and dorsal spinal nerve roots displayed Wallerian degeneration. There were no signs of hypoxic damage.

## Measurement of respiratory chain enzyme activities in fibroblasts and muscle

Activities of the respiratory chain complexes were determined in isolated fibroblast mitochondria and in muscle homogenate by standard spectrophotometric methods [10]. The complex I activity was clearly reduced with 12% residual activity of the lowest control value in muscle and 59% in fibroblasts. In muscle and fibroblast complexes II and III were also reduced (presumably secondary effects of the complex I deficiency) (see **Table 1**).

## Mutation analysis

Genomic DNA was extracted from skeletal muscle homogenate by proteinase K treatment followed by phenol/chloroform extraction. Mutation screen in candidate genes including NDUFAF2 was performed by high-resolution melting analysis using Idaho LightScanner Technology as described by Meisinger et al. in 2009 [11]. All exons of NDUFAF2 were PCR amplified by intronic primers (available on request) and altered melting curves compared were directly sequenced with BigDye Cycle sequencing kit (Applied Biosystems). Sanger sequencing of the entire mitochondrial DNA failed to detect a mutation. High-resolution melting profile analysis of exonic sequences from NDUFAF2 revealed a novel homozygous nonsense mutation c.9G>A introducing an early stop codon at position 3 of NDUFAF2 (p. Trp3X, **Fig. 2**).

## Discussion

Patients with complex I deficiencies present with a heterogeneous spectrum of clinical phenotypes, reflecting the complex structure and its function within the respiratory chain. So far, in more than 50% of the patients molecular diagnosis failed to detect the cause of disease. There are very few patients published with complex I deficiency due to mutations in assembly factors and only 4 patients published with a defect of *NDUFAF2*. We present a new case with a novel mutation in this assembly factor and point out the common clinical and neuropathological patterns of all so far known patients.

Ogilvie et al. (2005, **Table 2**) [12] reported on a girl with a severe childhood-onset progressive encephalopathy caused by mutation the NDUFAF2 gene. The clinical course was characterized initially by nystagmus and a wide-based gait at the age of 14 months. At the age of 3 years, a crisis during an infection was marked by acute ataxia, lethargy, decreased reflexes and signs of

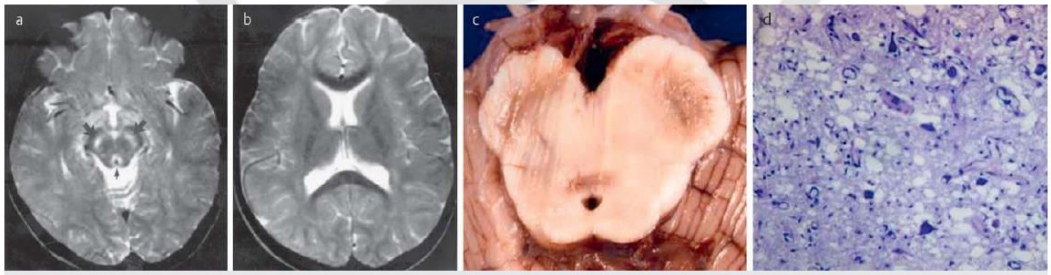


**Fig. 1** MRI of brain ($T_2$-weighted images) shows increased signal in the midbrain in the substantia nigra (**a**: gross arrow) and the periaqueductal region (**a**: fine arrow), corpus callosum, internal capsule and basal nuclei are spared (**b**). Autopsy shows softening and brown discolouration of substantia nigra and subaqueductal area of the midbrain (**c**), histopathology: spongy state, necrosis and capillary proliferation of pontine tegmentum (cresyl violet ×100) (**d**).

**Table 1** Respiratory chain enzyme activities in fibroblasts and muscle.

| Muscle | Patient* | Normal Range* | Patient** | Normal Range** |
|---|---|---|---|---|
| complex I (CI) | 2 | 30–65 | 0.02 | 0.17–0.26 |
| complex I+III (C13) | 7 | 27–58 | 0.05 | 0.13–0.19 |
| complex II (CII) | 39 | 53–100 | 0.29 | 0.30–0.36 |
| complex II+III (C23) | 12 | 41–75 | 0.09 | 0.20–0.28 |
| complex III (CIII) | 81 | 230–490 | 0.61 | 1.30–1.96 |
| complex V (CV) | 108 | 78–180 | 0.81 | 0.42–0.70 |
| cytochrome c oxidase (COX) | 113 | 205–739 | 0.85 | 1.24–2.38 |
| citrate synthase (CS) | 133 | 160–310 | | |
| **Fibroblasts** | **Patient*** | **Normal Range*** | **Patient**** | **Normal Range**** |
| complex I (CI) | 6 | 15–53 | 0.02 | 0.05–0.09 |
| complex I+III (C13) | 16 | 102–343 | 0.05 | 0.24–0.58 |
| complex II (CII) | 99 | 103–285 | 0.34 | 0.37–0.48 |
| complex II+III (C23) | 161 | 167–314 | 0.55 | 0.39–0.72 |
| complex III (CIII) | 298 | 283–1174 | 0.98 | 1.17–1.99 |
| complex V (CV) | 45 | 36–167 | 0.15 | 0.15–0.39 |
| cytochrome c oxidase (COX) | 381 | 392–939 | 1.35 | 1.30–1.68 |
| citrate synthase (CS) | 297 | 242–590 | | |

* Units/g protein
** Units/unit citrate synthase

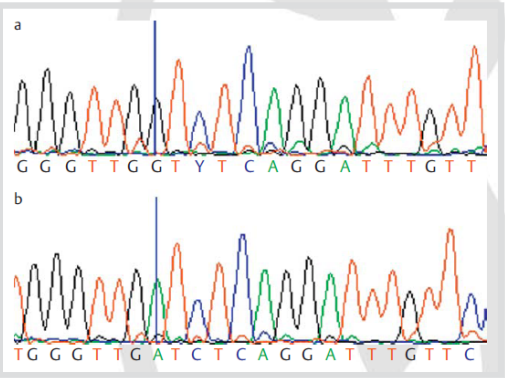


**Fig. 2** DNA sequence analysis of the patient revealed a homozygous missense mutation (c.9G>A) resulting in a premature stop codon instead of a tryptophan at position 3 (p.Trp3X). **a**: control; **b**: patient.

optic atrophy. At the age of 8 years she worsened with dysphagia, sleep apnoea and respiratory failure. She remained comatose and died at the age of 13 years. Neuropathology proved Leigh disease. Cerebral cMRI exhibited symmetric lesions in the mamillothalamic tracts, substantia nigra, medial lemniscus, medial longitudinal fasciculus and spinothalamic tracts. Basal ganglia and thalamus were not affected. Blood lactate, liver function and echocardiogram were normal almost all the time, so the authors stated that clinical presentation, laboratory and cMRI findings did not resemble typical Leigh syndrome.

Barghuti et al. (2008) added 2 more patients who were identified by homozygosity mapping [1]. The clinical course (see **Table 2**) was marked by apnoea, nystagmus, optic nerve atrophy (observed in 1 patient) and early death 4 and 13 months after the first crisis, respectively. Both patients had normal blood lactate levels and very similar cMRI changes of the brainstem as the patient from Ogilvie et al. Therefore, Barghuti et al. postulated that patients with NDUFAF2 mutations display a unique pattern of degenerative changes in neuroradiology.

Our case resembles the findings in the above-mentioned patients. There was a moderate delay in psychomotor development and nystagmus prior to the first crisis with severe apnoea during an infection. The second crisis within 3 weeks led to death due to cardiorespiratory arrest. The patient had normal or close to normal blood lactate levels (in CSF lactate was considerable increased) and cMRI findings were very similar to those of the so far described patients. The diagnosis of Leigh disease was proven by neuropathology (**Fig. 1c, d**). It can be speculated that other clinically similar patients with NDUFAF2 mutations might have been missed for diagnosis on intensive care ward units. The severe clinical picture with acute fulminant and lethal respiratory failure without lactate elevation and the atypical neuroradiological presentation might have misled to other diagnostic considerations than mitochondrial encephalopathy.

Very recently, Hoefs et al. (2009) published a girl with an NDUFAF2 mutation [7], who presented with nystagmus at the age of 3 months, followed by feeding difficulties, renal tubular acidosis, muscular hypotonia and dyskinetic movements. She died at age of 1 year due to respiratory failure (**Table 2**). Plasma lactate levels were moderately elevated and cMRI revealed high signal abnormalities in thalamus, brainstem and spinal cord. This case shows that neuroradiology findings in patients with NDUFAF2 mutations are not always as unique as postulated by Barghuti et al. [1].

All NDUFAF2 patients harboured loss of function mutations with a truncated protein useless in complex I assembly [7,8,12].

Median complex I residual activity was 31% of the lowest control value with a range of 12–55% in the 5 patients suggesting that mutated NDUFAF2 has a great influence on proper complex I function, although the residual activity does not correlate to the patient's clinical course. Nevertheless, there are specific features which have been observed in patients with NDUFAF2 mutations.

Patients expired at a median age of 2 years (range from 1 up to 13 years). Clinical hallmarks are nystagmus, optic nerve atrophy, apnoea and respiratory failure as signs of the mainly affected

**Table 2** Information of patients with complex I deficiency due to mutations in NDUFAF2.

| Reference | This report | Ogilvie et al. 2005 | Barghuti et al. 2008 | Barghuti et al. 2008 | Hoefs et al. 2009 |
|---|---|---|---|---|---|
| gender | male | female | female | male | female |
| mutation | c.9G>A, p.W3X homozygous | c.182C>T, p.R45X homozygous | c.1A>T, p.M1L homozygous | c.1A>T, p.M1L homozygous | c.114C>G, p.Y38X homozygous |
| consanguinity | no | no | yes | yes | yes |
| age of onset | 14 months | 12 months | 20 months | 8 months | 3 months |
| age of death | 24 months | 13 years | 2 years | 21 months | 12 months |
| death by | apnoea | respiratory failure | apnoea | not commented | respiratory failure |
| first symptoms | nystagmus, motor delay | nystagmus, wide-based gait | apnoea | motor delay, nystagmus | nystagmus |
| following symptoms | apnoea, pale optic disc, optic atrophy (histology) | ataxia, absent deep tendon reflexes, optic atrophy, weakness, dysphagia, sleep apnoea, respiratory failure, coma | myoclonic seizures, muscular hypotonia, horizontal and rotatory nystagmus, dysmetria, ataxia | optic atrophy; age 18 months: episodes of flaccidity and apnoea, remained ventilator dependent | muscular hypotonia, motor delay, renal tubular acidosis, feeding difficulties |
| lactate (serum) | normal – 4.2 mmol/L (<2.1 mmol/L) | normal | normal | normal | 3–5 mmol/L (<2.3 mmol/L) |
| lactate (CSF) | 6 mmol/L (<1.8 mmol/L) | 4.2 mmol/L (1.4–3.9 mmol/L) | normal | normal | n. d. |
| complex I activity* | 12% | 55% | 49% | 31% | 21% |
| **cMRI findings ($T_2$-w):** | | | | | |
| telencephalon (including basal ganglia) | basal nuclei spared, patchy lesions in cerebral white matter | cortex, subcortical white matter relatively spared | cortex, subcortical white matter relatively spared | no changes reported | no changes reported |
| diencephalon including thalamus | spared | mamillothalamic tract | mamillothalamic tract | mamillothalamic tract | symmetrical, bilateral lesions of thalamus |
| mesenzephalon, pons, cerebellum, medulla oblongata, spinal cord | substantia nigra, periaqueductal region, pontine tegmentum, spinal trigeminal nucleus | substantia nigra, medial lemniscus, medial longitudinal fasciculus, spinothalamic tracts, cerebellar white matter | substantia nigra, medial lemniscus, medial longitudinal fasciculus, spinothalamic tracts, cerebellar white matter | substantia nigra, periaqueductal grey matter, medial lemniscus, medial longitudinal fasciculus, spinothalamic tracts, cerebellar white matter | cerebral peduncles, brainstem, spinal cord |

* Percentage residual activity of lowest control value in skeletal muscle

Abbreviations: n. d. = no data

brainstem. The clinical course in all patients was marked by an early onset and progressive course. Furthermore, 4 of the 5 patients presented with acute episodes of encephalopathy during infections and were stable in the interval. On cMRI, brainstem lesions without changes in thalami and basal ganglia on $T_2$-weighted images are found in 4 out of 5 patients. Plasma lactate was normal in 3 of 5 patients and in the remaining only slightly elevated. CSF lactate levels were normal in 2 and elevated in 2 patients.

In conclusion, the diagnosis of a complex I assembly defect due to an NDUFAF2 mutation should not be missed. The lack of cMRI changes in thalamus and basal ganglia and normal or close to normal plasma and CSF lactate levels might mislead to other than mitochondrial diseases.

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## References

1 *Barghuti F, Elian K, Gomori JM et al.* The unique neuroradiology of complex I deficiency due to NDUFA12L defect. Mol Genet Metab 2008; 94: 78–82
2 *Bénit P, Lebon S, Chol M et al.* Mitochondrial complex I deficiency in humans. Current Genomics 2004; 5: 137–146
3 *Berger I, Hershkovitz E, Shaag A et al.* Mitochondrial complex I deficiency caused by a deleterious NDUFA11 mutation. Ann Neurol 2008; 63: 405–408
4 *Carroll J, Fearnley IM, Skehel JM et al.* Bovine complex I is a complex of 45 different subunits. J Biol Chem 2006; 281: 32724–32727
5 *Distelmaier F, Koopman W, van den Heuvel L et al.* Mitochondrial complex I deficiency: from organelle dysfunction to clinical disease. Brain 2009; 132: 833–842
6 *Dunning CJ, McKenzie M, Sugiana C et al.* Human CIA30 is involved in the early assembly of mitochondrial complex I and mutations in its gene cause disease. EMBO J 2007; 26: 3227–3237
7 *Hoefs SJ, Dieteren CE, Rodenburg RJ et al.* Baculovirus complementation restores a novel NDUFAF2 mutation causing complex I deficiency. Hum Mutat 2009; 30: 728–736
8 *Janssen RJ, Distelmaier F, Smeets R et al.* Contiguous gene deletion of ELOVL7, ERCC8 and NDUFAF2 in a patient with a fatal multisystem disorder. Hum Mol Genet 2009; 18: 3365–3374
9 *Loeffen JL, Smeitink JA, Trijbels JM et al.* Isolated complex I deficiency in children: clinical, biochemical and genetic aspects. Hum Mutat 2000; 15: 123–134
10 *Mayr JA, Paul J, Pecina P et al.* Reduced respiratory control with ADP and changed pattern of respiratory chain enzymes as a result of selective deficiency of the mitochondrial ATP synthase. Pediatr Res 2004; 55: 988–994
11 *Meisinger C, Prokisch H, Gieger C et al.* A genome-wide association study identifies 3 loci associated with mean platelet volume. Am J Hum Genet 2009; 84: 66–71
12 *Ogilvie I, Kennaway NG, Shoubridge EA.* A molecular chaperone for mitochondrial complex I assembly is mutated in a progressive encephalopathy. J Clin Invest 2005; 115: 2784–2792
13 *Pagliarini DJ, Calvo SE, Chang B et al.* A mitochondrial protein compendium elucidates complex I disease biology. Cell 2008; 134: 112–123
14 *Saada A, Edvardson S, Rapoport M et al.* C6ORF66 is an assembly factor of mitochondrial complex I. Am J Hum Genet 2008; 82: 32–38
15 *Saada A, Vogel RO, Hoefs SJ et al.* Mutations in NDUFAF3 (C3ORF60), encoding an NDUFAF4 (C6ORF66)-interacting complex I assembly protein, cause fatal neonatal mitochondrial disease. Am J Hum Genet 2009; 84: 718–727
16 *Schaefer AM, Taylor RW, Turnbull DM et al.* The epidemiology of mitochondrial disorders – past, present and future. Biochim Biophys Acta 2004; 1659: 115–120
17 *Sugiana C, Pagliarini DJ, McKenzie M et al.* Mutation of C20orf7 disrupts complex I assembly and causes lethal neonatal mitochondrial disease. Am J Hum Genet 2008; 83: 468–478

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# 8 Publications

Articles

Tobias B Haack, Florence Madignier, **Martina Herzer**, Eleonora Lamantea, Katharina Danhauser, Federica Invernizzi, Johannes Koch, Martin Freitag, Rene Drost, Ingo Hillier, Birgit Haberberger, Johannes A Mayr, Uwe Ahting, Valeria Tiranti, Agnes Rötig, Arcangela Iuso, Rita Horvath, Marketa Tesarova, Ivo Baric, Graziella Uziel, Boris Rolinski, Wolfgang Sperl, Thomas Meitinger, Massimo Zeviani, Peter Freisinger, Holger Prokisch (2012) 'Mutation screening of 75 candidate genes in 152 complex I deficiency cases identifies pathogenic variants in 16 genes including NDUFB9.' J Med Genet 49: 2. 83-89 Feb

**M Herzer**, J Koch, H Prokisch, R Rodenburg, C Rauscher, W Radauer, R Forstner, P Pilz, B Rolinski, P Freisinger, J A Mayr, W Sperl (2010). 'Leigh disease with brainstem involvement in complex I deficiency due to assembly factor NDUFAF2 defect.' Neuropaediatrics **41**(1): 30-34

John F O'Toole, Yangjian Liu, Erica E Davis, Christopher J Westlake, Massimo Attanasio, Edgar A Otto, Dominik Seelow, Gudrun Nurnberg, Christian Becker, Matti Nuutinen, Mikko Kärppä, Jaakko Ignatius, Johanna Uusimaa, Salla Pakanen, Elisa Jaakkola, Lambertus P van den Heuvel, Henry Fehrenbach, Roger Wiggins, Meera Goyal, Weibin Zhou, Matthias T F Wolf, Eric Wise, Juliana Helou, Susan J Allen, Carlos A Murga-Zamalloa, Shazia Ashraf, Moumita Chaki, Saskia Heeringa, Gil Chernin, Bethan E Hoskins, Hassan Chaib, Joseph Gleeson, Takehiro Kusakabe, Takako Suzuki, R Elwyn Isaac, Lynne M Quarmby, Bryan Tennant, Hisashi Fujioka, Hannu Tuominen, Ilmo Hassinen, Hellevi Lohi, Judith L van Houten, Agnes Rotig, John A Sayer, Boris Rolinski, Peter Freisinger, Sethu M Madhavan, **Martina Herzer**, Florence Madignier, Holger Prokisch, Peter Nurnberg, Peter K Jackson, Peter Jackson, Hemant Khanna, Nicholas Katsanis, Friedhelm Hildebrandt (2010). 'Individuals with mutations in XPNPEP3, which encodes a mitochondrial protein, develop a nephronophthisis-like nephropathy.' J Clin Invest **120**(3): 791-802

Talks

14. Pädiatrisches Stoffwechsel-Symposium, 19.-22.03.2009, Salzburg, Österreich
*„Verbesserte molekulare Diagnostik für Patienten mit Mitochondriopathie – High-throughput Mutationsscreen"*

Posters

39. Neuropädiatrie Kongress, 23.-26.04. 2009, Graz, Österreich
*„Verbesserte molekulare Diagnostik für Patienten mit Mitochondriopathie"*

41th European Human Genetics Conference (EHGC) of the European Society of Human Genetics (ESHG), 23.-26.05.2009, Wien, Österreich
*'Improved molecular diagnosis for patients with mitochondriopathy'*

11th International Congress of Inborn Errors of Metabolism (ICIEM), 29.08.-02.09.2009, San Diego, California, USA
*'Improved molecular diagnosis for patients with mitochondriopathy'*