Biochemical Diagnostics for

Mitochondrial (Encephalo)myopathies

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Biochemical Diagnostics for Mitochondrial (Encephalo)myopathies

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Abbreviations

AcCoA	Acetylcoenzyme-A
ANT	Adenine Nucleotide Translocator
ATP	Adenosine triphosphate
ADP	Adenosine diphosphate
bp	Base pair
BSA	Bovine serum albumin
c	Cytochrome *c*
CNS	Central nervous system
CO_2	Carbondioxide
CoQ_{10}	Coenzyme Q_{10}
COV	Coefficient of variation
PCr	Phosphocreatine
CS	Citrate synthase
cyt	Cytochrome
DGUOK	Deoxyguanosine kinase
DNA	Deoxyribonucleic acid
DNP	Dinitrophenol
EFG1	Elongation factor G1
EFTu	Elongation factor Tu
$FADH_2$	Flavin adenine dinucleotide (reduced form)
FH	Fumarate hydratase (fumarase)
Fp	Flavoprotein
Ip	Iron-sulfur protein
LHON	Leber’s Hereditary Optic Neuropathy

MC	Mitochondrial carrier
MEGS	Mitochondrial Energy Generating System
MELAS	Mitochondrial Encephalomyopathy, Lactic Acidosis and Stroke-like episodes
MERRF	Myoclonic Epilepsy with Ragged Red Fibres
MILS	Maternally Inherited Leigh Syndrome
MNGIE	Myo-Neuro-Gastro-Intestinal Encephalomyopathy
mtDNA	Mitochondrial DNA
NAD	Nicotinamide adenine dinucleotide
NADH	Nicotinamide adenine dinucleotide (reduced form)
NARP	Neurogenic muscle weakness, Ataxia and Retinitis Pigmentosa
nDNA	Nuclear DNA
OXPHOS	Oxidative Phosphorylation
PDH	Pyruvate dehydrogenase
PDHc	Pyruvate dehydrogenase complex
POLG	mtDNA polymerase γ
RC	Respiratory chain
SDH	Succinate dehydrogenase
RNA	Ribonucleic acid
rRNA	Ribosomal RNA
SCC	Succinate:cytochrome *c* oxidoreductase
TCA-cycle	Tricarboxylicacid cycle
TK	Thymidine kinase
TP	Thymidine phosphorylase
tRNA	Transfer RNA

CHAPTER 1

Introduction

1.1. The Mitochondrial Energy Generating System

In 1962 Luft and colleagues described the first patient with a mitochondrial disorder (Luft *et al.,* 1962). Since that time a large number of patients suffering from uni- or multisystem diseases associated with mitochondrial dysfunction have been described (Table 1). In the majority of these patients, mitochondrial dysfunction is manifested by a decreased activity of the mitochondrial energy generating system, the MEGS. Until now mitochondrial dysfunction was mostly described as an OXPHOS disorder. However, OXPHOS only involves the activities of the RC and complex V.

Although most patients with a primary mitochondriopathy indeed suffer from a deficiency in one of these enzymes, patients may also suffer from deficiencies in other enzymes or proteins e.g. PDHc, TCA-cycle enzymes, ANT or mitochondrial carrier proteins. Apart from the proteins directly involved in the MEGS, a large number of so-called "regulatory" proteins are involved in the transport of MEGS proteins into the mitochondrion, folding and unfolding of these proteins, assembly of enzyme complexes and post-translational modifications. A deficiency in one of these "regulatory" proteins can indirectly cause a deficiency in one or more of the MEGS enzymes. With respect to diagnostic biochemical analysis of patients suspected of a mitochondrial disorder, it is important to have a screening procedure that traces deficiencies in the enzymes of the MEGS as well as deficiencies in the "regulatory" proteins. An important part of this thesis is focussed on such a biochemical screening method in muscle tissue of patients suspected of a mitochondrial disorder.

First, a historical overview is presented of the evolution and the discovery of the mitochondrion and how the puzzle of the MEGS was unravelled during the last century. Furthermore, the development of the presently used methods to trace enzymatic deficiencies in the MEGS is described.

Table 1. **Clinical symptoms and diseases associated with mitochondrial dysfunction**.

CNS	seizures; spasticity; transient paraparesis; lethargy; coma; psychomotor retardation; extrapyramidal signs; ataxia; dyspraxia; central hypoventilation; deceleration or acceleration of head growth; (cortical) blindness; (perceptive) deafness; autism; developmental delay; mental retardation; dementia; atypical cerebral palsy; migraine; stroke and stroke-like events; neuropathic pain and weakness; acute and chronic inflammatory demyelinating polyneuropathy; absent deep tendon reflexes; neuropathic gastrointestinal problems; aberrant temperature regulation; Huntington's disease; Alzheimer's disease; Parkinson's disease; bipolar disorder; schizophrenia; anxiety disorders; Kearns-Sayre syndrome*; LHON*; MELAS*; MERRF*; Leigh syndrome*; Alpers syndrome*; NARP*; MNGIE*; MILS*.
Heart	hypertrophic and dilated cardiomyopathy; cardiac conduction defects; cardiovascular disease.
Muscle	exercise intolerance; easy fatiguability; muscle weakness; hypothonia; hyperthonia; cramps; ptosis; ophthalmoplegia.
Kidney	tubular dysfunction.
Liver	hepatic failure; hypoglycemia; gluconeogenic defects.
Eyes	optic neuropathy; retinitis pigmentosa; cataract.
Ears	sensorineural hearing loss.
Endocrine	diabetes; delayed puberty; hypothyroidism; exocrine pancreas dysfunction; primary ovarian dysfunction.
Intestinal	diarrhoea (villous atrophy); intestinal pseudoconstriction.
Other	failure to thrive; short stature; respiratory problems; pancytopenia; anaemia; cancer; aging and senescence; metabolic syndrome*.

*Inherited conditions in which mitochondrial dysfunction is implicated.

1.2. Evolution of the mitochondrion

About 2 billion years ago the ambient oxygen tension of the earth's atmosphere increased rapidly from 1% to 15% of the present level. This happened in about 200 million years. Many believe that the origins of mitochondria as organelles in primitive eukaryotes can be associated with this environmental trauma and it is generally accepted now that the respiratory system in eukaryotic lineages was introduced by an endosymbiotic α-proteobacterium (Kurland and Andersson, 2000; Hackstein *et al.,* 2006). This endosymbiotic

theory of mitochondrial origin arose in the early nineteenth century (Wallin, 1925) and was given new life some decades later (Margulis, 1970).

1.3. Discovery of the mitochondrion; a morphological history

The study on mitochondria started some 150 years ago when Albert Kölliker in 1857 discovered "sarcosomes" (mitochondria) in muscle cells. In 1889, Richard Altmann described a technique to dye mitochondria. He observed several granula in the cytoplasm of a variety of cells and assumed these were elementary organisms living in the cytoplasm of the cells. He postulated their genetic and metabolic autonomy and called them "bioblasts". In 1897, Carl Benda called this organelle mitochondrion (from Greek "mito"= screw-thread and "khóndrion"=grain, granula) because under a light-microscope these organelles looked like screw-thread shaped grains.

1.4. Mitochondrial biochemistry: from Warburg to MEGS

The main and well-known function of mitochondria in each cell is to produce energy in the form of ATP, but today we know that mitochondria are also involved in a number of other biochemical processes (Table 2).

Table 2. **Cellular processes in which mitochondria are involved.**

- ATP production
- Apoptosis
- Cellular proliferation
- Regulation of the cellular redox state
- Heme synthesis
- Steroid synthesis
- Glucose homeostasis/insulin secretion in the pancreas
- Heat production
- Detoxification (Urea cycle)
- Cellular ion homeostasis (Ca^{++})
- Production of radical oxygen species

ATP is produced by a very efficient set of enzymatic reactions: the MEGS. The MEGS can be divided in five major biochemical pathways: (*1*) production of AcCoA from oxidation of pyruvate or fatty acids by PDHc and β-oxidation, respectively. (*2*) Oxidation of AcCoA by the TCA-cycle to CO_2 and reduction equivalents NADH and $FADH_2$. (*3*) Oxidation of NADH

and $FADH_2$ and transport of electrons through the RC, (and simultaneously pumping protons out of the mitochondrion by the RC) resulting in the reduction of O_2 to H_2O. (*4*) Production of ATP, by restoration of the proton equilibrium by pumping protons back into the mitochondrial matrix, by complex V (F_1F_0-ATP synthase). (*5*) Transport of substrates by specific mitochondrial carrier proteins and transport of ATP out of the mitochondrion into the cytosol by ANT.

Biochemical and physiological studies that elucidated all these mitochondrial pathways started in the early 20th century. In 1912 Otto Warburg (Nobel-prize laureate 1931) described for the first time an "Atmungsferment" that could be inhibited by cyanide. He assumed that the mitochondrial RC-activity was catalyzed by one enzyme. Between 1918 and 1922, Otto Meyerhof in collaboration with Archibald Hill (both Nobel-prize laureates in 1922) proved that in muscle tissue, in the absence of oxygen, glycogen is converted into lactic acid and in the presence of oxygen, part of that lactic acid is converted to carbon dioxide and water. In 1929 Fiske and Subbarow discovered phosphocreatine (PCr) (Fiske, Subbarow, 1929) and a major breakthrough came from the discovery of ATP by Lohmann (Lohmann, 1929). However, the function of these compounds was not fully understood at that time. In 1941 Lipmann (Nobel-prize laureate 1953) described them as the carriers of phosphate-bound energy (Lipmann, 1941). From 1925 till 1940 Keilin discovered the cytochromes and purified cytochrome *c*. He proved that these proteins are involved in substrate oxidation and oxygen consumption (Keilin, 1925; Keilin, 1929; Keilin 1939; Keilin, 1940). In 1939 Kalckar described the stimulation of glucose oxidation under aerobic conditions by malic or fumaric acid under simultaneous consumption of oxygen and production of ATP, in rabbit- and cat-kidney extracts (Kalckar, 1939). Another breakthrough was made in 1940 when Krebs (Nobel-prize laureate in 1953) described the citric acid cycle and the oxidation of pyruvate under aerobic conditions in pigeon breast muscle (Krebs, 1940(1); Krebs, 1940(2)). In 1943 Ochoa (Nobel-prize laureate 1959) described the oxidation of pyruvate in the presence of succinate and simultaneously consumption of oxygen and production of ATP, in cat heart extracts. He also noticed that under these circumstances for each mole O_2 reduced, three moles ATP were produced, resulting in a P/O ratio of 3 (Ochoa, 1943). Between 1948 and 1950 all these findings were pieced together by Lehninger and co-workers. They subsequently proved that ATP production was coupled to electron transport between NADH and oxygen (Friedkin and Lehninger, 1949), that the TCA-cycle and the oxidation of pyruvate and fatty acids are localized inside the mitochondria (Kennedy and Lehninger,1949), that each oxidative step in the TCA-cycle revealed a P/O ratio of approximately 3, except for the

oxidation of succinate to fumarate (Lehninger and Wagner Smith, 1949) and that ATP production from oxidation of pyruvate is completely abolished by DNP (an uncoupler of the oxidative phosphorylation) while pyruvate oxidation itself is not impaired (Barkulis and Lehninger, 1951). At the same time Slater measured RC activities spectrophotometrically in horse-heart mitochondrial fractions. He measured NADH:O_2 oxidoreductase and NADH:cytochrome *c* oxidoreductase (he named it dihydrocozymase:cytochrome *c* reductase) by measuring NADH (he named it cozymase) oxidation at 340nm, and SCC by measuring cytochrome *c* reduction at 550nm (Slater, 1950(1); Slater, 1950(2); Slater, 1950(3)). He also noticed that besides the cytochromes, another enzyme was involved in the oxidation of NADH and called it diaphorase. He proved the existence of two kinds of diaphorases. One that oxidizes NADH and transfers electrons via methylene-blue directly to cytochrome *c*, an enzyme still known as diaphorase or NADH:cytochrome b_5 oxidoreductase (Fischer et al.1986). A second that oxidizes NADH but needs an additional unknown factor between diaphorase and cytochrome *c* (and for SCC between cytochrome *b* and cytochrome *c*), today known as complex I and coenzyme Q_{10}, respectively (Slater, 1950(3)). Between 1960 and 1969, Hatefi and collaborators studied the enzymatic properties of complex I and isolated the complex from beef heart mitochondria (Hatefi et al., 1960; Hatefi et al., 1969).

At that time it was clear that in the presence of oxygen, pyruvate, derived from oxidation of glucose via the glycolytic pathway, is oxidized to CO_2 by PDHc and the TCA-cycle and NADH produced by the PDHc and the TCA-cycle is oxidized to NAD^+ and H_2O by the RC with simultaneous production of ATP. In 1961 Mitchell (Nobel-prize laureate 1978) postulated “The coupling of phosphorylation to electron and hydrogen transfer by a chemi-osmotic type of mechanism” (Mitchell, 1961). During electron transport H^+ ions are pumped outward across the inner mitochondrial membrane yielding a gradient of H^+. The H^+ gradient is the driving force for the phosphorylation of ADP by an ATPase system. This last part of the aerobic synthesis of phosphate bound energy in mitochondria was elucidated between 1978 and the early nineties by Boyer and Walker (Nobel-prize laureates 1997) who independently of each other elucidated the function and structure of the mitochondrial F_1F_0-ATP synthase, also known as complex V of the OXPHOS system.

1.5. Mitochondrial biochemical diagnostics

Biochemical studies on the oxidative metabolism were initiated by Warburg, introducing the manometric measurements of CO_2 production and oxygen consumption. For a long time these

manometric measurements were the generally used methods to study aerobic oxidation of substrates. Substrates and products were measured by chemical methods. Because these methods were far from sensitive, huge amounts of cells or mitochondria were needed to perform these experiments. In 1953, an important step forward was made with the introduction of the Clark oxygen electrode. From that time onwards, mitochondrial oxygen consumption could be measured continuously in a small volume and with relatively small amounts of mitochondria by a polarographic method (Leland *et al*., 1953). Another breakthrough was made when ^{14}C labeled substrates became available. This made it possible to study decarboxylating enzymes by measuring $^{14}CO_2$ production rates (Kobayashi, 1962). The reaction vessels needed to measure these reactions were improved by Fox and these vessels are still used to date (Fox, 1971). In 1970 the first patient was described with a defect in pyruvate decarboxylase. This was proven by measuring $^{14}CO_2$ production rate from oxidation of [1-^{14}C] pyruvate in intact leucocytes (Blass *et al*., 1970). In 1974 the first patient was published with decreased $^{14}CO_2$ production rates from oxidation of [1-^{14}C] pyruvate in intact liver and muscle mitochondria (Willems *et al*., 1974). Between 1976 and 1980 a unique set of incubations was developed with sub-mitochondrial fractions from muscle tissue, based on measuring $^{14}CO_2$ production rates from ^{14}C labeled substrates and on measuring ATP production rates from oxidation of pyruvate (Janssen *et al.*, 2006) as described in Chapter 3 of this thesis. With this set of incubations MEGS-capacity can be measured and in case of decreased oxidation rates it can be predicted in which part of the MEGS a deficiency is located. To localize the enzyme deficiency it was necessary to develop reliable and sensitive assays for measuring OXPHOS-enzymes and PDHc because the majority of patients suffering from a mitochondrial disorder and decreased MEGS-capacity has a defect in one of these enzymes.

A PDHc assay based on measuring $^{14}CO_2$ production rate from [1-^{14}C]pyruvate in cell-free extracts from fibroblasts and other tissues was developed by Blass and coworkers and this method is still used to date (Blass *et al*., 1970; Blass *et al*., 1972).

As mentioned before, RC-enzymes were already measured by Slater (Slater, 1950(1);Slater, 1950(2); Slater, 1950(3)). However, the utility of these combined assays for diagnostic purposes is limited. NADH: O_2 oxidoreductase measures the whole RC but is rate-limited by complex IV. So the activity is only decreased in case of a complex IV deficiency. In muscle tissue of a number of patients with a complex I deficiency, NADH: O_2 oxidoreductase activity was only decreased when complex I activity was strongly decreased (unpublished own observations). NADH: cytochrome *c* oxidoreductase is a combined assay for complex I and

III but is rate-limited by complex I, so complex III deficiencies can be missed. SCC is a combined assay for complex II and III but is rate-limited by complex II. In muscle tissue of a number of patients with decreased complex III activity, SCC activity was normal (unpublished own observations). For those reasons single enzyme assays for complex I, II and III were developed (Fischer *et al*., 1986; Fischer *et al*., 1985; Bentlage *et al*., 1996). In 1951, a reliable and sensitive assay for complex IV was developed which is still used today (Cooperstein and Lazarow, 1951). A complex V assay was described in 1986 (Buckle et al., 1986). The principle of this assay is used for most assays developed later. Recently the assay was improved to make it more reliable for diagnostic purposes (Morava *et al*., 2006). The SCC assay still has its merit to trace patients with CoQ_{10} deficiency. Decreased SCC activity in fibroblasts or muscle tissue in combination with normal activities for complex II and III is indicative for CoQ_{10} deficiency. When SCC activity clearly increases in the presence of decylubiquinone (an artificial substrate for CoQ_{10}; stimulation >2.2 in muscle and >4.5 in fibroblasts), this is strongly indicative for a CoQ_{10} deficiency (López *et al.,* 2006).

Mitochondrial enzyme activities expressed on protein base are only a good measure if measured in highly purified mitochondrial fractions. In tissue homogenates or sub-mitochondrial fractions, mitochondrial protein is only a very small part of the total protein content. When the amount of mitochondria in tissue increases or decreases or mitochondrial enzyme activities are up-regulated, total-protein content does not increase or decrease proportionally. For that reason mitochondrial enzyme activities must be expressed on a mitochondrial marker enzyme. The mostly used mitochondrial marker enzyme is CS.

CS activity can be measured easily and sensitively and no genetic CS defects have been found so far. The mostly used CS assay today is that described by Srere (Srere, 1969). A disadvantage of CS as a mitochondrial marker enzyme is its localization in the mitochondrial matrix space and its weak bound inside the mitochondrion. So, it can only be used as a marker enzyme in crude homogenates of fresh or frozen tissues and in sub-mitochondrial or mitochondrial fractions of fresh tissues. When tissues have been frozen the mitochondrial inner membrane is disrupted and CS partially leaks out of the mitochondrion. So in frozen tissues, CS activity in sub-mitochondrial fractions will be relatively high because it leaks out of the mitochondria that still remain in the *600g* pellet, into the supernatant. In isolated mitochondrial fractions of frozen tissues, the activity is relatively low because it will partially be washed out of the mitochondrion during the isolation procedure. In sub-mitochondrial fractions or mitochondrial fractions from frozen tissues complex IV or complex II can be used as mitochondrial marker enzymes, as long as their activities are not decreased.

1.6. Mitochondrial molecular genetic diagnostics

mtDNA, properties and defects

Because of its bacterial origin, the mitochondrion is the only animal-cell organelle that contains its own DNA. During evolution most of the bacterial DNA has been transferred to the eukaryotic cell nuclear genome but part of it has remained in the bacteria and so led to the development of mitochondria (Kurland and Andersson, 2000). The mtDNA was discovered in 1963 (Nass and Nass, 1963(1); Nass and Nass, 1963 (2)). The human mtDNA consists of 16,569 bp and its sequence and organization were first published in 1981 (Anderson *et al.*, 1981). This sequence was revised in 1999 (Andrews *et al.,* 1999). The mitochondrial genome encodes 13 mitochondrial proteins, all subunits of OXPHOS-enzymes (ND1-ND6 and ND4L of complex I, cytochrome *b* of complex III, COI, II and III of complex IV and ATPase 6 and 8 of complex V), the *12S* and *16S* rRNAs and 22 tRNAs. In humans, mtDNA is maternally inherited because the mother transmits her oocyte mtDNA to all her offspring and her daughters transmit their mtDNA to the next generation. Paternal mtDNA does not contribute to mitochondrial inheritance (Bandelt *et al.*, 2005). The first published pathogenic mutations in the structural mtDNA genes were the 11778G>A mutation in the *ND4* gene causing LHON (Wallace *et al.* 1988) and the 8993T>G mutation in the *ATPase 6* gene causing NARP/MILS (Holt *et al.* 1990). The latter, together with the 8993T>C mutation (de Vries *et al.*, 1993), are the most frequently encountered mutations in patients suffering from complex V deficiency. In 1990 the first mutations in the m.tRNA genes were published: the 3243A>G mutation in $tRNA^{LEU(UUR)}$ causing MELAS (Goto *et al.* 1990; Kobayashi *et al.* 1990), and the 8344G>A mutation in $tRNA^{LYS}$ causing MERRF (Shoffner *et al.* 1990). To date more than 250 pathogenic mtDNA mutations (associated with variable clinical phenotypes) have been described, most of them (137) located in the mtDNA structural genes of the OXPHOS-enzymes (MITOMAP 2005).

nDNA defects in mitochondrial disease

Complex I, the largest OXPHOS complex with a relative molecular mass of about 980 kD, consists of 45 subunits identified so far (Carroll et al., 2006), 38 encoded by the nDNA. About 30% of the patients with an OXPHOS disorder suffer from a complex I deficiency mostly caused by a mutation in a nuclear encoded gene. In 1991 the sequence of the first nuclear complex I gene (*NDUFS1*) was published (Chow *et al.* 1991). Between 1991 and 1998 the sequences of all 34 nuclear encoded complex I subunits known at that moment, were

published (Triepels *et al.,* 1998). In 1998 also the first mutation in one of the nuclear complex I genes (*NDUFS4*) was published (van den Heuvel *et al.,* 1998). To date mutations in ten nuclear complex I genes and in two complex I assembly genes have been identified (see Table 3).

Complex II, with a relative molecular mass of about 130 kD consists of 4 subunits all encoded by the nDNA: a flavoprotein (Fp; SDH A) and an iron-sulfur protein (Ip; SDH B) that constitute the catalytic domain, and two subunits that anchor complex II in the mitochondrial membrane (SDH C and D). The complex is partially located in the TCA-cycle and partially in the RC. Mutations in all the genes encoding the four subunits have been described, with a strikingly diverse clinical phenotype. In 1995 a mutation was described in the *SDHA* gene, the very first mutation reported in a nuclear gene encoding a subunit of a RC-complex (Bourgeron *et al*., 1995). The complex II genes *SDHB, C* and *D* have been described as tumour suppressor genes (Astuti *et al*., 2001; Astuti *et al.*, 2001; Baysal *et al*., 2000; Niemann *et al*., 2000). Mutations in complex II genes causing complex II deficiency are rare. To date only 5 mutations in the *SDHA* gene have been described causing complex II deficieny, whereas 93 involved in tumour pathogenesis (Bayley *et al*., 2005).

Complex III, with a relative molecular mass of 500 kD, consists of 11 subunits, one encoded by the mtDNA (cyt *b*). Patients with isolated complex III deficiency are relatively rare. To date, 22 different mutations in the *cyt b* gene have been described (MITOMAP 2005) but only one in a nuclear gene of complex III. Two mutations have been described in a complex III assembly gene (see Table 3).

Complex IV, with a relative molecular mass of about 200 kD, consists of 13 subunits, 10 encoded by the nDNA. To date 47 mutations in the mtDNA encoded complex IV genes are known (MITOMAP, 2005). Strikingly, no mutations in the nuclear complex IV genes have been identified so far but only mutations in a number of complex IV assembly genes (see Table 3).

Complex V, with a relative molecular mass of 600 kD, consists of 14 subunits of which 12 are encoded by the nDNA. The most frequently encountered mutations in patients suffering from complex V deficiency are the mtDNA 8993T>G/C mutations in the *ATP6* gene. No mutations have been found in the nuclear encoded complex V genes so far, but only a mutation in a complex V assembly gene (see Table 3). However, there is striking evidence that more mutations in genes of nuclear genetic origin causing complex V deficiency must exist (Sperl *et al*., 2006, and unpublished own observations).

CoQ_{10} is the RC electron-transfer intermediate between complexes I and II and complex III. CoQ_{10} deficiency also causes severe mitochondrial dysfunction. Recently mutations in three genes of the CoQ_{10} biosynthesis pathway have been described (see Table 3).

PDHc is a multienzyme complex and consists of four catalytic subunits, E1α and E1β (together forming the PDHcE1 enzyme or PDH), E2 and E3, and an E2/E3 binding protein. PDH is regulated by two regulatory enzymes, PDH phosphatase and PDH kinase (Lissens *et al*., 2000). Mutations have been described in two PDHc genes and in the PDH phosphatase gene (see Table 3).

Fumarate hydratase (FH, fumarase) is a homo-tetramer enzyme of the TCA-cycle. FH is localized both in mitochondria and in the cytosol. FH deficiency is rare. The first patient with FH deficiency was described in 1986 (Zinn *et al*., 1986), and so far no more than 20 patients suffering from FH deficiency were published (Deschauer *et al*., 2006). Mutations in the *FH* gene have been described with the same strikingly diverse clinical phenotypes as for some mutations in the complex II genes. The first *FH* mutation in a patient with FH deficiency was described in 1994 (Bourgeron *et al.*, 1994). To day at least 14 mutations with autosomal recessive inheritance have been described causing FH deficiency and severe mitochondrial dysfunction (Deschauer *et al*., 2006). At least 20 mutations with autosomal dominant inheritance have been described as tumour suppressor gene mutations (Alam *et al*., 2003).

As mentioned in chapter 1.1., a lot of "regulatory" proteins are involved in the construction of the OXPHOS complexes like the proteins of the mitochondrion's own protein-synthesis machinery. Mutations in two "regulatory" proteins have been described (see Table 3).

A final interesting group of nDNA defects are those mutations that lead to structural mtDNA defects like point mutations, multiple large scale mtDNA deletions and tissue specific mtDNA depletion, with autosomal recessive or dominant inheritance. The first disease described with this kind of genotype was MNGIE (Hirano *et al*., 1994), a disease caused by a thymidine phosphorylase deficiency and associated with mutations in the nDNA encoded *TP* gene (Nishino *et al*., 1999). In the last years, mutations in a number of genes encoding proteins that control mtDNA maintenance have been described, all associated with mtDNA defects described above (see Table 3).

***Table 3*. Nuclear gene defects causing mitochondrial diseases.**

Enzyme/coenzyme	Mutated gene	Reference[a]
Complex I	***NDUFS4***	van den Heuvel *et al.* 1998
	NDUFS8	Loeffen *et al.* 1998
	NDUFV1	Schuelke *et al.* 1999
	NDUFS7	Triepels *et al.* 1999
	NDUFS1	Benit *et al.* 2001
	NDUFS2	Loeffen *et al.* 2001
	NDUFV2	Benit *et al.* 2003
	NDUFS3	Benit *et al.* 2004
	NDUFS6	Kirby *et al.*2004
	NDUFA1	Fernandez-Moreira *et al.* 2007
	B17.2L ‡	Ogilvie *et al.* 2005
	NDUFAF1‡	Sugiana *et al. 2006*
Complex II	***SDHA***	Bourgeron *et al.* 1995
	SDHB	Astuti *et al.* 2001*
	SDHC	Niemann *et al.* 2000*
	SDHD	Baysal *et al.* 2000*
Complex III	***QP-C***	Haut *et al.* 2003
	BCS1L‡	de Lonlay *et al.* 2001
		De Meirleir *et al.* 2003
Complex IV	*SURF1*‡	Zhu *et al. 1998*
		Tiranti *et al.* 1998
	SCO1‡	Valnot *et al.* 2000 (1)
	SCO2‡	Papadopoulou *et al.* 1999
	COX10‡	Valnot *et al.* 2000 (2)
	COX15‡	Antonicka *et al.* 2003
	LRPPRC‡	Mootha *et al.* 2003
Complex V	*ATP12*‡	De Meirleir *et al.* 2004
CoQ_{10}	***COQ2***	Quinzii *et al.* 2006
	PDSS1	Mollet *et al.* 2007
	PDSS2	López *et al.* 2006
PDHc	***E1α***	Lissens *et al.* 2000
	E3	Shaag *et al.* 1999
		Grafakou *et al.* 2003
	PDP1	Maj *et al.* 2005
Fumarate hydratase	***FH***	Bourgeron *et al.* 1994
		Alam *et al.* 2003*
Regulatory enzymes:		
Elongation factor G1	***EFG1***	Coenen *et al.* 2004
Elongation factor Tu	***EFTu***	Valente *et al.* 2007
Elongation factor EFTs	***TSFM***	Smeitink *et al.* 2006

mtDNA maintenance enzymes:		
ANT	***ANT1***	Palmieri *et al.* 2005
TWINKLE (primase/helicase)	***C10orf2***	Spelbrink *et al.* 2001
mtDNA polymerase γ	***POLG***	Naviaux *et al.* 2004 Ferrari *et al.* 2005
Deoxyguanosine kinase	***DGUOK***	Mandel *et al.* 2001
Mt thymidine phosphorylase	***TP***	Nishino *et al.* 1999
Mt thymidine kinase	***TK2***	Saada *et al.* 2001
Succinyl-CoA synthase - (ADP forming)	***SUCLA2***	Elpeleg *et al.* 2005

Structural genes for mitochondrial enzymes or enzyme subunits are printed in bold.
References describing tumour suppressor genes are marked with an asterisk.
‡ Assembly genes for OXPHOS enzymes.
[a] First published article.

1.7. Scope of this thesis

The major goal of this thesis was to evaluate and improve the reliability of the biochemical methods used to search for disturbances in the MEGS. After thirty years, biochemical research for mitochondrial disorders is still important and becomes more important, despite the great and fast developments in the molecular genetic field. Knowing the whole human genome sequence still does not explain the whole biochemical functioning of a mitochondrion, not to mention a cell. The significance of a great number of known, or until now unknown, genes involved in mitochondrial functioning can only be elucidated together with solid biochemical studies.

An other goal was to develop new assays for complex I and II that were at least as reliable and sensitive as the present methods, but more appropriate to measure these enzymes in large numbers of samples.

Along with postnatal diagnostics for mitochondrial diseases, enzymatic prenatal diagnostics for these diseases became more and more important since for a lot of OXPHOS disorders the molecular genetic defects are still unknown. For that reason assays were developed to measure OXPHOS-enzymes in human embryonic cells.

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CHAPTER 2

Some practical aspects of providing a diagnostic service for respiratory chain defects

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Abstract

The oxidative phosphorylation system (OXPHOS) is organized into five multi-protein complexes, comprising four complexes (l-lV) of the respiratory chain and ATP synthase (complex V). OXPHOS has a vital role in cellular energy metabolism and ATP production. Enzyme analysis of individual OXPHOS complexes in a skeletal muscle biopsy remains the mainstay of the diagnostic process for patients suspected of mitochondrial cytopathy. Practical guidelines are presented to provide optimal conditions for performance of laboratory investigations and a reliable diagnosis. A fresh muscle biopsy is preferable to a frozen muscle sample because the overall capacity of the OXPHOS system can be measured in a fresh biopsy. In about 25% of patients referred for muscle biopsy to our centre, reduced substrate oxidation rates and ATP+creatine phosphate production rates were found without any defect in complexes I-V and the pyruvate dehydrogenase complex. Investigation of frozen muscle biopsy alone may lead to false-negative diagnoses in many patients. In some patients, it is necessary to investigate fibroblasts for prospective diagnostic purposes. An exact diagnosis of respiratory chain defects is a prerequisite for rational therapy and genetic counseling. Provided guidelines for specimen collection are followed, there are now reliable methods for identifying respiratory chain defects.

Metabolic background to respiratory chain defects

Mitochondria are cytoplasmic cell organelles in which the energy-rich compounds ATP and creatine phosphate (CrP) are synthesized (see Fig. 1). During glycolysis, pyruvate is produced from glucose in the cytoplasm of the cell. Under anaerobic conditions, pyruvate is converted into lactate by lactate dehydrogenase (LDH). Under aerobic conditions, pyruvate is transported into the mitochondria, where it is converted into acetyl-coenzyme A (CoA) by the pyruvate dehydrogenase (PDH) complex. Acetyl-CoA is also formed from fatty acids by β-oxidation. The PDH complex is regulated by two enzymes: pyruvate dehydrogenase phosphatase, which dephosphorylates the inactive El-subunit of the complex, leading to activation; and pyruvate dehydrogenase kinase, which rephosphorytates the active El-subunit of the complex, leading to inactivation. The PDH complex is also regulated by the NADH/NAD^+ ratio and the acetyl-CoA/CoA ratio. Increases of these ratios result in feedback inhibition of the PDH complex. Acetyl-CoA from pyruvate and fatty acids is also oxidized in the citric acid cycle. During the oxidation of pyruvate and in the citric acid cycle, NADH and FADH2 are produced. These reduced co-enzymes are oxidized to NAD^+ or FAD by complex I and complex II, respectively, of the mitochondrial respiratory chain. The electrons are transferred from complex I and complex II to the redox carrier coenzyme Q_{l0} and are further transported via complex III and cytochrome *c* to complex IV. In this final step, oxygen is reduced to H_20. The energy released during the electron transfer reactions is conserved in the form of an electrochemical proton gradient by the transport of protons across the mitochondrial inner membrane. At the level of complexV, the protons are transported back into the mitochondrion and the released energy is used to synthesize the energy-rich compounds ATP and CrP. The process by which oxidation of substrates by the mitochondrial respiratory chain is coupled to the phosphorylation of ADP into ATP is called oxidative phosphorylation.

Mitochondrial cytopathies are a group of disorders caused by defects in one or more of the enzymes involved in mitochondrial energy metabolism. These defects are frequently localized in the PDH complex and/or in one or more of the respiratory chain complexes.

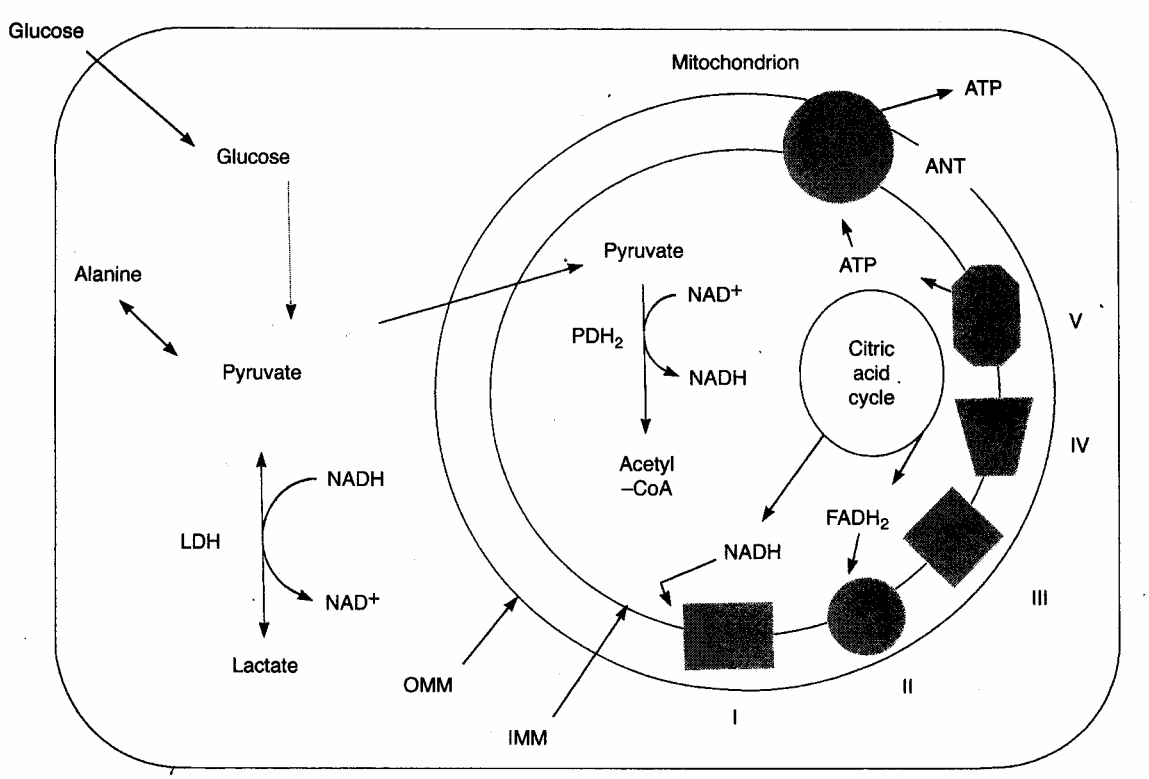

Fig. 1. Schematic representation of the biosynthesis of ATP via the oxidation of pyruvate.
LDH = lactate dehydrogenase:ANT =adenine nucleotide translocater; PDHc, pyruvate dehydrogenase complex;
CoA = coenzyme A; 0MM = outer mitochondrial membrane; 1MM = inner mitochondrial Membrane

What are the consequences of a defect in a respiratory chain complex?

A defect in one or more of the respiratory chain complexes results in a disturbed oxidation rate of NADH to NAD^+ (in cases of complex I, III and IV defects) or a disturbance of the oxidation rate of $FADH_2$ to FAD (in cases of complex II defects). Let us assume that a patient is suffering from an isolated complex I deficiency. This leads to an increase in NADH and decrease in NAD^+. The increased NADH/ NAD^+ ratio exerts a feedback inhibition on the PDH complex, leading to an increase in mitochondrial pyruvate. Pyruvate is thereafter transported into the cytosol and further metabolized into lactate by LDH or converted into alanine by transamination. Therefore, the final result of a complex I deficiency is an intracellular increase of lactate, pyruvate and alanine with a concomitant increase of these metabolites in body fluids [blood and urine and frequently also in cerebrospinal fluid (CSF)].

Apart from an absolute increase in lactate and pyruvate concentrations, respiratory chain defects are also frequently associated with an increased ratio of lactate/pyruvate in blood and CSF. This phenomenon can be explained by a shift of the LDH-catalyzed reaction in favour of lactate caused by the increased ratio of NADH/NAD^+.

Clinical presentation

The clinical abnormalities in patients suffering from a mitochondrial disorder are very heterogeneous and often non-specific. The symptomatology varies in age of onset (from birth to adulthood) and course (rapidly progressive or static). In some patients only one tissue seems to be affected, whereas other patients seem to suffer from a multisystem disorder.

In the majority of the patients, muscular and/or neurological complaints are the main presenting symptoms. Some symptoms are age-dependent (e.g. failure to thrive in neonates and exercise intolerance in adulthood); others (e.g. hypotonia, retardation) can present at any age. Many patients, particularly children, who meet the morphological, biochemical and/or molecular biological criteria for a mitochondrial disorder cannot be classified into one of the above entities. An additional complication is that, in a few patients, the clinical picture gradually changes from one well defined clinical phenotype into another.

No single clinical feature is diagnostically specific or distinctive. A patient is suspected to suffer from a mitochondrial disorder if demonstrating at least two chronic and unexplained symptoms from the extended list in Table 1, preferably occurring in two unrelated organs.[1,2]

Initial biochemical investigations

Patients suspected of suffering from a mitochondrial cytopathy on clinical grounds should undergo the following initial biochemical investigations:

- Blood, urine and CSF lactate, pyruvate and alanine measurement, and calculation of the lactate/ pyruvate ratio in blood and CSF.[3,4]
- Measurement of urinary amino acids.

Table 1. **The clinical symptoms most frequently found in mitochondrial disorders**

Central nervous system	Seizures
	Hypotonia/hypertonia
	Spasticity
	Transient paraparesis
	Lethargy/coma
	Psychomotor retardation/regression
	Extrapyramidal signs
	Ataxia (episodic)
	Dyspraxia
	Central hypoventilation
	Deceleration/acceleration of head growth
	Blindness (cortical)
	Deafness (perceptive)
Skeletal muscle	Exercise intolerance/easy fatiguability
	Muscle weakness
Heart	Cardiomyopathy (hypertrophic or dilated)
	Conduction abnormalities
Eyes	Ptosis
	Restricted eye movements
	Strabismus
	Cataract
	Pigmentary retinopathy
	Optic atrophy
Liver	Hepatic failure
Kidney	Tubular dysfunction
Endocrine	Diabetes insipidus
	Delayed puberty
	Hypothyroidism
	Diabetes mellitus
	Exocrine pancreas dysfunction
	Primary ovarian dysfunction
Gastrointestinal	Diarrhoea (villous atrophy)
	Intestinal pseudoconstriction
Other	Failure to thrive
	Short stature
	Pancytopenia
	Anaemia

A generalized aminoaciduria may occur in respiratory chain defects due to the latter's effect on the proximal tubular cells of the kidney, which leads to a decreased reabsorption of amino acids and thus increased excretion.

Analysis of organic acids in the urine may provide additional diagnostic information because, apart from lactate, citric acid cycle intermediates such as 2-oxoglutarate, succinate, fumarate and malate may be excreted in increased amounts in the urine of

patients with a respiratory chain defect. The finding of a normal concentration of lactate in blood, urine and CSF does not always exclude a respiratory chain defect. Where there is a strong suspicion of such a defect on clinical grounds, a glucose tolerance test is recommended with simultaneous determination of blood lactate.[5] Under normal conditions, lactate concentrations in blood remain nearly constant or increase only slightly, but in a respiratory chain defect a pathological increase may occur.

Definite biochemical investigations

Muscle biopsy collection guidelines

To optimize the performance of biochemical investigations on a muscle biopsy, it is essential to follow the guidelines summarized in Table 2. It is very important to obtain a muscle sample of sufficient size, enabling the whole diagnostic programme to be performed. At least 300 mg of a fresh muscle sample is required. If a smaller amount of tissue is sent to our centre, the material cannot be handled as a fresh muscle sample, and the measurements of enzyme activities are limited.

Table 2. **Practical guidelines for biochemical examinations of muscle**

Muscle biopsy	Quadriceps or soleus
Sample size	>50 mg frozen tissue, >300 mg fresh tissue
Quality of the sample	Small amount of connective tissue and fatty tissue
Transport conditions	Melting ice (fresh sample), dry ice (frozen sample)
Age of patient at date of biopsy	> 1 month (if possible)
Medication	Avoid medications preceding biopsy (e.g. anti-epileptics, vitamins, coenzyme Q, etc.)

The quality of the biopsy is also important and should be critically evaluated before processing. The sample must be free of connective tissue and fatty tissue as far as possible. The specimen collection and transport conditions are critical. For fresh samples our rules are the following. The biopsy should be taken under general anaesthesia (needle biopsy using local anaesthesia with lidocaine should be avoided). The sample must be placed immediately in a vial containing ice-cold sucrose EDTA Tris-HC1 heparin (SETH) buffer,

cooled by a sufficient amount of melting ice and sent to the diagnostic laboratory by courier. For frozen samples, the biopsy must be frozen immediately in liquid nitrogen, without using isopentane, and should be transported in a vial that is placed in a sufficient amount of dry ice to keep the muscle sample in a frozen state until arrival in the laboratory. Performance of a muscle biopsy for diagnostic purposes should be avoided in the first month of life, unless life-threatening events have occurred or reliable reference ranges are available for this age group. Medication should be avoided as much as possible before performing the biopsy to avoid false positive diagnosis due to secondary inhibitory effects of medication (e.g. sodium valproate) or enhancement of subnormal enzyme activities leading to a false negative diagnosis (e.g. vitamins, coenzyme Q, etc.)

Biochemical approach to definite diagnosis

After performance of the clinical and laboratory investigations, patients can be selected for further biochemical and molecular genetic investigations. Although the most reliable biochemical diagnostic approach consists of examination of a muscle biopsy because most of the defects are expressed in this tissue[5], missed diagnoses can occur. This is because of tissue-specific expression of some defects and because the absence of a respiratory chain defect in muscle does not completely exclude such a defect in other tissues (e.g. liver); this occurs rarely, however. A fresh muscle sample is preferable to a frozen muscle sample. Investigation of a fresh muscle biopsy, which contains functionally intact mitochondria, allows measurement of the overall capacity of the oxidative phosphorylation system. Moreover, the activities of the PDH complex and respiratory chain enzymes can subsequently be measured. If only a frozen muscle sample is available, only individual enzyme activities can be determined rather than the complete chain. A muscle biopsy can only be handled as a fresh sample if the specimen arrives in the diagnostic centre within 3 h of collection. In other cases, it should be frozen immediately in liquid nitrogen and sent to the diagnostic laboratory in dry ice.

Table 3 summarizes the possible biochemical diagnostic procedures that are available for fresh and frozen muscle samples. In a fresh muscle sample, substrate oxidation rates of radiochemically labelled compounds ([1-^{14}C]pyruvate, [U-^{14}C]malate and [1,4-^{14}C]succinate) can be determined, or oxygen consumption rates from substrate oxidation can be measured.

Table 3. **Biochemical investigations that are possible in fresh and frozen muscle biopsies**

	Fresh sample	Frozen sample
Measurement of oxidation rates (pyruvate, malate, succinate)	+	-
Measurement of production rate of ATP+CrP from oxidation of pyruvate and malate	+	-
Measurement of PDH complex and respiratory chain enzyme activities	+	+

CrP = creatine phosphate; PDH = pyruvate dehydrogenase.

From these observations, preliminary conclusions can be drawn concerning where a defect in the OXPHOS system may lie (e.g. in the PDH complex, the citric acid cycle, complex V or adenine nucleotide translocater). Measurement of the ATP+CrP production rate from substrates (e.g. pyruvate) is very important in establishing a defect in any of the above systems. The ultimate diagnosis can be made by measurement of the individual respiratory chain enzyme activities, complex V and PDH complex. In a frozen muscle sample only individual enzyme activities can be determined, because the mitochondrial inner membrane is partially destroyed and, as a consequence, no information can be obtained concerning the overall oxidative phosphorylation capacity. Finally, in addition to the advantages described above, investigation of a fresh muscle sample is also preferred because, in our experience, in about 25% of patients in whom oxidation rates of substrates and ATP+CrP production rate are clearly reduced, no defect in complexes I-V and the PDH complex can be established. Thus, investigation of a frozen muscle sample alone leads to a false negative diagnosis in these patients.

The primary defect in patients with a clear disturbance in the oxidative capacity of the mitochondria, without a deficiency in any of the relevant complexes, remains to be elucidated. We are currently using proteomics technology in these patients in an attempt to identify the primary defect at the protein level.

Investigation of fibroblasts

In some patients it is necessary to investigate fibroblasts for diagnostic purposes. The indications for biochemical investigations in fibroblasts are:

- If no muscle sample is available.
- If prenatal diagnosis is required.
- To clarify the results obtained in muscle tissue if no clear-cut diagnosis can be made.
- If molecular-genetic investigations are required.
- For research purposes.

Fibroblasts are less suitable than fresh muscle for investigating respiratory chain disorders for the following reasons:

- A defect that is present in muscle is not always expressed in fibroblasts.
- Exclusion of a defect in fibroblasts does not exclude the diagnosis with regard to muscle.
- A specific pattern of abnormalities demonstrated in fibroblasts may not be reflected in muscle tissue.
- Enzyme deficiencies found in muscle are generally more pronounced than in fibroblasts.

It is an absolute prerequisite to demonstrate a respiratory chain enzyme deficiency both in muscle tissue and in fibroblasts if prenatal diagnosis is required. In our opinion, if a defect is not expressed in fibroblasts, a reliable prenatal diagnosis is not possible. In such cases, even investigation of chorionic villi or amniocytes in which the relevant enzyme appears to be present with a normal activity does not exclude the possibility that the fetus might, nevertheless, be affected. Thus, if genetic counseling in a family with an affected child is required, we strongly recommend performance of skin and muscle biopsies for diagnostic purposes.

Fibroblasts are also suitable for molecular-genetic analysis. For this reason, fibroblasts should always be stored from every patient who might become a candidate for molecular-genetic studies. Finally, we recommend performance of a skin biopsy from every patient who is suspected of dying from a mitochondrial cytopathy because future diagnostic assays can be applied to subsequent relatives.

Until now, at least in our centre, diagnostic biochemical procedures in fibroblasts have been restricted to determination of activities of respiratory chain enzymes and of the PDH complex activity. We are now developing assays enabling us to measure, in a comparable manner to muscle, the overall oxidative capacity of the mitochondria by measurement of the substrate oxidation rates and the production rate of ATP+CrP. In the very near future this method will be applied as a routine diagnostic approach, thus extending the diagnostic possibilities in fibroblasts.

Prenatal diagnosis of respiratory chain defects

There is an increasing demand for prenatal diagnosis in families with a child who has been diagnosed with a respiratory chain defect. Prenatal diagnosis can only be performed reliably in families in which the index patient has been proven to suffer from a multisystem disorder. This means that a defect has been proven in at least two different tissues. In most cases, muscle and fibroblasts are the tissues of choice. If a defect is not expressed in fibroblasts, prenatal diagnosis is not possible at the moment. Development of new diagnostic tests (e.g. measurement of overall oxidative capacity of mitochondria in fibroblasts or application of proteomics) may lead to increased prenatal diagnostic possibilities. A further prerequisite for performance of prenatal diagnosis is the absence of a mitochondrial DNA mutation in the affected child. In the presence of such a mutation, performance of reliable prenatal diagnosis is nearly impossible because the percentage of heteroplasmy in a certain patient may vary considerably between the various tissues such as muscle, blood and fibroblasts. This means that the finding of a normal activity of the specific enzyme in the chorionic villi does not exclude a deficiency of the enzyme in other fetal cells. Therefore, mitochondrial DNA mutations have to be excluded in the index case before considering prenatal diagnosis.[6] However, this limitation excludes prenatal diagnosis in only a relatively small number of families because mitochondrial DNA mutations occur only rarely. We are able to perform prenatal diagnosis in families with a proven deficiency of complex I and/or complex IV and of the PDH complex. In the very near future we will be able to also include complex II deficiency in our prenatal diagnostic programme. Prenatal diagnosis of the above defects can be performed both in chorionic villi and in amniocytes. We prefer examination of native chorionic villi because the prenatal diagnosis can be performed at a considerably earlier stage of the pregnancy

(around the 10th week) as compared with amniocytes. Moreover, chorionic villi can be investigated without cultivation of the cells, in contrast to amniocytes. This reduces the time of investigation of the prenatal diagnostic procedure considerably. Clearly, prenatal diagnosis can only be performed in fetal cells that are not contaminated with maternal cells. Until recently we performed prenatal diagnosis only by measurement of the activities of the individual enzyme complexes.[6] At the moment we are also able to perform prenatal diagnosis by mutation analysis in cases where the defect has been characterized at the nuclear DNA level. At present we perform prenatal DNA analysis in families with established mutation(s) in nuclear genes of complex I,[7] the E1α gene of the PDH complex and the SURF-1 gene of cytochrome oxidase. This approach can also be applied to other nuclear genes responsible for OXPHOS deficiencies. In our institute, prenatal diagnosis is restricted to families in which a specific defect has been detected in muscle and fibroblasts of at least one patient in that family or in families in which the genetic defect in the nuclear DNA has been established.

An important feature in the final interpretation of the biochemical measurements concerns the observed residual activity of the deficient enzyme(s). An important question is: has an enzyme deficiency associated with a relatively high residual activity any pathological significance? In our view, the answer to this question can be deduced from our results obtained in fibroblasts. For example, we detected a complex I deficiency in fibroblasts in 55 patients. The residual complex I activity varied between 18% and 89% of the lowest control value. In 13 of these patients a mutation was found in one of the nuclear encoded subunits of complex I. The residual complex I activity varied in these patients from 32% to 82%. This means that, at least in fibroblast, patients with a proven mutation in a nuclear encoded complex I gene may exhibit a relatively high residual enzyme activity, even exceeding 80%. The same holds for prenatal diagnostic investigations. We made a prenatal diagnosis in 23 pregnancies in 15 families in which complex I deficiency was established. In four of these pregnancies complex I activity was reduced in chorionic villi. The residual activity in these latter cases varied between 30% and 81%. Two of these pregnancies were continued and in both cases a severe complex I deficiency was found in the newborn child. The complex I activity in the chorionic villi of these two pregnancies showed a relatively high residual activity of 75% and 81% of the lowest control value, respectively. Thus, it can be concluded that, at least for complex I, high residual activity found in chorionic villi should be considered as pathological for the unborn child.

Conclusion

Much progress has been made in the biochemical diagnosis of mitochondrial respiratory chain defects. The postnatal diagnosis of these defects should preferably be performed in a fresh muscle sample. Practical guidelines have been presented for optimal performance of a reliable diagnostic approach. Attention has been paid to the prenatal diagnostic possibilities of respiratory chain disorders. The increasing demand for prenatal diagnosis of these disorders prompted us to evaluate the present and future biochemical and molecular-genetic possibilities.

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CHAPTER 3

Measurement of the Energy-Generating Capacity of Human Muscle Mitochondria: Diagnostic Procedure and Application to Human Pathology

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Abstract

Background: Diagnosis of mitochondrial disorders usually requires a muscle biopsy to examine mitochondrial function. We describe our diagnostic procedure and results for 29 patients with mitochondrial disorders.

Methods: Muscle biopsies were from 43 healthy individuals and 29 patients with defects in one of the oxidative phosphorylation (OXPHOS) complexes, the pyruvate dehydrogenase complex (PDHc), or the adenine nucleotide translocator (ANT). Homogenized muscle samples were used to determine the oxidation rates of radiolabelled pyruvate, malate, and succinate in the absence or presence of various acetyl-CoA donors and acceptors, as well as specific inhibitors of tricarboxylic acid cycle or OXPHOS enzymes. We determined the rate of ATP production from oxidation of pyruvate.

Results: Each defect in the energy generating system produced a specific combination of substrate oxidation impairments. PDHc deficiencies decreased substrate oxidation reactions containing pyruvate. Defects in complexes I, III and IV decreased oxidation of pyruvate plus malate, with normal to mildly diminished oxidation of pyruvate plus carnitine. In complex V defects, pyruvate oxidation improved by addition of carbonyl cyanide 3-chlorophenyl hydrazone, whereas other oxidation rates were decreased. In most patients, ATP production was decreased.

Conclusions: The proposed method can be successfully applied to the diagnosis of defects in PDHc, OXPHOS complexes, and ANT.

Non-standard abbreviations

MEGS, mitochondrial energy generating system ; PDHc, pyruvate dehydrogenase complex; TCA, tricarboxylic acid cycle ; OXPHOS, oxidative phosphorylation ; RC, respiratory chain; ANT, adenine nucleotide translocator; mtDNA, mitochondrial DNA; CCCP, carbonyl cyanide 3-chlorophenyl hydrazone; CS, citrate synthase ; SDH, succinate dehydrogenase ; CrP, phosphocreatine; 2-ODHc, 2-oxoglutarate dehydrogenase complex ; and SCC, succinate: cytochrome c oxido reductase (complex II + III).

The human mitochondrion contains at least 100 different proteins directly involved in the mitochondrial energy generating system (MEGS) (1). MEGS enzymes are localized in the mitochondrial matrix space [pyruvate dehydrogenase complex (PDHc) and tricarboxylic acid (TCA) cycle] and in the mitochondrial inner membrane [oxidative phosphorylation (OXPHOS) complexes]. Oxidation of pyruvate or fatty acids yields acetyl-CoA which is oxidized in the TCA cycle, yielding NADH and $FADH_2$, which in turn are oxidized by the respiratory chain (RC) and complex V to yield ATP (2). Complete oxidation of 1 mole of pyruvate delivers 15 moles of ATP. The adenine nucleotide translocator (ANT) transports ATP out of the mitochondrion. Deficiencies have been found in all OXPHOS complexes, PDHc, the TCA cycle (3), and ANT. Pathogenic mutations have been identified in the nuclear encoded structural genes of complex I, II, III and PDHc (4-11), and in the mitochondrial DNA (mtDNA) (12). Here we describe a diagnostic procedure to examine the MEGS in detail by measurement of substrate oxidation rates and ATP production rates in intact mitochondria from a muscle biopsy.

Although fibroblasts or lymphocytes can be used for measurement of MEGS capacity, a muscle biopsy is preferred because a deficiency in muscle tissue is not always seen in other cell types (2). The reverse situation is also possible: one of our patients with a mtDNA ND6 mutation complex I deficiency in fibroblasts, showed normal complex l activities in muscle and liver tissue (13). The MEGS capacity can be measured either by oxygen consumption assays in isolated mitochondria (14), permeabilized single muscle fibres (14), and in cultured fibroblasts (8), or by measuring $^{14}CO_2$ production rates from oxidation of [1-^{14}C] pyruvate and carboxyl-^{14}C-labelled TCA cycle intermediates. We developed a unique set of incubations with 3 carboxyl ^{14}C-labelled substrates, in combination with measurement of ATP production in intact muscle mitochondria that gives maximum information about the MEGS capacity. Control values were obtained from muscle tissue of

43 healthy individuals. The results from muscle biopsies from 29 patients with deficiencies in PDHc and OXPHOS enzymes illustrate the rationale of our approach.

Materials and methods

Materials

[1-^{14}C]Sodium pyruvate (0.4-1.1 GBq/mmol) and L-[1,4(2,3)-^{14}C] malate ([U-^{14}C] malate, 1.5-2.3 GBq/mmol) were obtained from Amersham Life Sciences. [1,4-^{14}C] Succinate (0.55-1.11 GBq/mmol) and carbonyl cyanide 3-chlorophenyl hydrazone (CCCP) were from ICN. Sodium pyruvate, acetyl-D,L-carnitine hydrochloride, L-malate, L-carnitine hydrochloride, creatine, sodium-m-arsenite, atractyloside and p^1,p^5-di(adenosine-5') pentaphosphate were obtained from Sigma. Succinate and malonate were from Fluka, and ADP was from Roche. All other chemicals were of the highest purity commercially available. Glass incubation vials (20 ml) with injection caps and rubber septa, hydroxide of hyamine 10-X (hyamine) and Insta Fluor were from Packard BioScience.

Collection of muscle biopsies

Musculus semitendinosus samples were obtained after informed consent from 22 otherwise healthy individuals undergoing arthroscopic anterior cruciate ligament reconstruction using semitendinosus tendon; specimens were scraped from the semitendinosus tendon. Musculus quadriceps biopsies from 6 healthy volunteers were obtained after informed consent by needle biopsy under local anaesthesia of the skin with lidocaine. Because lidocaine can influence some OXPHOS enzymes (15, 16), a minimal dose of lidocaine was used and biopsies were taken at some distance from the incision. Control muscle samples [musculus quadriceps from 7 children (age range, 2-11 years)] and 8 adults were taken surgically from patients with minimal suspicion of a mitochondrial myopathy, definitively excluded in subsequent clinical examinations. Substrate oxidation rates, ATP production, and OXPHOS enzyme activities were within the values measured in the healthy individuals. Fiber typing and enzyme histochemical studies revealed no differences between musculus semitendanosus and musculus quadriceps. Musculus quadriceps samples from patients (needle or open biopsies) were taken as described above. For all

patients and controls, the muscle biopsy used for this study was the first and only biopsy that was taken.

Homogenisation of muscle tissue

After biopsy, muscle tissue was immediately put in ice-cold SETH buffer (0.25 mol/L sucrose, 2 mmol/L EDTA, 10 mmol/L Tris, $5x10^4$ U heparin/L, pH 7.4) and transported to the laboratory within 3 h. Fat and connective tissue were removed. Muscle tissue was minced with a Sorvall TC2 tissue chopper, homogenized in SETH buffer, and centrifuged at 600*g* (17). A portion of the 600*g* supernatant was used for measuring oxidation rates and ATP production rates. The remaining 600*g* supernatant was frozen in 100-µl aliquots in liquid nitrogen and kept at –80 ^{0}C for enzymatic measurements. Citrate synthase (CS) activity was measured according to Srere (18) with minor modifications. Protein concentrations were measured according to Lowry et al. (19) with minor modifications.

Incubations

Incubations were performed in a shaking water-bath at 37 ^{0}C in 20 ml glass incubation vials closed with injection caps and rubber septa. The vials for measuring ^{14}C-labelled substrate oxidations, contained a small plastic tube with 0.2 ml of hyamine. The incubation time was 20 min. Incubation volume was 0.5 ml, containing 30 mmol/L potassium phosphate, 75 mmol/L potassium chloride, 8 mmol/L Tris, 1.6 mmol/L EDTA, 5 mmol/L $MgCl_2$, 0.2 mmol/L p^1,p^5-di(adenosine-5') pentaphosphate (myo-adenylate kinase inhibitor), and where indicated, 2.0 mmol/L ADP, 1 mmol/L pyruvate, 1 mmol/L malate, 1 mmol/L succinate, (with or without) 8,3 KBq of [1-^{14}C]pyruvate, (with or without) 8.3 KBq [U-^{14}C]malate, (with or without) 8.3 KBq [1,4-^{14}C]succinate, 5 mmol/L L-carnitine, 2 mmol/L acetyl-D,L-carnitine, 2 mmol/L sodium arsenite, 5 mmol/L malonate, 2 µmol/L CCCP and 10 µmol/L atractyloside, pH 7.4 . To regenerate ADP, by creatine kinase in the 600g supernatant, 20 mmol/L creatine was added to all ADP-containing incubations.
[1-^{14}C]Pyruvate solution was made fresh and the purity was checked by comparing the concentrations measured by radioactivity counting and an enzymatic assay with lactate dehydrogenase and NADH. Oxidation rates of [U-^{14}C]malate were measured in the presence of malonate [inhibitor of succinate dehydrogenase (SDH)] to prevent the oxidation of [2,3-^{14}C] malate to proceed beyond 1 TCA cycle. The end product, [2,3-^{14}C]

succinate, is transported from the mitochondrion and does not interfere with the substrate oxidation reactions. ATP production was measured in incubations containing pyruvate, malate, creatine, and ADP, in both the absence and presence (blank reaction) of arsenite. Incubations were started with 50 µl of 600g supernatant and stopped by addition of 0.2 ml of 3 mol/L perchloric acid through the rubber septum via a hypodermic syringe. Incubations were kept on ice for 1 h, to trap the $^{14}CO_2$ in the hyamine. The hyamin was mixed with 5 ml Insta Fluor and counted in a Wallac 1400 LSC. Incubations for ATP production measurements were kept on ice for 15 min and then centrifuged (5 minutes at 14000*g* and 2 ^{0}C) in an Eppendorf 5402 centrifuge, after which 0.5 ml of the supernatant was neutralized with 0.6 ml of ice-cold 1 mol/L $KHCO_3$. The mixtures were kept on ice for 15 min and frozen at –20 ^{0}C.

ATP and phosphocreatine measurements

Samples were thawed, put on ice for 5 min, and centrifuged (2 minutes at 14000*g* and 2 ^{0}C) in an Eppendorf 5402 centrifuge. ATP and phosphocreatine (CrP) were measured in the supernatant according to Lamprecht et al. (20) with minor modifications.

RESULTS

The results of the biochemical examination of control muscles are given in Table 1. The intraassay variation (CV) for substrate oxidation rates and ATP production was determined by assaying 4 different 600*g* supernatants prepared from the same muscle biopsy (Table 1). The CV for the CS activity measurement was 7.6%. Substrate oxidation rates and the ATP production were expressed on CS base. The influence of an acetyl-CoA trap on the oxidation rate of [1-^{14}C]pyruvate, of an acetyl-CoA donor on the oxidation rate of [U-^{14}C]malate, and of malonate on the oxidation rate of [U-^{14}C]malate + pyruvate and ATP production is given in Table 2. We tested the validity of the methods by examining muscles of 29 patients with deficiencies in either one of the OXPHOS complexes, PDHc, or ANT. In 24 patients the genetic defect was established. An overview of these patients is given in Table 3, and results of the biochemical examinations are shown in Table 4.

Table 1. **Oxidation rates of [1-^{14}C] pyruvate, [U-^{14}C] malate, [1,4-^{14}C] succinate; ATP production rate from the oxidation of pyruvate + malate; incubation ratios; and intraassay variations in control muscle biopsies.[a]**

		Rate of oxidation					
Incubation	**Substrate**	**Mean**	**sd**	**Mean ±2sd**	**Range**	**n**	**cv %**
1	[1-^{14}C]pyruvate+malate	5,72	1,15	3,43-8,01	3,45-7,99	43	7,4
2	[1-14C]pyruvate+carnitine	6,09	1,08	3,92-8,26	4,21-8,34	43	4,8
3	[1-14C]pyruvate+malate without ADP	0,99	0,35	0,28-1,69	0,45-2,31	43	4,9
4	[1-14C]pyruvat + malate-ADP+CCCP	5,69	1,08	3,53-7,86	3,76-8,37	41	3,7
5	[1-14C]pyruvate+malate-ADP+atractyloside	0,50	0,16	0,18-0,81	0,23-1,10	42	5,7
6	[U-14C]malate+pyruvate+malonate	6,33	1,30	3,73-8,94	3,28-8,80	42	6,3
7	[U-14C]malate+acetylcarnitine+malonate	3,93	0,72	2,50-5,36	1,97-5,24	37	6,5
8	[U-14C]malate+acetylcarnitine+arsenite	2,21	0,39	1,43-3,00	1,10-3,02	37	6,2
9	[1,4-14C]succinate+acetylcarnitine	3,15	0,55	2,05-4,24	2,03-4,18	34	6,8
10	ATP+CrP from oxidation of pyruvate+malate	56,2	10,2	35,9-76,5	36,0-81,7	41	12,1
		Ratios					
	ATP/pyruvate ratio	9,8	1,6	6,6-13,1	6,6-12,1	41	
	Incubation 1/3 (ADP stimulation)	6,0	1,5	3,0-8,9	3,5-8,5	43	
	Incubation 2/1	1,1	0,1	0,8-1,3	0,9-1,5	43	
	Incubation 4/1	1,0	0,1	0,7-1,3	0,7-1,3	41	
	Incubation 3/5	2,0	0,5	1,1-3,0	1,3-3,2	42	
	Incubation 6/1	1,1	0,1	0,8-1,4	0,9-1,3	42	
	Incubation 7/6	0,6	0,1	0,4-0,8	1,4-1,0	37	
	Incubation 7/8	1,8	0,2	1,5-2,1	1,5-2,1	37	

[a] Substrate oxidation rates and ATP production rates were measured in a total of 43 control muscle samples, as described in the *Materials and Methods* section, in the presence of ADP unless indicated otherwise. Oxidation rates for ^{14}C-labeled substrates were calculated as nmoles $^{14}CO_2$ / (h · mU CS). The ATP production rate was calculated as nmoles (ATP + CrP) / (h · mU CS). The mean (SD) age of the controls was 28.9 (15.5) years (range, 2.2-57.6 years). The intraassay variations (CV), as described in the *Results* section, are given in column 7.

***Table 2*. Substrate oxidation experiments in control muscle biopsies testing the influence of malonate on the malate oxidation rate and ATP production rate, and the effect of cosubstrates on the malate and pyruvate oxidation rates.**[a]

Incubation	Mean (SD), %	n
[U-^{14}C]malate+pyruvate without malonate	104 (4)	9
ATP+CrP from oxidation of pyruvate+malat+malonate	87 (5)	7
[U-^{14}C]malate+malonate without pyruvate	6 (1)	4
[1-^{14}C]pyruvate without malate	20 (3)	4

[a] The [U-^{14}C] malate oxidation rates in the absence of either malonate or pyruvate were related to those in the presence of either pyruvate or malonate, which were set to 100%. The ATP production rate in the presence of malonate was related to that in the absence of malonate, which was set to 100%. The oxidation rate of [1-^{14}C] pyruvate in the absence of malate was related to that in the presence of malate, which was set to 100%. All incubations were performed in the presence of ADP and as described in the *Materials and Methods* section.

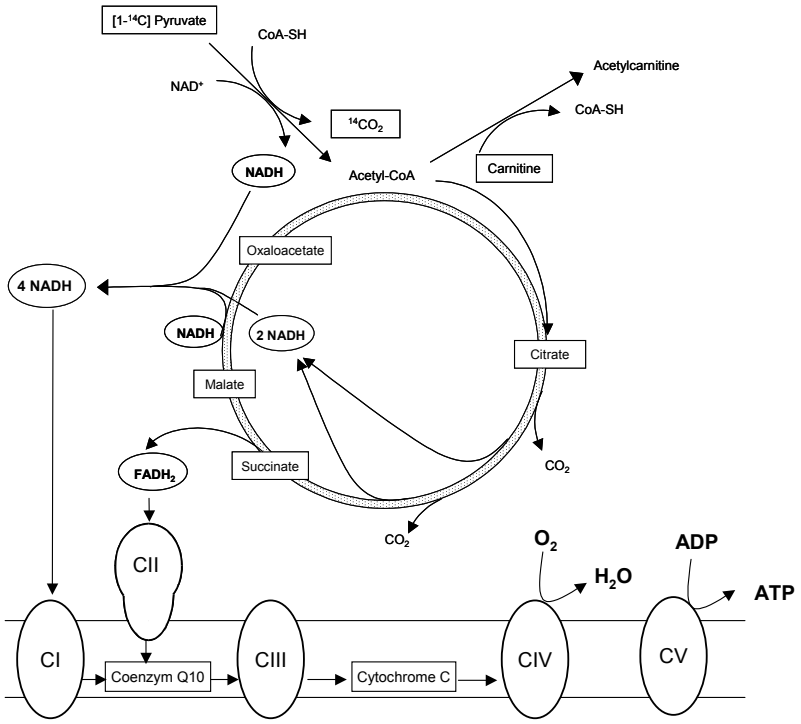

Fig. 1. Schematic representation of the oxidation of [1-^{14}C] pyruvate in the presence of ADP and with either malate or carnitine as acetyl-CoA acceptor.

Malate is converted into oxaloacetate, which subsequently traps acetyl-CoA by the CS-catalysed formation of citrate . In the presence of carnitine, acetyl-CoA is converted into acetylcarnitine and coenzyme-A (CoA-SH) by the mitochondrial enzyme carnitine-acetyltransferase, which is endogenously present in sufficient amounts in muscle tissue. In addition, the production of ATP from the oxidation of pyruvate is shown. CI, CII, CIII, CIV, and CV, complexes I, II, III, IV, and V, respectively.

Table 3. **Genetic and clinical data of the 29 patients examined in this study**

Patient[a] (sex)	Enzyme deficiency[b]	Genetics[c]	Age at biopsy (death)[d]	Clinical course	Reference
1(F)	Complex I (m: 20%; f: 26%)	*NDUFS2* 1336G>A (D446N)	5m (8m)	Leigh syndrome	
2 (M)	Complex I (m: 14%; f: 60%)	*NDUFS4* 316C>T (premature stop)	4.5m (4.5m)	Leigh syndrome	(28)
3 (M)	Complex I (m: 23%; f: 59%)	*NDUFS7* 364G>A (V122M)	3.75y (5y)	Leigh syndrome	(29)
4 (F)	Complex I (m: 43%; f: 38%)	*NDUFS7* 364G>A (V122M)	40y	Spastic hemiparese; poor eyesight; Blefaropasmus; exercise intolerance	
5 (M)	Complex I (m: 34%; f: 24%)	*NDUFS7* 364G>A (V122M)	2.25y	Leigh syndrome, progressive ataxia	
6 (M)	Complex I (m: 29%; f: 63%)	*NDUFS8 (*C.H.)[e] 236C>T (P79L) 305G>A (R102H)	2.3m (2.5m)	Leigh syndrome	(30)
7 (M)	Complex I (m: 39%; f: 77%)	*NDUFV1(*C.H.)[e] 175C>T (premature stop) 1268C>T (T423M)	9m (17m)	Brain atophy; psychomotor retardiaon; Myoclonic epilepsy	(31)
8 (M)	Complex I (m: 51%; f: 37%)	mtDNA: *ND2* T4681C (L71P) (m: 100%; f: 100%)	1.5y (12y)	Leigh syndrome*	
9 (F)	Complex I (m: 66%; f: normal)	mtDNA: *ND4L* A10750G(m: 40%; bl: 40%)	46y	Exercise intolerance; migraine; disturbed cognitive Function; progressive spasticity	
10 (F)	Complex I (m: 43%; f: 85%)	mtDNA: *ND5* G13513A (m: 65%; f: 25%; bl: 26%)	22y	Leigh syndrome	
11 (F)	Complex I m: 13%; f : normal)	mtDNA: A3243G MELAS (m: 40%; f: 30%)	20y	Muscle cramps; exercise intolerance, insufficient kidney function	
12 (F)	Complex I+III CI m: 18% CIII m: 84%	mtDNA: A3243G MELAS (m: 81%)	21y	Psychomotor retardation; convulsions; Sensory deafness	
13 (F)	Complex II (m: 42%; f: 38%)	Genetic defect unknown	11m	Leigh syndrome	
14 (M)	Complex II (m: 8%; f: 10%)	Genetic defect unknown	9m	Leigh syndrome	

Patient[a] (sex)	Enzyme deficiency[b]	Genetics[c]	Age at biopsy (death)[d]	Clinical course	Reference
15 (F)	Complex III m: 7%; f: normal)	*MT-CYB* G15243A (G166E) (m: 100%; f: 0%)	10.9y	Psychomotor retardation; exercise intolerance; muscle weakness; hypertrichosis	
16 (F)	Complex IV m:35%)	*SURF1 (C.H.)*[e] 312insAT TCTGCCAGCC del	2.5y	Leigh syndrome	
17 (M)	PDHc (m: 83% f:) PDHc-E1[e] m: 2%	*PDHA1* 784G>C (V262L)	11.75y	Leigh syndrome	
18 (F)	PDHc m: 68%; f: 82%) PDHC-E1 m: 64%	*PDHA1* del926 AAGTAAG	1.6y	Leigh syndrome	
19 (F)	PDHc m: 89%; f: 85%) PDHC-E1 m: 29%	*PDHA1* ins926 AAGTAAG	1.2y	Leigh syndrome	
20 (F)	PDHc m: 24%; f: 36%)	*PDHA1* 430G>A (G144S)	6m	Psychomotor retardation; hypotonia	
21 (F)	PDHc m: 51%; f: normal) PDHc-E1 m: 31%	*PDHA1* dupl859-862 (TACC)	1.1y	Psychomotor retardation; convulsions; lissencephaly	
22 (F)	PDHc m: 80%; f: normal) PDHc-E1 m: 46%	*PDHA1* 1133G>A (R378H)	14.2y	Leigh syndrome	
23 (F)	PDHc m: 116%; f: 98%) PDHc-E1 m: 81%	*PDHA1* 924G>T (Q308H)	1.3m	Psychomotor retardation; convulsions; hypotonia; spasticity; microcephaly	
24 (M)	Complex V (m: 77%)	*MT-ATP6* T8993G (m: >95%)	4y	Leigh syndrome	
25 (F)	Complex V (m: 50%)	*MT-ATP6* T8993G (m: 90%)	10m	Leigh syndrome	
26 (F)	Complex V (m: 49%)	*MT-ATP6* T8993C(m: >95%)	9.7y	Leigh syndrome	
27 (M)	ANT	Genetic defect unknown	1y	Sengers-like syndrome	(32)
28 (F)	ANT	Genetic defect unknown	1.7m	Sengers-like syndrome	(32)
29 (M)	ANT	Genetic defect unknown	5.2y	Exercise intolerance; motor retardation	(33)

Table 3.

[a] Patients 1-5 and 17 carried a homozygous mutation. Patients 6, 7, and 16 carried a compound heterozygous mutation (F, Female; M, male).

[b] Measured enzymatic activities in muscle tissue (m) and cultured fibroblasts (f) are expressed as a percentage of the lowest control value.

[c] Affected genes and the mutation. In the case of a mtDNA mutation, the percentage heteroplasmy in muscle tissue (m), fibroblasts (f), and blood (bl), is given. Human genes: *NDUFS2,* NADH dehydrogenase (ubiquinone) Fe-S protein 2, 49kDa (NADH-coenzyme Q reductase); *NDUFS4,* NADH dehydrogenase (ubiquinone) Fe-S protein 4, 18kDa (NADH-coenzyme Q reductase); *NDUFS7,* NADH dehydrogenase (ubiquinone) Fe-S protein 7, 20kDa (NADH-coenzyme Q reductase); *NDUFS8,* NADH dehydrogenase (ubiquinone) Fe-S protein 8, 23kDa (NADH-coenzyme Q reductase); *NDUFV1,* NADH dehydrogenase (ubiquinone) flavoprotein 1, 51kDa; *MT-ND2,* mitochondrially encoded NADH dehydrogenase 2; *MT-ND4L,* mitochondrially encoded NADH dehydrogenase 4L; *MT-ND5,* mitochondrially encoded NADH dehydrogenase 5; MELAS, mitochondrial encephalomyopathy, lactic acidosis, and stroke like episodes; *MT-CYB,* mitochondrially encoded cytochrome *b*; *SURF1,* surfeit 1; *PDHA1,* pyruvate dehydrogenase (lipoamide) alpha 1; *MT-ATP6,* mitochondrially encoded ATP synthase 6.

[d] Age of each patient at the time of the muscle biopsy. If the patient died as a result of the mitochondrial disease, the age at the time of death is given in parentheses.

[e] C.H., compound heterozygous; MELAS, mitochondrial encephalomyopathy, lactic acidosis, and stroke like episodes; PDHc-E1, enzymatic activity of the E1 subunit of PDHc.

DISCUSSION

Any disturbance of the MEGS, apart from complex II deficiency, will lead to a lower pyruvate oxidation rate and ATP production. We measured pyruvate oxidation in the presence of or carnitine, which was added to remove acetyl-CoA (Fig 1) to prevent inhibition of PDHc by accumulation of its product. PDHc is regulated by the ATP/ADP, $NADH/NAD^+$, and acetyl-CoA/CoA ratios (2). Therefore, a defect in the TCA cycle or RC will lead to a decreased oxidation rate for [1-^{14}C] pyruvate + malate as a result of an increase in the acetyl-CoA/CoA or $NADH/NAD^+$ ratio, repectively. A disturbance in complex V or ANT leads to a decreased oxidation rate for [1-^{14}C] pyruvate + malate as the result of an increase in the $NADH/NAD^+$ or ATP/ADP ratio, respectively. In incubation 2, in which acetyl-CoA is removed by carnitine acetyltransferase, 1 mole of pyruvate oxidized yields one mole NADH, whereas incubation 1 yields 4 moles of NADH and one mole of $FADH_2$ (Fig. 1). In case of an OXPHOS or ANT deficiency, the oxidation rate in incubation 2 will be less decreased than in incubation 1 , giving an increased ratio of

incubation 2 to incubation 1. A PDHc deficiency will lead to equally diminished oxidations rates in incubation 1 and 2, and therefore the ratio of incubation 2 to incubation 1 remains ~1. Incubation 3 is performed to determine the ADP stimulation factor (ratio of incubation 1 to incubation3), a measure for the coupling state of oxidation and phosphorylation in mitochondria. In incubation 4, addition of CCCP makes the pyruvate oxidation independent from ADP, complex V and ANT. A deficiency in complex V or ANT will lead to a higher oxidation rate in incubation 4 than in incubation 1 and therefore an increased incubation 4/incubation 1 ratio. We found normal PDHc activities in 52 patients with normal oxidation rates in incubation 2 and 99 patients with normal oxidation rates in incubation 4, all showing decreased oxidation rates in incubation 1 (data not shown), which indicates that a normal oxidation rate in incubation 2 or 4, combined with a decreased oxidation rate in incubation 1, excludes a PDHc deficiency. Incubation 5 measures the pyruvate oxidation rate in the presence of atractyloside. In the absence of exogenous ADP (incubation 3), the oxidation of pyruvate is dependent on endogenous ADP inside the mitochondrion. Because ATP cannot be transported through the mitochondrial membrane , atractyloside will further inhibit this residual substrate oxidation, giving a high ATP/ADP ratio and feedback inhibition of PDHc. In case of an ANT deficiency, addition of atractyloside will have little or no effect on this oxidation rate. A decreased oxidation rate in incubation 1 and equal oxidation rates in incubations 3 and 5, which gives a decreased incubation3/incubation 5 ratio, are indicative of an ANT deficiency.

Oxidation of malate and succinate strongly depends on an acetyl-CoA donor. Without pyruvate, the oxidation rate of [U-^{14}C] malate is only 6% of that with pyruvate (Table 2). We measured malate and succinate oxidation rates in the presence of pyruvate or acetylcarnitine (Fig. 2). Incubation 6 measures the TCA cycle activity except for SDH and fumarase. Disturbances in PDHc, TCA cycle enzymes (except SDH and fumarase), OXPHOS and ANT will produce a decreased oxidation rate in incubation 6. In theory, during oxidation of 1 mole pyruvate , one mole of malate is oxidized, yielding twice as much $^{14}CO_2$ in incubation 6 as incubation 1.

Table 4. **Substrate oxidation rates, ATP production rate, and incubation ratios in patients with a RC deficiency, PDHc deficiency, Complex V deficiency, and ANT deficiency.[a]**

	Patient	inc 1	inc 2	inc 3	inc 4	inc 5	inc 6	inc 7	inc 8	inc 9	ATPprod[c]
Respiratory chain deficiencies[b]											
1	Complex I *(NDUFS2)*	15,7	32,3	26,3	13,9	28,0	11,1	30,3	31,2	23,9	21,2
2	Complex I *(NDUFS4)*	6,8	12,8	26,3	3,7	24,0	6,6	13,0	21,7	11,8	9,1
3	Complex I *(NDUFS7)*	19,4	28,7	40,4	19,9	72,0	15,5	26,7	27,1	21,3	20,8
4	Complex I *(NDUFS7)*	61,7	94,4	90,9	100,5	92,0	55,3	86,0	95,0	81,2	37,4
5	Complex I *(NDUFS7)*	18,5	37,4	31,3	26,4	16,2	19,7	39,4	52,5	29,6	29,4
6	Complex I *(NDUFS8)*	11,7	23,0	51,5	9,1	52,20	9,3	17,0	38,0	12,1	11,4
7	Complex I *(NDUFV1)*	15,9	24,3	49,5	20,0	nd[d]	13,7	nd	nd	11,5	14,6
8	Complex I *(MT-ND2)*	35,3	72,2	94,9	51,5	110,0	30,0	74,3	nd	nd	34,0
9	Complex I *(MT-ND4L)*	69,4	90,5	66,7	80,5	66,0	77,3	101,5	92,8	85,7	77,0
10	Complex I *(MT-ND5)*	27,3	58,1	61,6	33.9	62,0	22,3	49,4	65,6	43,3	28,3
11	Complex I *(MELAS A3243G)*	5,1	8,4	22,2	6,5	36,0	7,4	14,2	40,7	27,4	13,9
12	Complex I *(MELAS A3243G)*	15,4	23,3	57,6	16,7	132,0	12,5	27,7	46,2	26,4	17,3
13	Complex II	68,0	78,5	62,6	71,5	44,0	73,0	91,1	116,7	68,5	81,3
14	Complex II	27,6	36,9	46,5	28,1	22,0	24,5	37,9	41,2	8,9	19,4
15	Complex III *(MT-CYB)*	14,7	4,6	44,4	26,4	40,0	13,6	30,8	48,0	14,0	nd
16	Complex IV *(SURF1)*	55,2	69,3	54,5	68,4	56,0	49,8	69,0	68,3	61,1	52,0
	(PDHc deficiencies)										
17	PDHc *(PDHA1)*	22,6	21,2	69,7	15,5	38,0	21,2	83,0	92,8	89,2	23,3
18	PDHc *(PDHA1)*	14,7	115,3	23,2	14,2	14,0	nd	nd	nd	nd	14,8
19	PDHc *(PDHA1)*	21,5	21,8	30,3	17,8	18,0	19,7	43,3	58,8	26,8	16,5
20	PDHc *(PDHA1)*	11,9	14,9	18,2	13,4	8,0	16,1	34,9	44,3	32,8	10,5
21	PDHc *(PDHA1)*	14,2	14,3	38,4	12,7	50,0	16,1	54,2	70,6	44,6	25,4
22	PDHc *(PDHA1)*	39,0	39,1	62,6	39,4	70,0	42,2	102,5	104,1	93,0	42,
23	PDHc *(PDHA1)*	26,7	25,6	55,6	41,1	34,0	26,7	50,9	51,1	40,1	21,4
	(Complex V deficiencies)										
24	Complex V *(MT-ATP6)*	14,2	nd	13,1	17,4	nd	25,8	33,8	34,8	30,9	20,1
25	Complex V *(MT-ATP6)*	18,2	30,9	34,3	27,6	50,0	nd	nd	nd	nd	13,2
26	Complex V *(MT-ATP6)*	57,3	87,7	100,0	89,8	108,0	49,0	84,7	71,0	75,5	51,6
	(ANT-deficiencies)										
27		10,3	11,7	12,1	16,5	14,0	111,8	18,3	21,3	15,3	8,4
28		3,3	2,8	3,0	4,6	6,0	nd	nd	nd	nd	3,4
29		10,7	21,5	23,2	62,4	44,0	8,2	19,3	36,7	7,0	5,9
	Control range	60 - 140	69 - 137	45 – 233	66 - 147	46 - 220	52 - 139	50 - 133	50 - 137	65 - 133	64 - 145

Patient		ATP/Pyr ratio	ADP-stim[c]	Ratio 2/1	Ratio 4/1	Ratio 3/5	Ratio 6/1	Ratio 7/6	Ratio 7/8
	Respiratory chain deficiencies[b]								
1	Complex I *(NDUFS2)*	13,2	3,4	2,2	0,9	1,9	0,8	1,7	1,7
2	Complex I *(NDUFS4)*	13,0	1,5	2,0	0,5	2,2	1,1	11,2	1,1
3	Complex I *(NDUFS7)*	10,5	2,8	1,6	1,0	1,1	0,9	1,1	1,8
4	Complex I *(NDUFS7)*	5,9	3,9	1,6	1,6	2,0	1,0	1,0	1,6
5	Complex I *(NDUFS7)*	15,7	3,4	2,2	1,4	3,9	1,2	1,2	1,3
6	Complex I *(NDUFS8)*	9,5	1,3	2,1	0,8	2,0	0,9	1,1	0,8
7	Complex 1 *(NDUFV1)*	9,00	1,9	1,6	1,3	nd	1,0	nd	nd
8	Complex 1 *(MT-ND2)*	9,5	2,2	2,2	1,5	1,7	0,9	1,5	nd
9	Complex I *(MT-ND4L)*	10,9	6,	1,4	1,2	2,0	1,2	0,8	1,9
10	Complex 1 *(MT-ND5)*	10,2	2,6	2,3	1,2	2,0	0,9	1,4	1,3
11	Complex 1 *(MELAS A3243G)*	27,0	1,3	1,8	1,3	1,2	1,6	1,2	0,6
12	Complex I *(MELAS A3243G)*	11,1	1,5	11,6	1,1	0,9	0,9	1,4	1,1
13	Complex II	11,7	6,3	1,2	1,0	2,8	1,2	0,8	1,14
14	Complex II	6,9	3,5	1,4	1,0	4,2	1,0	1,0	1,6
15	Complex III *(MT-CYB)*	nd	1,9	1,8	1,8	2,2	1,0	1,4	1,1
16	Complex IV *(SURF1)*	9,2	5,9	1,3	1,2	1,9	1,0	0,9	1,8
	(PDHc deficiencies)								
17	PDHc *(PDHA1)*	10,2	1,9	1,0	0,7	3,6	1,0	2,4	1,6
18	PDHc *(PDHA1)*	9,9	3,6	1,1	1,0	3,3	nd	nd	nd
19	PDHc *(PDHA1)*	7,5	4,2	1,1	0,8	3,3	1,0	1,4	1,3
20	PDHc *(PDHA1)*	8,7	3,8	1,3	1,1	4,5	1,5	1,3	1,4
21	PDHc *(PDHA1)*	17,6	2,1	1,1	0,9	1,5	1,3	2,1	1,4
22	PDHc *(PDHA1)*	10,6	3,6	1,1	1,0	1,8	1,2	1,5	1,8
23	PDHc *(PDHA1)*	7,8	2,8	1,0	1,5	3,2	1,1	1,2	1,8
	(Complex V deficiencies)								
24	Complex V *(MT-ATP6)*	13,9	6,4	nd	1,2	nd	2,0	0,8	1,7
25	Complex V *(MT-ATP6)*	7,0	3,1	1,8	1,5	1,4	nd	nd	nd
26	Complex V *(MT-ATP6)*	8,8	3,3	1,6	1,6	1,8	0,9	1,1	2,1
	(ANT-deficiencies)								
27		7,9	5,1	1,2	1,6	1,7	1,3	1,0	1,5
28		10,2	5,5	0,9	1,4	1,0	nd	nd	nd
29		5,4	2,7	2,1	5,8	1,0	0,9	1,5	0,9
	Control range	6,6 – 12,1	3,5 – 8,5	0,9 – 1,5	0,7 – 1,3	1,3 – 3,2	0,9 – 1,3	0,4 – 1,0	1,5 – 2,1

Table 4.

[a]Incubations were performed as described in the *Materials and Methods* section. Incubations are numbered as in Table 1. The activities and ratios, including the respective control values, are expressed as percentage of the mean control values given in Table 1. The patient numbering is as in Table 3.

[b] Terms in parentheses indicate gene defects. Human genes: *NDUFS2,* NADH dehydrogenase (ubiquinone) Fe-S protein 2, 49kDa (NADH-coenzyme Q reductase); *NDUFS4,* NADH dehydrogenase (ubiquinone) Fe-S protein 4, 18kDa (NADH-coenzyme Q reductase); *NDUFS7,* NADH dehydrogenase (ubiquinone) Fe-S protein 7, 20kDa (NADH-coenzyme Q reductase); *NDUFS8,* NADH dehydrogenase (ubiquinone) Fe-S protein 8, 23kDa (NADH-coenzyme Q reductase); *NDUFV1,* NADH dehydrogenase (ubiquinone) flavoprotein 1, 51kDa; *MT-ND2,* mitochondrially encoded NADH dehydrogenase 2; *MT-ND4L,* mitochondrially encoded NADH dehydrogenase 4L; *MT-ND5,* mitochondrially encoded NADH dehydrogenase 5; MELAS, mitochondrial encephalomyopathy, lactic acidosis, and stroke like episodes; *MT-CYB,* mitochondrially encoded cytochrome *b*; *SURF1,* surfeit 1; *PDHA1,* pyruvate dehydrogenase (lipoamide) alpha 1; *MT-ATP6,* mitochondrially encoded ATP synthase 6.

[c] ATP production rate from oxidation of pyruvate + malate.

[d] ND, not determined; MELAS, mitochondrial encephalomyopathy, lactic acidosis, and stroke like episodes.

[e] Stimulation of the oxidation rate of pyruvate + malate by ADP (incubation 1/incubation 3 ratio).

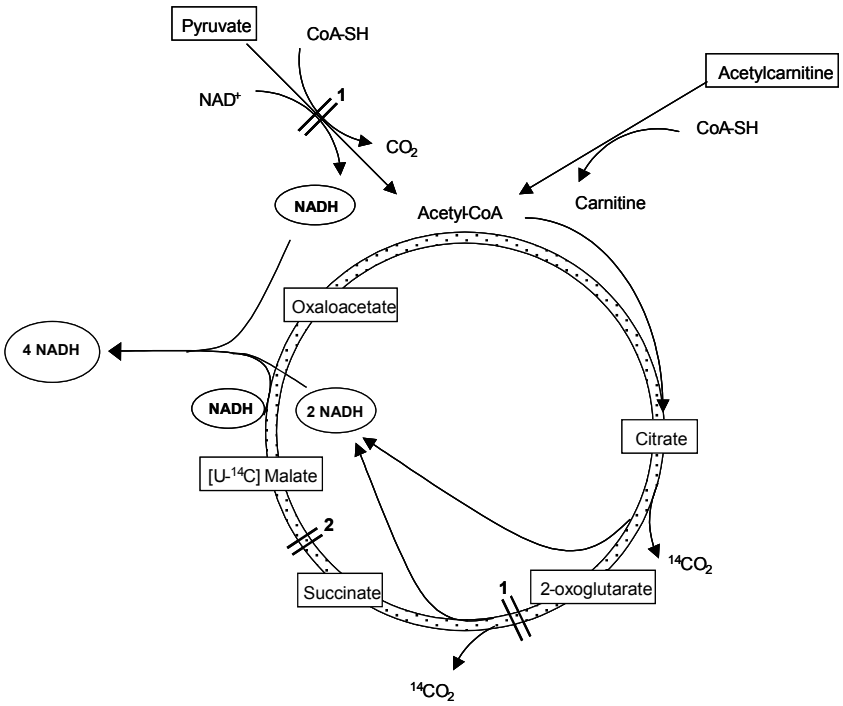

Fig 2.. Schematic representation of the oxidation of [U-^{14}C] malate in the presence of either pyruvate or acetylcarnitine as acetyl-CoA donor.

Inhibition sites of arsenite (///1) on PDHc and 2-ODHc and malonate (///2) on SDH are indicated. CoA-SH, coenzyme A.

However, the ratio between incubations 6 and 1 is lower (Table 1) because of partial transport of 2-oxoglutarate out of the mitochondrion (A. Janssen, unpublished observations), thereby decreasing the amount of $^{14}CO_2$ formed. Malonate, added to incubations 6 and 7, prevents $FADH_2$ production by SDH, producing, in theory, 13 instead of 15 moles of ATP from oxidation of 1 mole of pyruvate. We found that addition of malonate indeed lowered the amount of ATP produced to 87% of the amount produced in the absence of malonate (Table 2), indicating that malonate specifically inhibits SDH in our incubations. Incubation 7 yielded 2 moles $^{14}CO_2$ from oxidation of 1 mole of [U-^{14}C] malate. Incubation 8 yielded only 1 mole of $^{14}CO_2$ because 2-oxoglutarate dehydrogenase complex (2-ODHc) is inhibited by arsenite,giving an incubation 7/incubation 8 ratio of ~2 (Table 1). , In case of a 2-ODHc deficiency, this ratio will be close to 1, because the reaction will not proceed beyond the formation of 2-oxoglutarate (Fig. 1). Incubations 7 and 8 are performed in the presence of acetylcarnitine, as acetyl-CoA donor, and are therefore independent on PDHc. By contrast, incubation 6 is dependent on PDHc. Therefore, a PDHc deficiency will lead to an increased ratio of incubation 7 to incubation 6. Because [U-^{14}C] malate oxidation rates are independent of SDH and fumarase (Fig. 1), we included incubation 9, in which $^{14}CO_2$ production from oxidation of succinate is measured. A decreased oxidation rate of [1,4-^{14}C] succinate combined with moderately decreased or normal oxidation rates of [U-^{14}C] malate is indicative of an SDH or fumarase deficiency.

The ATP production and ATP / pyruvate ratio are a measure of the efficiency of the total MEGS. In theory, the maximum value for the ATP / pyruvate is 15. In practice, we obtained a mean value of 9.8 (Table 1), which is most likely attributable to export of 2-oxoglutarate and succinate from the mitochondria.

The majority of complex I deficient patients had decreased substrate oxidation rates and ATP production. The ratio of incubation 2 to incubation 1 was increased in 11 of 12 patients. In patients 4, 5, and 8, the ratio of incubation 4 to incubation 1 was increased, suggesting that CCCP inhibits the proton-motive activity of complex I in these patients. In 9 of 11 complex I deficient patients the ratio incubation 7 to incubation 6 was increased which is indicative of a decreased PDHc activity. We measured PDHc activity in 7 of these patients and found that it was decreased in 5 patients [patients 1 (80% of the lowest control value), 2 (83%), 5 (74%), 6 (80%), and 12 (68%)]. In 6 of 10 patients, the ratio of incubation 7 to incubation 8 was decreased, indicative of decreased 2-ODHc activity. We found decreased 2-ODHc activity in 2 of these patients: patient 2 (49% of the lowest

control value) and patient 11 (47% of the lowest control value). Complex I deficiency probably leads to a high NADH/NAD^+ ratio, inhibiting PDHc and 2-ODHc (2).

Complex I deficient patients 3, 4, and 5, who carry the same mutation, showed variability in clinical phenotype correlating with the biochemical phenotype (Table 3), suggesting that additional factors influence the phenotype of these patients. Patient 9 showed normal substrate oxidation rates and ATP production, but diminished complex I activity (Table 3). The mtDNA A10750G mutation in the *ND4L* gene, established in this patient, has been described as a polymorphism (21). Our data seem to support this, because the MEGS capacity is not affected, although the diminished complex I activity in muscle tissue still needs to be elucidated.

This study includes two genetically uncharacterized complex II-deficient patients with dissimilar biochemical characteristics. Patient 13 had a complex II deficiency in muscle tissue as well as in cultured fibroblasts. Succinate cytochrome *c* oxidoreductase (complex II + III) (SCC) activity was decreased in muscle tissue (60% of the lowest control value) and borderline in cultured fibroblasts (98% of the lowest control value). She had normal ATP production, borderline oxidation rate in incubation 1, and normal oxidation rates for all other substrates. This is compatible with the observation that blocking of complex II with malonate had little or no effect on the oxidation rate of malate + pyruvate and ATP production (Table 2). We have no explanation for the normal oxidation rate of incubation 9. Patient 14 had a complex II deficiency in muscle tissue and cultured fibroblasts. SCC activity in muscle tissue and cultured fibroblasts was decreased (13% and 46% of the lowest control value, respectively). The oxidation rates for incubations 1, 2, 6, 7 and 8, and ATP production ranged from 19 to 41% of the mean control value, whereas the oxidation rate of incubation 9 was strongly decreased (9% of the mean control value).

Patient 15, with a complex III deficiency attributable to a mutation in the *MT-CYB* gene, had decreased substrate oxidation rates and disturbed incubation2 / incubation 1, incubation7 / incubation 6, and incubation7 / incubation 8 ratios.. The mutation has been described by Valnot et al. (22). The increased ratio incubation 4 to incubation 1 in patient 15 indicates that CCCP inhibits the proton-motive activity of complex III in this patient, similar to complex I in patients 4, 5, and 8, who have complex I deficiency. Patient 16, with complex IV deficiency attributable to a *SURF-1* mutation, displayed a relatively mild biochemical phenotype with oxidation rates and ATP production around 60% of the mean control value and normal incubation 2 / incubation 1, incubation 7 / incubation 6, and incubation 7 / incubation 8 ratios.

Seven patients with a PDHc deficiency (*PDHA1* gene mutation) were studied. The gene encoding the PDHc E1α subunit is located on the X chromosome. Although the affected females included in this study are heterozygous, they showed features of a PDHc deficiency attributable to X-linked inactivation (9). In all patients, the oxidation rates of incubations 1, 2 and 4 and the ATP production were decreased, with a normal incubation 2 / incubation 1 ratio. Six patients had decreased oxidation rates in incubation 6 and an increased incubation 7 / incubation 6 ratio. Therefore, decreased oxidation rates in all pyruvate-containing incubations, with a normal incubation 2 / incubation 1 ratio and an increased incubation 7 / incubation 6 ratio are indicative of a PDHc deficiency.

Three patients with complex V deficiency (Leigh/NARP T8993G/C mutation in the *MT-ATP6* gene) showed a decreased oxidation rate in incubation 1, decreased ATP production, decreased oxidation rate in incubation 6 in patients 24 and 26, and an increased incubation 4 / incubation 1 ratio in patients 25 and 26. The decreased oxidation rates are in agreement with observations in fibroblasts and platelets from patients carrying a T8993G/C mutation (23, 24). In 2 patients, the ratio of incubation 2 to incubation 1 was increased, indicating diminished RC activity. Three patients with ANT deficiency had decreased oxidation rates in incubation1 and 2 and ATP production, with an increased incubation 4 / incubation 1 ratio. In 2 patients, the oxidation rates in incubation 6 and 7 were decreased. In patients 28 and 29, the ratio of incubation 3 to incubation 5 was decreased. This indicates that the combination of decreased oxidation rates and decreased ATP production, with an increased incubation 4 / incubation 1 ratio and a decreased incubation3 / incubation 5 ratio, are indicative of an ANT deficiency.

A muscle biopsy is an invasive, uncomfortable procedure; it is therefore important to obtain maximal information from the biochemical examinations. A fresh muscle biopsy allows for measurement of both the MEGS capacity and individual enzyme activities. In each patient, 10 oxidation rates are measured and 8 ratios are calculated. From a statistical point of view, one of these values could be outside the 95% confidence interval. One deviating value, not fitting in the total biochemical picture, is not considered proof of mitochondrial dysfunction. Substrate oxidation rates and ATP production are always evaluated in the context of the total biochemical picture, whereas ratios are used as an additional diagnostic tool (25). We calculated 216 ratios in 29 patients. Six did not fit in our theory (ATP / pyruvate ratio in patients 5, 11, and 21; ratio of incubation 3 to incubation 5 in patients3, 11, and 12). Although ratios are derived values and should not be

regarded as diagnostic in their own right, this illustrates that the results agree with our theory about the substrate oxidations. We have observed decreased oxidation rates for one or more substrates and decreased ATP production rates in ~50% of the fresh muscle biopsies examined in our laboratory. In ~30% of these biopsies, the measured activities for all OXPHOS enzymes and PDHc have been normal. In a subset of these patients, the primary defect is probably caused by disturbances in uncharacterized proteins directly involved in the MEGS. The mitochondrial carrier proteins are among the likely candidates. Recently, the first patient with a pyruvate carrier deficiency was described (26). A small subset of patients in which we found secondary MEGS dysfunction are those with non-MEGS diagnoses, including spinal muscle atrophy, Duchenne muscular dystrophy, and Rett, Cockayne, and Joubert syndromes (27). This illustrates that decreased substrate oxidation rates and ATP production rate are not enough to diagnose primary MEGS dysfunction and that a definite diagnosis can be achieved only by combining biochemical, clinical, metabolic, and morphologic data (25). The method has been used in our center for many years and has demonstrated its merit in the diagnosis of patients suffering from a mitochondriopathy.

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CHAPTER 4

Muscle 3243A→G mutation load and capacity of the mitochondrial energy generating system

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Abstract

Objective: The mitochondrial energy generating system (MEGS) encompasses the entire chain of mitochondrial enzymatic reactions from oxidation of pyruvate to the export of ATP from the mitochondrion. It is investigated in intact muscle mitochondria by measuring the pyruvate oxidation and ATP production rates in the presence of malate or carnitine and ADP, which we refer to as the "MEGS-capacity". Presently little is known about MEGS pathology in patients with mutations in the mitochondrial DNA (mtDNA). As MEGS-capacity is an indicator for the overall mitochondrial function, we wanted to search for a correlation between MEGS-capacity and 3243A→G mutation load in muscle of patients with the MELAS syndrome.

Methods: In muscle tissue of 24 patients with the 3243A→G mutation and 43 normal controls we investigated the MEGS-capacity, the respiratory chain enzyme activities and the 3243A→G mutation load. To exclude coinciding mutations, all 22 mitochondrial tRNA genes (m.tRNAs) were sequenced in muscle tissue of the patients, if possible.

Results: We found highly significant differences between patients and healthy controls with respect to the MEGS-capacity and complex I, III, and IV activities. MEGS related measurements correlated considerably better with the mutation load than respiratory chain enzyme activities, especially in the subgroup with low mutation load. We did not find any additional mutations in the m.tRNAs of the patients.

Interpretation: The results show that MEGS-capacity, as a parameter for mitochondrial function, has a higher sensitivity than respiratory chain enzyme activities for detection of subtle mitochondrial dysfunction. This is important in the workup of patients with suspected mitochondriopathy, especially in cases with rare or new mtDNA mutations, and in patients with low mutation loads. Especially in these cases we suggest to determine the MEGS-capacity.

Introduction

The acronym MELAS was introduced in 1984 for a group of patients with mitochondrial myopathy, encephalopathy, lactic acidosis and stroke-like episodes.[1] In 1987, two MELAS patients were described with complex I deficiency.[2] In 1990, the 3243A→G mutation was detected as the molecular basis of the disease.[3,4] This mutation is found in more than 80% of the MELAS patients, but additional mutations were reported in m.tRNAs and mtDNA encoded subunits of complex I.[5] The phenotypic variability of the MELAS 3243A→G genotype is large and comprises hypertrophic cardiomyopathy,[6] diabetes mellitus with hearing loss,[7] intestinal pseudo-obstruction with MNGIE (mitochondrial neurogastrointestinal encephalopathy syndrome) phenotype,[8] MERRF (myoclonic epilepsy with ragged-red fibers) phenotype,[9] autistic spectrum disorders,[10] age-related maculopathy,[11] Nail-Patella syndrome,[12] bipolar disorder and schizophrenia,[13] Kearns-Sayre syndrome,[14] renal cell carcinoma,[15] and nephrotic syndrome with focal segmental glomerulosclerosis.[16]

In patients referred to our center, the 3243A→G transition is the most frequently encountered mtDNA mutation. We found this mutation in 57 (4.9%) of 1162 patients with suspected mitochondrial disease. A similar result was described in a study of 2000 patients with suspected mitochondrial disease in the United States, 49 of them carrying the 3243A→G mutation (2.5%).[17]

The mutation is located in the dihydrouridine loop of the m.tRNA$^{Leu(UUR)}$. The change of its tertiary structure renders it less reactive with its leucyl-tRNA synthetase and leads to a general reduction of mitochondrial protein synthesis.[18,19]

The m.tRNA$^{Leu(UUR)}$ catalyzes the incorporation of leucine into mtDNA encoded proteins to the following percentages (in brackets): mtND6 (89%), mtND3 (36%), mtATP8 (20%), mtCOX2 (15%), mtND2 (14%), mtCyb (14%), mtATP6 (11%), mtCOX1 (11%), mtND1 (9.5%), mtND4 (9.3%), mtCOX3 (8.8%), mtND5 (8.7%), and mtND4L (4.3%).[5] The m.tRNA$^{Leu(CUN)}$ catalyzes the incorporation of the remaining leucines.[5] In muscle tissue of patients with the 3243A→G mutation, decreased activities of respiratory chain enzyme complexes I, III and IV were measured and mutation load correlated with the cytochrome *b* and cytochrome *aa3* content in muscle.[20] Investigation of OXPHOS complexes in muscle specimens of patients with the 3243A→G mutation by blue native 2D-electrophoresis, showed almost complete absence of complex I, partial reduction of complex III and IV, and normal amounts of complexes II and V.[21] One patient was described with double mutations in the m.tRNAs.[22] Data on the correlation between 3243A→G mutation load and reduction of

mitochondrial function are conflicting. In one study of nine patients with the 3243A→G mutation no correlation was found between the mutation load and the *in vivo* function of muscle mitochondria.[23] A correlation was found *in vitro* using transmitochondrial cybrids with varying degrees of mutation load for the 3243A→G mutation.[24,25]

For the present study, we performed a set of functional measurements that had been shown to discriminate well between patients with mitochondrial enzyme deficiencies and controls.[26] To establish a genotype-(biochemical) phenotype relation, we measured pyruvate oxidation rates, ATP production rates as well a respiratory chain complex I, III and IV activities in muscle mitochondria from patients with the 3243A→G mutation along with a detailed analysis of the mtDNA (determination of the 3243A→G mutation load and sequence analysis of all m.tRNAs). The results were compared to the results measured in 43 controls that have been described previously.[26]

Materials and methods

Patients and controls

After informed consent of the patients or their caretakers, fresh *quadriceps* muscle biopsy specimens (n=24; 14 female, 10 male; age at the time of biopsy 0.5-57 years) were obtained for diagnostic purposes through open surgery or needle biopsy as described.[26] All patients and their physicians were asked to complete a patient questionnaire to detail the clinical phenotype.

A detailed description of the 43 individuals used as control group (age at the time of biopsy 2.2-58 years) has been published.[26] We had excluded the presence of the 3243A→G mutation in the muscle tissue of all individuals from the control group.

Biochemical methods

We studied MEGS-capacity by measuring oxidation rates of [1-^{14}C]pyruvate+malate in the presence and absence of ADP, [1-^{14}C]pyruvate+carnitine, and the ATP production rate from oxidation of pyruvate+malate in fully coupled muscle mitochondria as previously described.[26] A detailed description of the theory of these incubations has been published[26]. Complex I,

complex III, complex IV and citrate synthase (CS) activities were measured as described before.[27-30]

Molecular genetic methods

In 22 patients, we sequenced all the m.tRNAs. From patients #22 and #24 no appropriate muscle tissue was left for extraction of DNA. Additionally, we determined the m.12S rRNA and m.16S rRNA sequences of mtDNA from muscle in patients #5 and #9. A detailed description of the methods and oligonucleotides used for sequence analysis of m.tRNAs and the m.rRNA genes has been published.[31,32]

For quantification of the 3243A→G mutation we amplified the *MT-TL1* gene (m.tRNA$^{Leu(UUR)}$) from muscle mtDNA with a three primer protocol as described earlier[31], with the forward primer: 5'-ACT TCA CAA AGC GCC TTC CC-3', an M13(-21)-tailed reverse primer: 5'-TGT AAA ACG ACG GCC AGT GCA TTA GGA ATG CCA TTG CG-3' and the FAM-labeled M13(-21)-primer: FAM-TGT AAA ACG ACG GCC AGT-3'. In the presence of the mutation, the endonuclease *Apa* I cuts the ensuing 237 bp FAM-labeled PCR product into two fragments of 95 and 142 bp size. The fragments were separated on a GeneScanner (ABI PRISM® 3100) after adding ROX500 (Applied Biosystems) as an internal standard. The percentages of the 142 and 237 bp fragments were calculated through integration of the signal intensity with the GeneMarker Software (SoftGenetics, State College, PA). Each of the quantifications was performed in triplicate. Quantification of the 3243A→G mutation in muscle tissue of patient #22 was performed by restriction fragment length polymorphism.
In patient #9 additionally all the mtDNA encoded ND genes were sequenced as described,[33] because in this patient particularly complex I activity was severely decreased.

Statistical analysis

Measurements of MEGS parameters and respiratory chain enzyme activities in the patients were considered to be significantly decreased if they were two standard deviations below the mean of the controls.

We used the non-parametric Mann-Whitney U Test to determine the significance of the differences in biochemical data between controls and the entire patient group.

Further on, we subdivided the patient group into three subsets with increasing mutation loads (12-50%, 51-80% and 81-100%). For each subset we determined the significance of the differences between patients and controls for MEGS parameters and for respiratory chain enzyme activities. Differences were considered to be statistically significant if $p<0.01$.
The non-parametric Spearman rank correlation test was used to calculate the correlations of all the biochemical parameters versus the 3243A→G mutation load, and to calculate the correlation of all the respiratory chain enzyme activities versus the parameters representing MEGS-capacity. Correlations were considered to be relevant if $|Rho|>0.7$.
The relationship between the various biochemical parameters was evaluated by means of quadratic regression analysis using the model ($y=ax^2+xb+c$). Regression was considered to be relevant if $R^2>0.7$ (Figure 2)

We calculated the *sensitivity* and the *specificity* of MEGS capacity related measurements and of classic respiratory chain enzyme determinations for the ability to detect a mitochondrial disease from of patients with proven mitochondriopathies (total n=53; MELAS n=24 respiratory chain enzyme deficiency n=16; pyruvate dehydrogenase deficiency n=7; NARP/Leigh syndrome n=3; adenine nucleotide translocator n=3) and of normal controls (n=43) and their respective biochemical measurements are detailed in Table 1 or published previously.[26]

Results

Clinical phenotypes of the patients

The clinical phenotypes of our patients were heterogeneous. A detailed description is presented in Table 2. Patients #7 and #8 were siblings. Patients #1, #4 and #21 were members of the same family (a mother and her two daughters, respectively). Eight of 24 patients suffered from the "classic" MELAS symptoms: mitochondrial myopathy, encephalopathy, lactic acidosis and stroke-like episodes. Most frequent findings were lactic acidosis (20/23), ragged-red-fibers (15/24), exercise intolerance (14/24), seizures (14/24) and hearing impairment (12/24). The clinical picture of patients #1, #3, #6 and #16 did not hint towards a 3243A→G mutation at first sight. Patient #9 was remarkable because of the very young age of onset of the clinical symptoms.[34]

Table 1

Patient	m.3243A→G %	1: Pyruvate+malate	2: Pyruvate+carnitine	Ratio 2/1	3: Pyruvate+malate-ADP	4: ATP-production	ATP/Pyruvate	ADP-stimulation	Complex 1	Complex III	Complex IV
1	12	3,13	4,41	1,41	0,79	33,1	10,6	3,9	119	2825	2051
2	27	3,74	3,50	0,94	0,46	41,0	11,0	8,1	85	2669	1587
3	34	4,75	5,15	1,08	0,65	43,6	9,2	7,3	119	2375	1419
4	34	3,41	4,02	1,18	0,57	33,4	9,8	6.0	90	2014	1185
5	40	1,89	2,50	1,32	0,53	15,3	8,1	3,6	31	1896	1168
6	41	4,19	5,67	1,35	0,28	51,9	12,4	14,7	53	3905	1530
7	45	3,68	3,81	1,04	1,04	26,3	7,1	3,5	47	2075	1300
8	46	3,07	3,50	1,14	0,57	23,9	7,8	5,4	48	1634	1589
9	60	0,74	1,33	1,80	0,55	3,6	4,8	1,4	12	2000	606
10	75	4,75	5,73	1,21	0,73	44,7	9,4	6,5	69	2906	860
11	76	3,37	4,65	1,38	0,73	31,3	9,3	4,6	47	2527	1181
12	76	3,81	4,04	1,06	1,21	39,0	10,3	3,2	120	nd	1401
13	77	3,76	3,59	0,95	0,83	31,1	8,3	4,6	88	2731	1767
14	78	nd	1,11	nd	0,74	13,6	nd	nd	48	2981	1653
15	80	1,92	3,24	1,69	0,68	21,8	11,4	2,8	28	2760	1095
16	81	2,49	3,37	1,35	0,62	19,8	8.0	4.0	42	1798	1282
17	82	1,17	2,01	1,72	0,44	nd	nd	2,7	16	1609	1122
18	83	0,88	1,42	1,61	0,57	9,7	11,1	1,6	11	1740	875
19	84	1,01	1,69	1,67	0,35	9,5	9,4	2,9	19	2036	692
20	86	3,98	4,63	1,16	0,47	37,7	9,5	8,5	71	2632	1683
21	88	0,29	0,51	1,76	0,22	7,8	27,0	1,3	9	2131	812
22	90	0,77	1,94	2,52	1,06	6,1	8,0	0,7	58	nd	2238
23	92	0,82	1,39	1,70	0,36	5,6	6,9	2,3	11	1760	528
24	100	0,75	1,38	1,84	0,36	5,6	7,5	2,1	10	2109	660
Controls (observed range)		**3.45-7.99**	**4.21-8.34**	**0.91-1.48**	**0.45-2.31**	**36.0-81.7**	**6.6-12.1**	**3.5-10.1**	**53-163**	**2057-4964**	**1073-2938**
Controls (range mean±2SD)		**3.43-8.01**	**3.92-8.26**	**0.82-1.33**	**0.28-1.69**	**35.9-76.5**	**6.6-13.1**	**3.0-8.9**	**45-124**	**1894-4546**	**1068-2564**
n		**43**	**43**	**43**	**43**	**41**	**41**	**43**	**42**	**36**	**43**

Table 1. **Biochemical and molecular findings from muscle tissue of the patients with the 3243A→G mutation and the reference ranges (observed ranges and mean±2SD ranges). Pathologic values (below mean-2SD, ratio 2/1 above mean+2SD) are shaded grey. The percentage of mutation load is presented in the second column. Patient numbers are identical with Table 2.**

Incubations: 1: oxidation rate of [1-^{14}C]pyruvate+malate; 2: oxidation rate of [1-^{14}C]pyruvate+carnitine; 3: oxidation rate of [1-^{14}C]pyruvate+malate without ADP; 4: ATP production rate from oxidation of pyruvate+malate; Complex I: complex I activity; Complex III: complex III activity; Complex IV: complex IV activity. Ratio 2/1: ratio of incubation #2 to incubation #1; ATP/pyruvate: ratio ATP production to oxidation rate of incubation #1; ADP-stimulation: stimulation of ADP on the oxidation rate of [1-^{14}C]pyruvate+malate (ratio incubation 1 to incubation 3). Activities are expressed as nmoles $^{14}CO_2$/hour.mU CS (incubations 1, 2 and 3), nmoles ATP/hour.mU CS (incubation 4) and mU/U CS (C I, C III and C IV). CS, citrate synthase; nd, not done

Biochemical results

Rates of pyruvate oxidation and of ATP production, as well as ratios between oxidation rates, activities of the respiratory chain enzyme complexes I, III and IV and 3243A→G mutation load, measured in muscle tissue of the patients are presented in Table 1. Disturbances of the MEGS-capacity were more frequently found than decreased activities of respiratory chain enzyme complexes. The sensitivity of MEGS-capacity related parameters (e.g. pyruvate oxidation and ATP production rates) for prediction of a mitochondrial disease respective MELAS syndrome was much higher (65-85%) than classic respiratory chain enzyme measurements (9-42%). The sensitivity of sole respiratory chain enzyme measurements was much lower (Table 3). In eight patients with normal activities of the respiratory chain enzymes, the activities of one or more parameters of the MEGS-capacity were significantly decreased. In patient #10 with decreased complex IV activity, all MEGS-parameters were normal. In three patients all MEGS as well as respiratory chain enzyme activities were normal.

Table 2. **Clinical features of the patients with the 3243A→G mutation (the mutation load is presented in column 5).**

Patient number	Sex	Age of onset	Age at biopsy	% m.3243A>G	Encephalopathy	Myoclonic epilepsy	Stroke-like episodes	Ataxia	Progressive neuropathy	Headache	Cardiomyopathy	Cardiac arrhythmia	Renal failure	Ophthalmoplegia	Ptosis	Double vision	Deafness	Chronic diarrhea	Recurrent vomiting	Exercise intolerance	Muscle atrophy	Myalgia	Muscle weakness	Myopathy	Hyperthonia	Ragged-red-fibers	COX negative fibres	Diabetes mellitus	Depression	Short stature	Clinical course	Positive family history	Lactate in blood	Lactate in CSF
1	f	u	57.3	12	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	prog	yes	nd	nd
2	m	10,0	41.4	27	-	-	-	-	-	-	+	+	-	-	-	-	+	+	-	+	-	+	+	+	-	+	-	+	-	-	†44	no	5.5	nd
3	f	28,0	30.9	34	-	-	-	+	-	+	-	-	-	-	-	-	-	-	+	-	-	+	-	-	-	-	-	-	-	-	stat	no	N	2,2
4	f	13,0	26.3	34	-	-	-	-	-	+	-	-	-	-	-	-	-	-	+	+	-	+	+	-	-	-	-	-	+	-	stat	yes	N	nd
5	m	32,0	37.1	40	+	+	+	-	-	-	+	-	-	-	-	-	+	-	-	-	-	-	-	-	-	+	-	-	-	-	fluc	no	3,1	4,3
6	f	u	27.5	41	-	+	-	-	-	-	-	-	-	-	-	+	-	+	-	-	-	-	-	-	-	-	-	-	-	-	fluc	yes	N	N
7	f	15,0	46.2	45	-	-	-	-	-	-	-	-	-	+	-	+	+	-	-	-	-	+	+	-	-	+	+	-	+	-	stat	yes	2,4	3,3
8	m	33,0	48.5	46	-	-	+	+	+	-	-	-	-	+	+	-	+	-	-	+	-	+	+	-	-	+	+	+	-	-	prog	yes	2.7	nd
9	m	0,3	0.5	60	+	-	-	-	-	-	+	-	-	-	-	-	-	-	+	-	-	-	+	-	+	+	-	-	-	-	†3.5	yes	5.5-16.9	7,3
10	m	14,0	18.7	75	-	+	+	-	-	-	+	-	-	+	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	fluc	no	12.2	nd
11	f	u	5.7	76	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-	-	-	+	u	no	2,4	5,0
12	f	15,0	18.0	76	+	+	+	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	+	-	-	+	u	no	5,0	6,0
13	f	13,0	14.1	77	+	+	-	-	-	-	-	+	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	†15	no	2.6	nd
14	f	u	20.8	78	+	+	+	+	-	+	-	-	-	-	-	-	+	-	-	+	-	-	-	-	-	+	-	-	-	+	†21	yes	3,0	5.3
15	m	u	9.3	80	+	+	+	+	-	+	-	-	-	-	-	-	+	-	-	+	-	-	-	-	-	+	-	+	-	-	†16	yes	5.5	nd

Patient number	Sex	Age of onset	Age at biopsy	% m.3243A>G	Encephalopathy	Myoclonic epilepsy	Stroke-like episodes	Ataxia	Progressive neuropathy	Headache	Cardiomyopathy	Cardiac arrhythmia	Renal failure	Ophthalmoplegia	Ptosis	Double vision	Deafness	Chronic diarrhea	Recurrent vomiting	Exercise intolerance	Muscle atrophy	Myalgia	Muscle weakness	Myopathy	Hyperthonia	Ragged-red-fibers	COX negative fibres	Diabetes mellitus	Depression	Short stature	Clinical course	Positive family history	Lactate in blood	Lactate in CSF
16	f	31,0	35.0	81	-	-	-	-	+	-	-	-	+	-	-	-	-	-	-	+	+	-	+	+	-	+	+	-	-	-	fluc	yes	2.6	nd
17	f	u	27.6	82	+	+	+	-	-	+	+	-	-	+	-	-	+	-	+	+	-	-	+	+	-	+	-	+	-	-	stat	yes	3.1	7.4
18	f	10,0	21.7	83	-	+	+	-	-	+	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	+	+	-	-	-	fluc	no	4.2	4.9
19	m	u	0.5	84	-	+	-	-	-	+	-	-	-	-	+	-	-	-	-	+	-	-	-	-	-	+	+	-	-	+	prog	no	3.5	5.1
20	m	18,0	30.4	86	-	-	-	-	-	-	+	-	+	-	-	-	+	-	-	+	-	-	-	-	-	+	-	-	-	-	u	no	7.1	nd
21	f	4,0	19.8	88	-	-	+	-	-	-	-	-	+	-	-	-	-	-	-	+	-	+	-	-	-	-	-	-	-	-	prog	yes	10.5	nd
22	f	3,0	19.0	90	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	+	+	-	-	-	-	-	-	-	prog	yes	2.4-5.6	4.4
23	m	u	6.5	92	+	+	-	-	-	-	-	+	-	-	+	-	-	-	+	+	-	+	+	-	+	+	-	-	-	+	prog	no	10.9	3.8
24	m	15,0	21.8	100	+	+	+	+	-	+	-	-	-	-	-	-	+	-	+	+	+	-	+	-	-	+	-	-	-	-	stat	yes	3.6	6,0

In columns 3 and 4 the age (in years) is presented at the time of onset of clinical manifestation and at the time of the muscle biopsy. Abbreviations used (in alphabetical order): f, female; fluc, fluctuating; m, male; N, normal; nd, not done; prog, progressive; stat, static; u, unknown; †, age [years] at death. Normal value for blood-lactate is <1.7 mmol/l and for cerebrospinal fluid (CSF) <1.9 mmol/l. Some rare clinical features only found in a single individual patient were: cataract and nephritis in patient #2; irritable bowel disease in patient #4; dysphagia in patient #5; recurrent hypoglycemia in patient #6; Leigh syndrome, failure to thrive and feeding problems in patient #9; hypertrichosis in patient #12, recurrent infarctions of the gastrointestinal tract in patient #15, and nephrotic syndrome in patient #16.[16] Patient #21 had a renal transplantation at the age of 22 years because of severe renal failure.

Table 3. **Sensitivity and specificity of the various assays with respect to mitochondrial disease or the MELAS syndrome.**

Measurement	Test for	Sensitivity [%]	Specificity [%]
Pyruvate+malate+ADP	Mitochondriopathy in general	78	100
	MELAS syndrome	65	100
ATP production	Mitochondriopathy in general	85	100
	MELAS syndrome	74	100
Complex I	MELAS syndrome	42	100
Complex III	MELAS syndrome	23	100
Complex IV	MELAS syndrome	29	100

"Sensitivity" indicates how well the presence of a pathologic test recognizes the presence of the disease and "specificity" reflects the probability of a healthy person to have a normal test result.

Table 4. **Results of the comparison between the entire group of patients and three subgroups of patients with increasing percentages of mutation load, and the control group with respect to the MEGS parameters and respiratory chain enzymes activities.**

Subgroup size	**n=8**	**n=7**	**n=9**	**n=24**
Controls	**n=43**	**n=43**	**n=43**	**n=43**
Mutation load	**12-50%**	**51-80%**	**81-100%**	**overall**
	p	**p**	**p**	**p**
Pyruvate+malate	<0.0001	0,0005	<0.0001	<0.0001
Pyruvate+carnitin	0,0001	0,0003	<0.0001	<0.0001
ATP production	0,0001	<0.0001	<0.0001	<0.0001
Complex I	0,3083	0,3450	<0.0001	0,0001
Complex III	0,0890	0,0695	<0.0001	<0.0001
Complex IV	0,0172	0,0020	0,0009	<0.0001
Ratio 2/1	0,6980	0,0474	<0,0001	<0,0001
Pyr+mal-ADP	0,0024	0,1441	0,0002	<0,0001
ATP/Pyruvate	0,7455	0,6439	0,2853	0,3140
ADP stimulation	0,7580	0,0095	0,0004	0,0010

The calculation was performed with the non-parametric Mann-Whitney U-test. Significant values are shaded grey.

We found highly significant differences for all the biochemical parameters (except for the ATP to pyruvate ratio) between patients and controls (Table 4). In all three subgroups with increasing mutation loads we found highly significant differences between patients and controls with respect to the MEGS parameters, but not with respect to the respiratory chain enzyme activities that only showed significant differences at mutation loads above 80% (Table 4).

	Mutation load	1: Pyruvate+malate	2: Pyruvate+carnitine	Ratio 2/1	3: Pyruvate+malate-ADP	4: ATP production	ATP/pyruvate ratio	ADP stimulation	Complex IV	Complex I
1: Pyruvate+malate	-0,78									
2: Pyruvate+carnitine	-0,76	0,94								
Ratio 2/1	0,58	-0,67	-0,45							
3: Pyruvate+malate-ADP	-0,52	0,67	0,62	-0,49						
4: ATP production	-0,80	0,89	0,90	-0,51	0,50					
ATP/pyruvate ratio	-0,15	-0,01	0,07	0,11	-0,20	0,33				
ADP stimulation	-0,47	0,54	0,50	-0,49	-0,08	0,54	0,25			
Complex IV	-0,56	0,67	0,60	-0,58	0,66	0,58	-0,09	0,28		
Complex I	-0,56	0,39	0,40	-0,41	0,43	0,43	0,22	0,34	0,40	
Complex III	-0,58	0,58	0,53	-0,43	0,50	0,60	0,19	0,39	0,56	0,52

Fig. 1. Spearman rank correlation matrix for all the measured biochemical parameters.

A correlation was considered relevant if |Rho|>0.70. The relevant relationships are shaded grey. None of the single respiratory enzyme measurements reached relevance with respect to the mutation load.

In a non-parametric Spearman rank correlation test between mutation load and biochemical parameters only the measurements representing MEGS-capacity had relevant Rho values above 0.7 (Figure 1). None of the isolated respiratory chain enzyme activities correlated with the mutation load. With the help of quadratic regression analysis we only found a relevant relation between complex I activity and the oxidation rate of pyruvate+malate and a nearly relevant relation between complex I activity and ATP production (Figure 2).

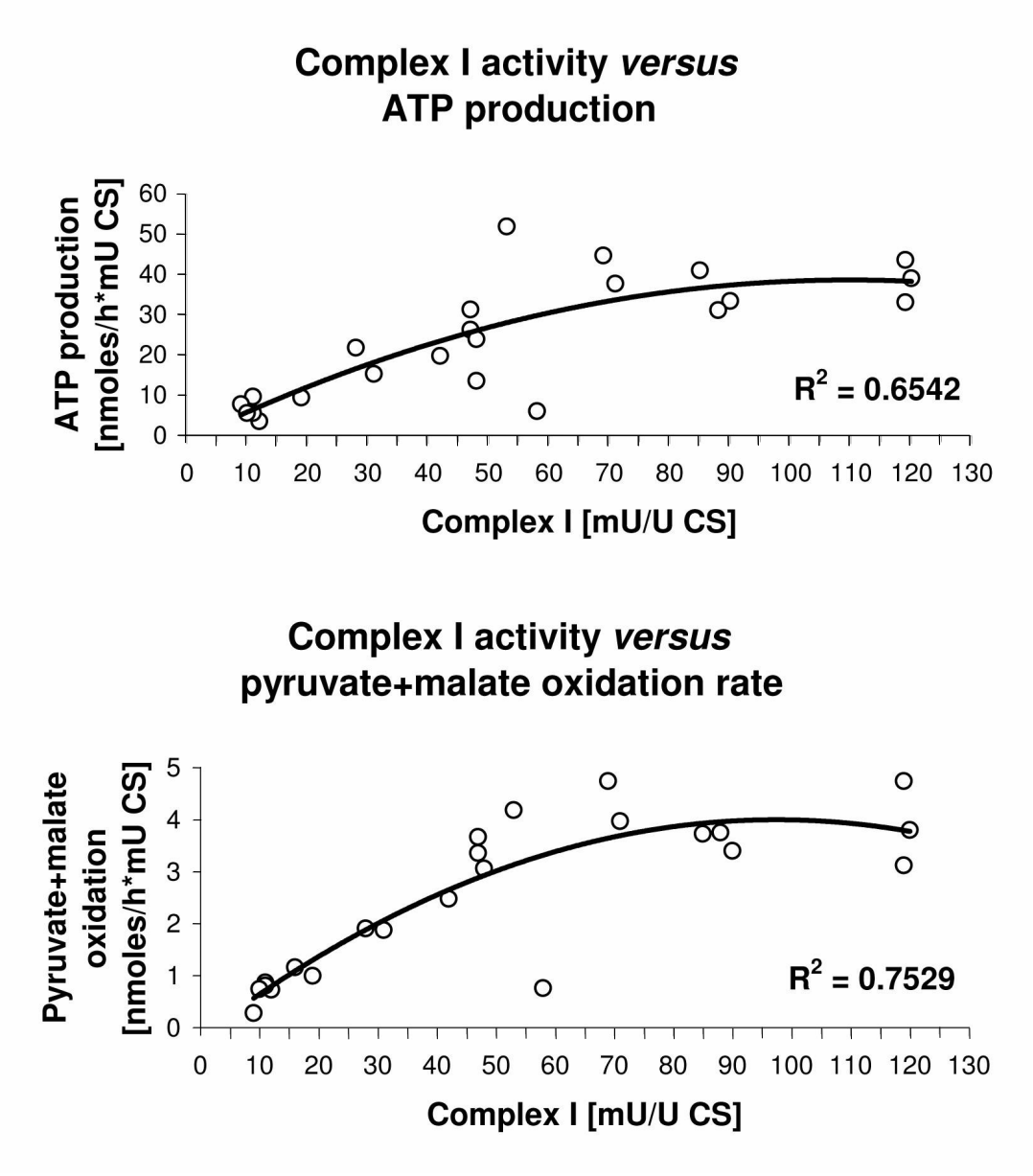

Fig. 2 *XY Scatter plots and quadratic regression analyses between complex I activity and MEGS-related parameters. There is a significant link between complex I activity and the oxidation rate pyruvate+malate and a nearly significant link between complex I activity and the ATP production rate.*

The biochemical results of patients #5, #9 and #20 were not as predicted from their mutation load (Table 1). Patient #5 (40% mutation load) showed a clearly decreased MEGS-capacity and complex I activity. Patient #9 (60% mutation load) showed strongly decreased MEGS-capacity and complex I and IV activities. Patient #20 (86% mutation load) showed normal MEGS-capacity and respiratory chain enzyme activities. In patient #21, we presently have no explanation for the high ATP production rate in relation to the pyruvate oxidation rate, and as a consequence the increased ATP/pyruvate ratio.

Molecular genetic results

As an explanation for the deviating results in patients #5 and #9, we hypothesized that besides the 3243A→G mutation, they might harbor another mutation in one of the m.tRNAs, the m.rRNAs or the m.*ND* genes. Therefore, we sequenced all 22 *m.tRNA* genes in mtDNA from native muscle tissue in 22 of the patients. From patients #22 and #24 only crude muscle homogenate was left and kept at -80^{0}C. DNA extracted from these samples was appropriate for quantification of the 3243A→G mutation, but PCR-amplification of the sequences of all m.tRNAs was impossible due to disrupted mtDNA that had been severely sheared during homogenization. The same applied to the sequences of m.tRNA^{His}, m.tRNA$^{Ser(AGU/C)}$ and m.tRNA$^{Leu(CUN)}$ in patient #20, and of m.tRNA^{Gly}, m.tRNA^{Arg}, m.tRNA^{His}, m.tRNA$^{Ser(AGU/C)}$ and m.tRNA$^{Leu(CUN)}$ in patient #19. In the mtDNA of patients #5 and #9 we also sequenced the 12S and 16S m.rRNAs and in patient 9 all m.*ND* genes. The results (Table 5) were compared with data from the mtDB database (http://www.genpat.uu.se/mtDB/index.html).[35]

In patients #1, #4 and #21 (mother and her 2 daughters), a new homoplasmic m.12302C→T transition was found in the m.tRNA$^{Leu(CUN)}$ that was absent in 2704 controls in the mtDB database. The m.12308A→G polymorphism found in patients #10 and #14, is located in the m.tRNA$^{Leu(CUN)}$ and defines the mtDNA haplogroup U.[36] The same polymorphism was found in patients #12 and #23, in combination with a m.12372G→A polymorphism. The combination of these polymorphisms defines the mtDNA super-haplogroup U/K.[37] Besides the polymorphisms described in Table 5, no additional mutations were found in the m.tRNAs in 22 patients, the m.rRNAs in patients #5 and #9, or the m.*ND* genes in patient #9.

Table 5. mtDNA polymorphisms found in our patients with 3243A→G mutation.

Transition/ deletion	mtDNA coding region	Prevalance in mtDB	Patient number												
			1	2	3	4	5	6	9	10	12	13	14	21	23
m.750G>A	*12S rRNA*	22/2704					Hom		Hom						
m.1438A>G	*12S rRNA*	2620/2704					Hom		Hom						
m.1719G>A	*16S rRNA*	111/2704					Hom								
m.2706A>G	*16S rRNA*	2178/2704					Hom								
m.3010G>A	*16S rRNA*	550/2704							Hom						
m.3796A>G	*m.ND1*	10/2704							Hom						
m.4295A>G	*m.tRNA-Ile*	5/2704								Hom					
m.4769A>G	*m.ND2*	2674/2704							Hom					Hom	
m.8348A>G	*m.tRNA-Lys*	3/2704							Hom						
m.10034T>C	*m.tRNA-Gly*	37/2704		Hom	Hom										
m.10463T>C	*m.tRNA-Arg*	127/2704						Hom							
m.12302C>T	*m.tRNA-Leu2*		Hom			Hom								Hom	
m.12308A>G	*m.tRNA-Leu2*	347/2704								Hom	Hom		Hom		Hom
m.12354T>C	*m.ND5*	7/2704									Hom				
m.12372G>A	*m.ND5*	390/2704									Hom				Hom
m.15907A>G	*m.tRNA-Thr*	20/2704									Hom				
m.15924A>G	*m.tRNA-Thr*	106/2704		Hom	Hom								Hom		
m.15928G>A	*m.tRNA-Thr*	132/2704						Hom							
m15940T>C	*m.tRNA-Thr*	3/2704										Het (85%)			
15940 del T	*m.tRNA-Thr*	20/2704													Hom

Patient numbers are the same as in Supplementary Table 1. Hom: homoplasmic; Het: heteroplasmic (mutation load in parentheses). Prevalence is the number of sequences carrying the respective polymorphism and the total number of sequences in the mtDB database (http://www.genpat.uu.se/mtDB/index.html).

Discussion

In this study we investigated 24 MELAS patients with the 3243A→G mutation. Assessment of a patient was always started by measuring MEGS-capacity and respiratory chain enzymes in muscle tissue. When the results of these investigations, in combination with the clinical phenotype, pointed towards a potential mtDNA defect, mtDNA studies were initiated. In patients with a clinical phenotype pointing towards a mtDNA defect, the mutation load of a 3243A→G mutation in blood does not reflect that in muscle or brain tissue and the mutation even can be absent in blood.[38] For that reason we always measure the mutation load in combination with MEGS-capacity in muscle samples of patients suspected from a mtDNA defect. In the interest of the patient it is important to know the consequences of the mutation load on biochemical functioning of the mitochondria.

We found relevant correlations between the mutation load and MEGS-capacity. Until now, genotype to biochemical phenotype relations in muscle tissue were investigated only by measuring respiratory chain enzymes. However, the measurement of the MEGS-capacity in parallel with respiratory chain enzymes, provides more information about the mitochondrial function. Measuring parameters of the MEGS-capacity provides a better discrimination between patients and controls. The sensitivities of the MEGS parameters to detect MELAS or a mitochondrial disease in general were much higher than those for the respiratory chain enzymes alone. Especially in diagnostically difficult cases, such as in patients with a low mutation load, the measurement of MEGS parameters will answer the question more confidently whether patient is affected with a mitochondrial disease or not. Only patient #10, with decreased complex IV activity, showed normal activities for all MEGS parameters.

Complex I deficiency is most frequently found in patients harboring the 3243A→G mutation, but deficiencies of complex III and IV and combined deficiencies have also been described to varying degrees.[20,39,40] However, in two of these studies[39,40] no complex III activity but succinate:cytochrome *c* oxidoreductase activity was measured, which mainly is a measure for complex II activity (own observations). In one of these studies a linear correlation was found between mutation load and complex I activity.[40] In contrast to this study, we found no correlation (Rho=-0.56) between mutation load and complex I activity. The same applied to the activities of complexes III and IV.

With two exceptions, the complex I activities were significantly decreased only in patients with a mutation load above 80% (Table 1). It is known that especially the ND6 and ND4 subunits of complex I are required for assembly of the complex.[41] The ND6 subunit depends

89% on m.tRNA$^{Leu(UUR)}$ for incorporation of leucine. In patients with a high 3243A→G mutation load, probably less ND6 will be produced, resulting in a disturbed complex I assembly which explains the decreased level of fully assembled complex I.[21] The influence of the ND6 subunit on complex I assembly had already been shown in one of our patients with an m.14487T→C mutation in the m.*ND6* gene.[42] Recently it was reported that tRNA$^{Leu(UUR)}$ harboring the 3243A→G mutation lacked the normal taurine-containing modification at the anticodon wobble position (τm^5U at position 3265), resulting in severely reduced UUG translation but no decrease in UUA translation.[43] The authors concluded that this finding could explain the translational defect of UUG-rich genes like m.*ND6* that leads to complex I deficiency observed in patients with the 3243A→G mutation. These findings may explain why complex I is more decreased than other respiratory chain enzymes in our patients with a mutation load above 80%.

In vivo mitochondrial function was studied by measuring concentrations of inorganic phosphate, phosphocreatine and ATP with ^{31}P-Magnetic Resonance Spectroscopy in calf muscles of patients with the 3243A→G mutation and no correlation was found between the mutation load and mitochondrial function.[23] *In vitro* studies showed decreased oxygen consumption in transmitochondrial cybrids harboring the 3243A→G mutation, that correlated with increasing mutation load and decreased sharply at a mutation load of about 90%.[25] These findings are in contrast to the results of our measurements where we did not find a sharp threshold, but a more gradual decrease in enzymatic activities and MEGS capacity . This probably relates to variation in nuclear genetic background of the patients and additionally to secondary pathologic changes in the muscle as compared to the relative uniformity of cybrids.

As shown in Table 1, complex I activities had a certain relation to the mutation load, especially at mutation loads above 80%. We calculated the correlation between complex I activity and MEGS-capacity (as represented by the pyruvate+malate oxidation rate and the ATP production rate) and found a relevant one for the oxidation rate of pyruvate+malate (R^2=0.75) and a nearly relevant one for the ATP production (R^2=0.65) (Figure 2). This means that the decreased complex I activity is the main cause for the decline of MEGS-capacity but does not explain it entirely. This might have several reasons: **(I)** Complex I activity is measured in disrupted micelles of the inner mitochondrial membrane, an artificial and isolated system with excess substrate which probably does not reflect the steady state flow through the respiratory chain *in vivo*. **(II)** Complex I assays only measure the electron flow, but not the proton pumping capacity of the enzyme complex, whose impairment might considerably contribute to the pathology. **(III)** The MEGS-capacity might also depend on supermolecular

structures termed as “respirasomes” or “respiratory strings”.[44] The derangement of respiratory chain complex stochiometry could thus impair the composition of these supermolecular structures leading to reduced overall respiratory chain function. For that reason, measurement of the pyruvate oxidation and the ATP production rates in coupled mitochondria are much closer to the *in vivo* situation. It is therefore not astonishing that these MEGS-related parameters correlate better with the mutation load because they measure combined proton pumping, coupled electron transport and ATP production. In line with these arguments is the fact that the ATP production rate showed the highest correlation with the mutation load (Rho=-0.80).

The ADP stimulation of the pyruvate+malate oxidation rate is a measure of the coupling state of mitochondria. In coupled mitochondria, ADP should lead to a 3.5-10.1 fold increase of pyruvate+malate oxidation (Table 1). In the majority of patients with a mutation load above 80%, the ADP-stimulation was decreased while the ATP/pyruvate ratio was normal. The latter indicates that despite the decreased ATP output, this production rate is always proportional to the oxidation rate of pyruvate. So, in the presence of ADP, ATP is produced during oxidation of pyruvate, but in the absence of ADP, pyruvate oxidation rate is relatively high in proportion to that in the presence of ADP. This indicates that the mitochondria were “loosely coupled”, a phenomenon originally described in Luft’s disease.[45] This “loose coupling” might be another hint towards a derangement of the mitochondrial “respirasomes”.

In patients #5, #9 and #20, biochemical findings deviated grossly from prediction according to their mutation load. In patients #5 and #9, no additional pathogenic mtDNA mutations were found in their m.tRNAs and m.rRNAs. In patient #9 also no additional mutations were found in the m.*ND* genes. So, in these patients the reason of the deviant biochemical phenotype remains unclear and might be ascribed to unknown factors in the genetic backgrounds of these patients. From our biochemical and molecular genetic results we conclude that the biochemical phenotype of our patients with 3243A→G mutations is diverse but only caused by this mtDNA mutation, possibly in combination with so far unknown nuclear genes.

In patients #1, #4 and #21, we found a new homoplasmic m.12302C→T transition in the $tRNA^{Leu(CUN)}$. The transition was neither known in MITOMAP nor in the mtDB database and could potentially disturb the $tRNA^{Leu(CUN)}$. However, the transition is homoplasmic, is located in the anticodon-loop, is not well conserved (most mammals have an A at this position) and does not contribute to Watson-Crick base pairing, all features pointing towards a polymorphism. For physicians it would be desirable to have a “gold standard” test to prove or to exclude a mitochondriopathy. Probably due to the complexity of mitochondrial function,

such a test unfortunately does not exist. By determining MEGS-capacity, we were able to lower the threshold for detection of biochemical abnormalities in the presence of the 3243A→G mutation from a mutation load of 80% (in the case of complex I activity) to 34% (in the case of the ATP production rate) (see Tables 1 and 3). We hope further improvement of the methods (simultaneous measurement of many parameters of the respiratory chain) will enable us to follow the sequence of events from a specific mtDNA mutation to the biochemical and ultimately to the clinical phenotype of the patients. A further challenge will be to identify modifying factors of the nuclear genetic background in those patients that deviated from the predicted biochemical phenotype. For those reasons it is not only important to know if a patient harbors a mtDNA mutation, but also to study the biochemical consequences of the mutation.

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CHAPTER 5

Prenatal Diagnostics in Oxidative Phosphorylation Disorders

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Abstract

In this chapter we describe about 18 years of experience with prenatal diagnosis in oxidative phosphorylation (OXPHOS) diseases in our centre. We start diagnostics of OXPHOS disorders in patients with a mitochondrial (encephalo)myopathy by preference by measuring oxidation rates of pyruvate, malate and succinate and ATP production rates from oxidation of pyruvate in a "fresh" muscle biopsy. In the same biopsy activities of the mitochondrial respiratory chain enzymes complex I, complex II, complex III and complex IV are also measured. When decreased substrate oxidation rates and ATP production rates give indication for suspicion on a complex V or a pyruvate dehydrogenase complex (PDHC) deficiency, activities of these enzymes are also measured.

In frozen muscle biopsies we only can measure the respiratory chain enzymes. In which cases now can we offer prenatal diagnosis? In about 30% of the muscle biopsies with clearly decreased substrate oxidation rates and ATP production rates, all respiratory chain enzymes, complex V and PDHC show normal activities. In these cases it is impossible at the moment to offer prenatal diagnosis. In the remainder of the biopsies with clearly reduced substrate oxidation- and ATP production rates, decreased activities are measured of one or more of the afore mentioned enzymes. The most frequently occurring deficiencies in fresh as well as in frozen muscle biopsies are complex I, complex IV or combined deficiencies of these enzymes. The next step is to search if the deficiency is also expressed in cultured fibroblasts and to exclude a mtDNA mutation as a cause of the deficiency. If the deficiency is also expressed in cultured fibroblasts and mtDNA mutations have been excluded we are willing to offer prenatal diagnosis. This chapter is aggravated on prenatal diagnosis for complex I, complex IV or a combined deficiency of these enzymes because the majority of the total number of requests for prenatal diagnosis that reach us concerns pregnancies in families in which the index patient was suffering from a deficiency of one of these (or both) enzymes.

Introduction

Mitochondrial (encephalo)myopathies are a group of diseases with a very heterogeneous clinical picture. The clinical picture of patients varies from very severely affected with early onset and often fatal outcome at young age to only mildly affected, with sometimes late onset and reaching adult age. In some patients only one tissue seems to be affected and others suffer from a multisystem disorder. In some patients only one enzyme involved in OXPHOS is deficient, in others a combined deficiency of two or more enzymes is found. In the majority of the patients muscular and/or neurological complaints are the main presenting symptoms. There are age-dependent symptoms as failure to thrive in neonates and exercise intolerance in adults, other symptoms as hypotonia and retardation can present at any age. No single clinical feature is specific for one clinical phenotype and in some patients the clinical picture of a specific phenotype can gradually change into another. A patient is suspected to suffer from a mitochondrial disorder if he/she suffers from at least two chronic and unexplained symptoms as described in Table 1, preferably occurring in two different organs.[1-2] As soon as a child has been diagnosed to suffer from a mitochondrial disorder, many parents raise the question if prenatal diagnosis is possible, especially if the parents have more affected children or when the mother had one or more spontaneous abortions before or after birth of an affected child.

Prerequisites for Offering Prenatal Diagnosis in OXPHOS Disorders

Prenatal diagnostics in mitochondrial disease can be a delicate case and susceptible to many pitfalls. The human mitochondrion contains at least 1000 different proteins[3] but only a relatively small number of them is directly involved in the primary task of the mitochondrion: production of the energy-rich compounds ATP and creatine phosphate from oxidation of several substrates. Only these proteins can be evaluated by measuring their enzymatic activity Other proteins are involved in the import, folding and defolding and assembly of proteins in the mitochondrion. The majority of the mitochondrial proteins is encoded by the nuclear DNA, synthesized in the cytoplasm and transported into the mitochondrion. Only 13 mitochondrial proteins, all subunits of the complexes of the mitochondrial respiratory chain and complex V, are encoded by the mitochondrial genome (mtDNA). These are the seven ND-subunits of complex I (NDI -ND6 and ND4L), the

cytochrome-b subunit of complex III, the cox-1, 2 and 3 subunits of complex IV (cytochrome *c* oxidase) and the complex V (F_1F_0 ATP-ase) subunits 6 and 8. In all these subunits mutations have been described causing mitochondrial disease.

Also mutations in the tRNA-genes of the mtDNA and deletions in the mtDNA have been described that can cause mitochondrial disease. If prenatal diagnosis for OXPHOS disorders is requested, it is important to exclude mtDNA mutations as a possible cause for the disorder. Mutations in the mtDNA have a very specific feature: the mutations can show heteroplasmy. This means that besides the mutated DNA there still is a part of normal (wild type) DNA present. If all the mtDNA is mutated, the mutation is called homoplasmic. The percentage of heteroplasmy varies between different tissues, mutant load in blood and cultured fibroblasts being mostly low as compared to high energy demanding tissues like muscle, liver and brain. For many mtDNA mutations a relation exists between the percentage heteroplasmy and the clinical phenotype, for instance MELAS (mitochondrial myopathy, encephalopathy, lactic acidosis, stroke like episodes), MERRF (myoclonic epilepsy, ragged red fibers) and NARP (neuropathy, ataxia, retinitis pigmentosa).[4-7] Only when the percentage mutated DNA exceeds a certain threshold value, the patient is affected and shows the clinical phenotype. Other mutations like LHON (Leber's hereditary optic neuropathy) lack such correlation.[8] Several mutations in the mitochondrial ND genes as well as mutations in the tRNA genes cause isolated complex I deficiency. Mutations in the mitochondrial complex IV genes and the cytochrome-b gene cause complex IV and complex III deficiency, respectively. The Leigh/NARP 8993 mutation, causing NARP or Leigh syndrome (subacute necrotizing encephalomyelopathy) is the major source of complex V deficiency (for an up to date overview of mtDNA mutations and matched clinical phenotypes look at MITOMAP: http://www.mitomap.org on the internet). So, if a patient with a mitochondrial (encephalo) myopathy is suffering from a complex I, III, IV or V or a combined deficiency of some of these complexes, searching for mtDNA mutations is inevitable when prenatal diagnostics is required. In case of an enzyme deficiency caused by a mtDNA mutation, prenatal diagnostics should strongly be dissuaded at this moment. When normal activities of the affected enzyme are measured in native chorionic villi, cultured chorionic cells or cultured amniotic cells of such fetus, enzymatic activity can still be decreased in muscle, liver or brain tissue of the fetus.

Table 1. **Clinical symptoms most frequently found in mitochondrial disorders.**

Central Nervous System	Seizures
	Hypotonia/hypertonia
	Spasticity
	Transient paraparesis
	Lethargy/coma
	Psychomotor retardation/regression
	Extrapyramidal signs
	Ataxia (episodic)
	Dyspraxia
	Central hypoventilation
	Deceleration/acceleration of head growth
	Blindness (cortical)
	Deafness (perceptive)
Skeletal Muscle	Exercise intolerance/easy fatiguability
	Muscle weakness
Heart	Cardiomyopathy (hypertrophic or dilated)
	Conduction abnormalities
Eyes	Ptosis
	Restricted eye movements
	Strabismus
	Cataract
	Pigmentary retinopathy
	Optic atrophy
Liver	Hepatic failure
	Hypoglycaemia
Kidney	Tubular dysfunction
Endocrine	Diabetes insipidus
	Diabetes mellitus
	Delayed puberty
	Hypothyroidism
	Exocrine pancreas dysfunction
	Primary ovarian dysfunction
Gastrointestinal	Diarrhea (villous atrophy)
	Intestinal pseudoconstriction
Other	Failure to thrive
	Short stature
	Pancytopenia
	Anaemia
	Migraine

The percentage of mutated mtDNA can be very low or even undetectable in the prenatally investigated cells, whereas high amounts of mutated mtDNA in muscle, liver or brain tissue can be present and therefore the fetus may be affected. Only if in native or cultured chorionic cells a severe deficiency of one or more respiratory chain enzymes has been measured, caused by a mtDNA mutation, one could conclude that the fetus would probably be affected because the percentage heteroplasmy increases from tissues like fibroblasts or chorionic cells towards muscle, liver or brain. However, more study has to be done to prove this conclusion.

Dahl et al.[9] performed a first study to come towards reliable prenatal diagnosis for both the LEIGH/NARP 8993 mutations (8993T>G and 8993T>C) and concluded that there is no substantial tissue variation in mutant load between fetal and adult tissue, implicating that the mutant load in a prenatal sample will represent the mutant load in other fetal tissues. Each of the two nucleotide 8993 mutations shows a strong correlation between mutant load and severity of the clinical picture. Their empirical data for calculating recurrence risk and predicting the clinical outcome of a given mutant load can be used cautiously for genetic counseling and prenatal diagnosis of nucleotide 8993 mutations. Harding et al.[10] performed prenatal diagnostics in two pregnancies of a mother with a severely affected child with LEIGH/NARP 8993 T>G mutation. Compared with the mutant load in the probandus, the native chorionic villi showed a higher proportion of mutant mtDNA on both occasions and this was reflected in several fetal tissues including muscle and brain.

It is important that in patients with an (encephalo)myopathy and decreased activities of one or more enzymes of the OXPHOS system, extensive examinations on the presence of mutations in mtDNA are performed to exclude mtDNA mutations. One method is sequencing the whole mitochondrial genome but this is expensive and time consuming. Besides, the mtDNA contains a lot of polymorfisms. A convenient method to test whether an enzyme deficiency is caused by a mutation in the mtDNA or by a mutation in the nuclear genome is the ρ^0 test as described by King and Atardi.[11] Patient cells are enucleated and fused with a ρ^0 cell line (a human cell line lacking mtDNA). If the enzyme deficiency still remains in the transmitochondrial cell line, the deficiency is caused by a mtDNA mutation. If the deficiency disappears it is caused by a mutation of nuclear origin. A rapid and sensitive method to search for mutations in the whole mitochondrial genome could be the Conformation Sensitive Gel Electroforesis (CSGE) as described by Finnilä et al.[12] After analysis of the mtDNA haplogroup of the patient to exclude polymorfisms, the whole mitochondrial genome is analyzed for mtDNA mutations. CSGE is based on the

separation of heteroduplexes containing single base-pair mismatches from homoduplexes in a polyacrylamide gel. Heteroduplexes are formed by heat denaturation and reannealing PCR fragments of mtDNA from patients and a control.

Another very important prerequisite for performance of prenatal diagnosis is testing the obtained chorionic villi on maternal contamination. Immediately after the chorionic biopsy has been taken the tissue has to be dissected accurately under a microscope to separate fetal from maternal tissue. Especially when chorionic cells must be cultured from native chorionic villi this has to be done very precisely. However this precaution is not enough because when only a few maternal cells are left in the material, they can overgrow the fetal cells especially when the fetal cells, for instance, show a complex I deficiency. In that case the "healthy" maternal cells may overgrow the fetal cells because of their advantage in oxidative energy metabolism. So, especially in cultured chorionic cells there is a substantial risk for maternal cell contamination (decidual cells) which can lead to false diagnosis. Milunsky and Cheney[13] proved the presence of maternal cells in 3 of 24 chorionic villus samples and concluded that microscopic examination of chorionic villi for apparent presence of maternal cells is not rigorous enough. Ledbetter et al.[14] found a rate of 1.8% maternal cell contamination in chorionic cell cultures. Wang et al.[15] reported a rate of 1.7% maternal cell contamination. In 10 long term cultures of chorionic cells we found one culture which consisted totally of maternal cells and this culture led to a wrong prenatal diagnosis for complex I deficiency (Schuelke et al.).[16]

The probandus in this family was a girl, the first child of healthy Caucasian first-degree cousins (family 1). She suffered from acrocyanosis, muscular hypotonia, nystagmus, bitemporal retinal depigmentation and severe lactic acidosis (plasma lactate concentration 24.l mmol/l, reference interval 0.5-2.2 mmol/l, cerebrospinal fluid lactate concentration 9.6 mmol/l, reference values <2, and a plasma lactate-to-pyruvate ratio of 57, reference value <20). In muscle tissue and cultured fibroblasts respiratory chain complexes I, II +III and IV were measured and in both tissues an isolated complex I deficiency was found. The child died at the age of 4 weeks from respiratory failure in the course of an uncontrollable metabolic crisis. As the mother became pregnant again the parents asked for prenatal diagnosis and we measured complex I activity in native chorionic villi and cultured chorionic cells. Complex I activity in native chorionic villi was normal, but decreased in cultured chorionic cells. This policy was adopted after we performed a prenatal diagnosis in an other family (family 2) where the probandus also suffered from complex I deficiency in muscle tissue and cultured fibroblasts. In the next pregnancy we measured a normal

complex I activity in native chorionic villi but a severely decreased activity in cultured chorionic cells of the fetus. The parents decided to continue the pregnancy and the child died at the age of 18 months from severe lactic acidosis and a hypertrophic cardiomyopathy. In cultured fibroblasts of the girl we found an isolated complex I deficiency. Some years later we found a homozygous 683G>A mutation in the *NDUFS2* gene of complex I in cultured fibroblasts of this patient and the probandus of this family. Until now the reason of this discrepancy between native chorionic villi and cultured chorionic cells has not been cleared.

In the second pregnancy of family 1 the parents opted for a high degree of security and decided to terminate the pregnancy. In muscle tissue from the aborted female fetus, however, we measured a normal complex I activity. At the time of the third pregnancy in family 1 we could offer prenatal diagnosis on base of enzymatic measurement of complex I activity as well as on mutation screening of complex I genes. In the probandus we had proven a homozygous C>T transition at nucleotide 632 of the *NDUFVI* cDNA. In native chorionic villi as well as in cultured chorionic cells of this fetus normal complex I activity was measured. In both fractions of this fetus and in cultured chorionic cells and muscle tissue from the fetus of the second pregnancy we searched for the mutation in the *NDUFV1* gene and both fetuses were found to be heterozygous for the mutation. Next we investigated the genetic identity of the cultured chorionic cells and the muscle tissue from the fetus of the second pregnancy, native chorionic villi and cultured chorionic cells of the third pregnancy and DNA from peripheral blood lymphocytes from both parents by analyzing nine different polymorphic short tetranucleotide repeat loci and the X-Y homologous gene amelogenin with the AmpF/STR^{TM} Profiler Plus Kit (Applied Biosystems). Native chorionic villi as well as cultured chorionic cells from the fetus of the third pregnancy were totally of fetal origin without any maternal contamination. However the cultured chorionic cells of the second pregnancy seemed to be totally of maternal, probably decidual origin. Because both parents were found to be heterozygous for the 632C>T mutation in the *NDUFV1* gene and cultured fibroblasts of both parents showed normal complex I activity, it was strange that the decidual cells of the mother showed a decreased complex I activity. In an attempt to clear up this discrepancy we measured complex I activity in 3 control decidual cell cultures but also when compared to these control cells, complex I activity in the decidual cells of the mother was clearly decreased (cultured decidual cells mother: 120 mU/U cytochrome *c* oxidase, controls: 220-300). Complex I activity in cultured decidual cells of controls was comparable with control

cultured chorionic cells, 220-300 (n=3) and 186-330 mU/U cytochrome *c* oxidase (n=13), respectively. So despite all efforts we could not find a satisfactory explanation for the enzymatic results obtained in the second pregnancy of this family.

Tissues to Be Used for Prenatal Diagnosis in OXPHOS Disorders

For a reliable prenatal diagnosis three kinds of fetal tissues are considered: native chorionic villi, cultured chorionic cells and cultured amniotic cells. Chorionic villi can be taken transcervical after 9 weeks of pregnancy or transabdominal after 11-13 weeks of pregnancy. Amniotic cells can be taken after 16 weeks of pregnancy.

In our institute we use the following policy. After the native chorionic villi sample has been taken the material is carefully made free from maternal contaminating tissue under a microscope. Always a blood sample of the mother is taken simultaneously for the test on maternal contamination. The pure fetal sample is centrifuged in an Eppendorf tube and the supernatant is discarded. Part of the material is cultured in Amnionmax tissue culture medium and the rest of the sample is immediately frozen in liquid nitrogen. This material can be used for DNA isolation for all kind of prenatal diagnosis on molecular genetic base and/or can be homogenized in 0.1 M potassium phosphate buffer, pH 7.4 and used for enzymatic analysis of complex I, complex IV or both. Part of the homogenate is used for testing on maternal contamination, for measuring Citrate Synthase (CS) activity as a mitochondrial marker enzyme, and protein content. If whatever goes wrong with one or more of the measurements or if there are doubts about the reliability of a certain test we still have the cultured chorionic cells in reserve to repeat the measurements. Especially these cultured cells must be tested on maternal contamination for reasons as mentioned before. From cultured chorionic cells a mitochondrial enriched fraction is prepared as described by Attardi et al.[17] with modifications as described by Bentlage et al.[18] This fraction can be used for enzymatic studies. We routinely measure complex I, complex III, Succinate: cytochrome *c* oxido reductase (SCC; Complex II+III) and complex IV activity in this fraction. CS activity and protein content are also measured. If something might go wrong with one of the assays on these cells or there is still doubt about the reliability of the results or when maternal contamination is proven in these cells, there still can be done an amnioncentesis to get amniocytes. From cultured amniocytes we also prepare a mitochondrial enriched fraction and the same enzymes are measured as in cultured

chorionic cells. When native chorionic villi are used for prenatal diagnostics on enzymatic base a diagnosis can be made within one week at most. With cultured chorionic cells or amniotic cells it takes between one or two weeks, beyond the time that is needed to culture the required amount of cells.

Methods for Prenatal Diagnosis in OXPHOS Disorders

To perform prenatal diagnostics on fetal tissue, in general two methods can be used: the molecular genetic way based on searching for mutations, that have already previously been established in the index patient, in the DNA of fetal tissue, or the enzymatic way based on measuring the activity of those enzymes proven to be deficient in the index patient, in fetal tissue. It is beyond doubt that the first mentioned way is the most reliable method for prenatal diagnostics. However, this method is only possible if a mutation has been established in a nuclear gene that encodes for the enzyme or protein found to be deficient in the index patient. For a major number of deficiencies in enzymes involved in the OXPHOS the genetic defect is still unknown. In those cases only the enzymatic approach is possible. A thorough explanation and information from the biochemist towards the doctor about the possibilities and limitations of this method is of utmost importance. In our opinion it is not correct to go for absolute certainty because in that case a lot of parents will be left out in the cold. Besides, every new case is a precept and has an added value to the reliability of the method. In our institute we offer prenatal diagnostics, especially when it has to be done on enzymatic base, only when the enzyme deficiency in the proband has been proven in at least two different tissues and by preference one of them being cultured fibroblasts. The necessity of a proven deficiency in cultured fibroblasts of the proband is generally accepted.[19] A deficiency in cultured fibroblasts is never an absolute deficiency. Although relatively high residual activities can be measured, for complex I for instance up to 80% of the lowest control value, our experience is that even in those cases prenatal diagnosis can still reliably be performed.

At the moment about 26 mutations in nuclear genes, directly or indirectly involved in the enzymatic activity of one or more enzymes of the OXPHOS, are known. These mutations vary from mutations in genes encoding for subunits of complex I, complex II or complex III[20] of the mitochondrial respiratory chain, to mutations in genes causing multiple mtDNA deletions or depletion, mutations in genes encoding for proteins involved in the assembly

of complex III and complex IV mutations in genes encoding for proteins involved in homeostasis and import of proteins over the mitochondrial inner membrane and one gene involved in transcription and translation of OXPHOS genes. For a review of these mutations see Van den Heuvel and Smeitink[21]. A mutation in one of these genes always leads to a severe disturbance in OXPHOS. If a mutation in one of these genes has been proven in a patient suffering from a deficiency in one or more enzymes of the OXPHOS, prenatal diagnosis can be offered on molecular genetic base. Special attention must be paid to some patients with complex I deficiency. Bénit et a1[11] described 4 patients with hypotonia, ataxia, psychomotor retardation, or Leigh syndrome with complex I deficiency in muscle tissue or in muscle and liver tissue but normal oxidation rates of NADH-generating substrates in cultured fibroblasts, suggesting a normal complex I activity in cultured fibroblasts. Complex I activity was not measured in cultured fibroblasts. Three of them were compound heterozygous for mutations in the *NDUFVI* gene encoding for the 51 kDa subunit of complex I and one of them was compound heterozygous for mutations in the *NDUFS1* gene encoding for the 75 kDa subunit of complex I. So possibly our rule that in patients with complex I deficiency in muscle tissue, the deficiency must also be proven in cultured fibroblasts before prenatal diagnostics can be offered, does not always fit.
In patients with this phenotype and complex I deficiency in muscle or muscle and liver tissue we currendy advice to sequence the *NDUFVI* and *NDUFSI* genes, especially when complex I activity in cultured fibroblasts is normal. Besides, it is advisable to screen both parents for mutations in these genes. When a mutation is found, prenatal diagnosis is probably only possible on molecular genetic base.

Results of Prenatal Diagnosis for OXPHOS Disorders in Our Center

Complex IV Deficiency

In 1985 we started our investigations for prenatal diagnosis for complex IV deficiencies and in 1988 we published for the first time two cases of prenatal diagnosis in a family where the index patient suffered from Leigh syndrome and died at the age of three years.[23] In cultured fibroblasts of this patient we measured a clear complex IV deficiency. In native chorionic villi and cultured amniocytes of the first fetus we measured a normal complex IV activity. After an uneventful pregnancy a healthy boy was born that showed no symptoms

of Leigh syndrome. In the second pregnancy we measured a clearly decreased complex IV activity in native chorionic villi and cultured amniocytes. The parents decided for an abortion and in cultured fibroblasts of the aborted fetus also a markedly decreased complex IV activity was measured.

From 1986 till June 2003 we diagnosed 47 pregnancies in 33 families for complex IV deficiency. In 35 cases a normal complex IV activity was measured in native chorionic villi, cultured chorionic cells, cultured amniocytes or a combination of one or more of these tissues, which resulted in an unaffected newborn in 24 cases; in 11 cases the outcome of the pregnancy is currently unknown for us. In 9 cases a clearly decreased complex IV activity was measured in native chorionic villi, cultured chorionic cells, cultured amniocytes or a combination of two of these tissues. In 3 of these cases the complex IV deficiency could be confirmed in fetal cultured fibroblasts from abortion tissue (2 cases) or in placenta tissue. In 3 cases no cultured fibroblasts of the aborted fetus were available. In 2 cases the parents decided to continue the pregnancy and in both cases an affected child was born which died at the age of 3 and 5 years, respectively. However, no cultured fibroblasts from both children were available. In one case a decreased complex IV activity was measured in cultured amniocytes. The parents decided to continue the pregnancy and a clinically unaffected boy was born. No cultured fibroblasts of the boy were available. In one case, apart from the 9 cases with clearly decreased complex IV activity in fetal cells, only a mildly decreased complex IV activity was measured in native chorionic villi (complex IV on CS 89% of the lowest control value). So we could not make a definite conclusion with respect to the fetus, affected or not. The parents decided to terminate the pregnancy. In cultured fibroblasts of the aborted fetus a normal complex IV activity was measured.

Two other cases of prenatal diagnosis for complex IV deficiency resulted in an unexpected outcome of the pregnancy. In one case a normal complex IV activity was measured in native chorionic villi, cultured chorionic cells and cultured amniocytes of the fetus but an intra-uterine death appeared, possibly a consequence of the amniocentesis. No cultured fibroblasts of this fetus were available. In one case a normal complex IV activity was measured in native chorionic villi. However, a clinically affected boy was born. In muscle tissue of the boy a normal complex IV activity, but surprisingly, a decreased pyruvate dehydrogenase complex activity was measured.

Complex I Deficiency

In the same period 36 pregnancies in 24 families were diagnosed for complex I deficiency. The first 23 pregnancies in 15 families have already been published.[24] In 27 pregnancies a normal complex I activity was measured which resulted in an unaffected new-born in 25 cases. In one case the parents decided not to continue the pregnancy and the fetus was aborted. No tissue of this fetus was available. In one case a spontaneous abortion appeared after intra-uterine death of the fetus and also from this fetus no tissue was available for further examinations. In 9 cases a decreased complex I activity was measured in native chorionic villi (7 cases) or cultured chorionic cells (2 cases). In one case the pregnancy ended in a spontaneous abortion after intra-uterine death of the fetus; no tissue was available to confirm the deficiency. In 3 cases out of 9 the parents decided to continue the pregnancy in spite of the complex I deficiency established in fetal cells and 3 affected children were born. The complex I deficiency could be confirmed in muscle tissue (first case), muscle tissue and cultured fibroblasts (second case) and cultured fibroblasts (third case) of the children. In 2 cases out of 9 the pregnancy was terminated. From one fetus no tissue was available, the other fetus was from the second pregnancy in the family that we already described[16] and complex I activity in fetal muscle tissue was normal. In the index-patient of the latter family we found the homozygous 632 C>T mutation in the *NDUFV1* gene as well as a severely decreased complex I activity in muscle tissue and cultured fibroblasts. In one case out of 9 the outcome of the pregnancy is unknown. Recently we diagnosed a pregnancy of twins. The index patient of this family suffered from a complex I deficiency in muscle tissue and cultured fibroblasts and we found a homozygous 686C>A mutation in the *NDUFS2* gene of complex I. In native chorionic villi of one fetus a normal complex I activity was measured and the mutation was not present. The other fetus showed a decreased complex I activity in native chorionic villi and the same homozygous mutation was proven in this tissue. The outcome of this pregnancy is still unknown. In one case out of 9 (mentioned already before in this chapter) a normal complex I activity was measured in native chorionic villi but a decreased one in cultured chorionic cells. The pregnancy was continued and an affected girl was born. Complex I activity was decreased in cultured fibroblasts of the girl. In her cultured fibroblasts and those of the index patient in this family we later found a homozygous 683G>A mutation in the *NDUFS2* gene of complex I (family A in ref. 25).

Combined Deficiencies of Complex I and Complex IV

In 14 pregnancies in 10 families we performed prenatal diagnosis for combined deficiency of complex I and complex IV. In 11 cases normal activities were measured for complex I as well as complex IV in native chorionic villi (10 cases) or amniocytes (one case). In 6 cases a healthy unaffected child was born, in 5 cases the outcome of the pregnancy is still not known. In one case a decreased complex IV with normal complex I activity was measured in native chorionic villi. The pregnancy was terminated but no tissue of the fetus was available. In one case a decreased complex IV activity was measured in native chorionic villi as well as in cultured chorionic cells. Complex I activity was normal in both fractions. The pregnancy ended with an intra-uterine death of the fetus in the 17th week of the pregnancy. No tissue of the fetus was available. In one case a decreased complex I activity was measured in native chorionic villi as well as in cultured chorionic cells. Complex IV activity was normal in both fractions. The pregnancy was terminated and in cultured fibroblasts of the fetus we measured normal activities for complex I as well as for complex IV. Until now we have no explanation for this discrepancy between chorionic tissue and fetal fibroblasts.

Prenatal Diagnosis Only on Molecular Genetic Base

In 3 pregnancies in 2 families we performed prenatal diagnosis only on molecular genetic base. In the index patient of family one we measured an isolated complex I deficiency in cultured fibroblasts and afterwards a homozygous 316 C>T mutation was found in the *NDUFS4* gene of complex I. The fetus was found to be heterozygous for the mutation as measured in native chorionic villi. The pregnancy was continued and a healthy unaffected child was born. In the other family we measured a decreased complex IV activity in cultured fibroblasts of the index patient and afterwards we found a homozygous 688 C>G mutation in the *SURF1* gene. Both parents were found to be heterozygous for the mutation in blood. In 2 pregnancies in this family the fetuses were found to be heterozygous for the mutation, the pregnancies were continued and resulted in two healthy unaffected new-borns.

Table 2. **Control values for respiratory chain enzymes in native chorionic villi,cultured chorionic cells and cultured amniocytes**

Native chorionic villi		**Mean ± SD**	**Range**
Complex IV *	(n= 29)	530 ± 200	270-920
CompIex I*	(n=21)	160±60	80-260
Cultured chorionic cell	(n= 13)		
CompIex IV*		688 ± 119	500-860
CompIex I**		264 ± 43	186-330
Complex III**		2591 ± 542	1620-3605
SCC**		334 ± 105	190-530
Cultured amniocytes	(n= 10)		
CompIex IV*		510 ± 173	250-840
CompIex I**		344 ± 93	220-530
Complex III**		2644 ± 701	1770-3600
SCC**		332± 100	240-590

*Values expressed in mU/U Citrate synthase; **values expressed in mU/U Cytochrome oxidase. Activities in native chorionic villi were measured in crude homogenate. Activities in cultured cells were measured in a mitochondrial enriched fraction. SCC: succinate: cytochrome *c* oxido reductase

General Considerations

A diagnosis is considered to be 100% correct when after measuring a normal activity for complex I and/or complex IV in fetal tissue, a healthy unaffected child is born, or in case a decreased activity of complex I and/or complex IV, measured in fetal tissue, has been confirmed in fetal abortion tissue or in tissue of an affected new-born. We made a 100% correct prenatal diagnosis for complex I in 30 out of 36 pregnancies (83%). For complex IV we made a 100% correct diagnosis in 27 out of 45 pregnancies (60%). For combined deficiency of complex I + complex IV we made a 100% correct diagnosis in 6 out of 14 pregnancies (43%) but in this group the number of pregnancies with unknown outcome was relatively high (5 of 14 or 36%). Considering only the group of combined deficiencies with known outcome of the pregnancy the score was 6 of 9 (67%). In our laboratory most of the demands for prenatal diagnosis are coming from abroad and in our experience most of the chorionic villi samples and amniocytes are taken in a university clinic after we made thorough appointments with the sender. However, after the surgery the patients return to their peripheral hospital and if relevant, the abortion is also done there. In those cases it is

mostly impossible to get cultured fibroblasts of the aborted fetus and it takes a lot of time and efforts to ascertain the outcome of the pregnancy.

Conclusion

Considering all the aspects we met in the last 18 years since we started with prenatal diagnosis for OXPHOS disorders, we think that with strict observance of some precautions, we can offer reliable prenatal diagnosis, especially for complex I and complex IV. We gathered control values for complex I and complex IV in native chorionic villi, cultured chorionic cells and cultured amniocytes. Recently we also gathered control values for complex III and SCC in cultured chorionic cells and cultured amniocytes to be able to offer also prenatal diagnosis for these enzymes (for reference values of complex I, III, IV and SCC, see Table 2). Until now we did not receive a request for prenatal diagnosis for these enzymes.

However, mitochondrial respiratory chain enzyme deficiencies are not the only cause of mitochondrial disease. The already mentioned mutations that have been proven in genes as those encoding for proteins involved in homeostasis and import of proteins over the mitochondrial inner membrane, can lead to disturbances in the mitochondrial energy metabolism that can not be detected with an enzymatic assay. Especially for these cases an assay that measures the overall capacity of the mitochondrial oxidative phosphorylation would be suitable and could also be used as a screening method for disturbances of the mitochondrial energy metabolism. Chowdhury et al.[26] described a polarographic method on digitonin-permeabilised cultured amniocytes that looks suitable. However, they did not test the method on amniocytes with for instance a proven defect in one of the mitochondrial respiratory chain enzymes. Finally, the whole mitochondrial proteome will probably be solved in the future by proteomics and this technique could provide a valuable tool in prenatal diagnosis for mitochondrial disease.

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CHAPTER 6

Spectrophotometric assay for complex I of the respiratory chain in tissue samples and cultured fibroblasts

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Abstract

Background: A reliable and sensitive complex I assay is an essential tool for the diagnosis of mitochondrial disorders, but current spectrophotometric assays suffer from low sensitivity, low specificity, or both. This deficiency is mainly due to the poor solubility of coenzyme-Q analogs, and reaction mixture turbidity caused by the relatively high concentrations of tissue extract that are often required to measure complex I.

Methods: We developed a new spectrophotometric assay to measure complex I in mitochondrial fractions and applied it to muscle and cultured fibroblasts.

The method is based on measuring 2,6-dichloroindophenol reduction by electrons accepted from decylubiquinol, reduced after oxidation of NADH by complex I. The assay thus is designed to avoid nonspecific NADH-oxidation because electrons produced in these reactions are not accepted by decylubiquinone, resulting in a high rotenone-sensitivity.

Results: The assay was linear with time and amount of mitochondria. The Km values for NADH and 2,6-dichloroindophenol in muscle mitochondria were 0.04 and 0.017 mmol/L, respectively. The highest complex I activities were measured with 0.07 mmol/L decylubiquinone and 3.5 g/L bovine serum albumin (BSA). The latter was an essential component of the reaction mixture, increasing the solubility of decylubiquinone and rotenone. In patients with previously diagnosed complex I deficiencies, the new assay detected the complex I deficiencies in both muscle and fibroblasts.

Conclusions: This spectrophotometric assay is reproducible, sensitive, and specific for complex I activity because of its high rotenone sensitivity, and it can be applied successfully to the diagnosis of complex I deficiencies.

Complex I (NADH:ubiquinone oxidoreductase, EC 1.6.5.3) is the first complex of the oxidative phosphorylation system (OXPHOS). It is the entry point for electrons into the respiratory chain (RC) by oxidation of NADH and transport of electrons to coenzyme-Q_{10}. Complex I also has proton transporting activity over the inner mitochondrial membrane to the intermembrane space. With a relative molecular mass of about 980000, it is the largest complex of the respiratory chain (RC). Complex I consist of 45 subunits (identified so far), forming a characteristic L-shape-configuration[1]. The hydrophilic peripheral arm stretches out into the mitochondrial matrix and catalyzes the NADH-oxidation and electron transport. The hydrophobic membrane arm is embedded in the inner mitochondrial membrane and contains the proton-transport activity. A deficiency of complex I is probably the most frequently encountered cause of mitochondrial disease, and mutations in several nuclear DNA-encoded and mitochondrial DNA-encoded subunits have been described to date[2]. In addition, mutations in the mitochondrial tRNAs, such as the m.3243A>G-mutation in the mitochondrial $tRNA^{LEU(UUR)}$, usually result in complex I deficiency[2]. The most commonly used technique for measuring complex I is a spectrophotometric assay measuring rotenone-sensitive NADH-oxidation at 340 nm in tissue-homogenate or mitochondria-enriched fractions from cultured fibroblasts[3,4]; however, the sensitivity and specificity of these assays are not optimal. One reason for this deficiency is the poor solubility of coenzyme-Q analogues. In addition, owing to the low sensitivity, relatively high concentrations of tissue extract are often required to detect complex I, resulting in turbidity of reaction mixtures. Here we describe a new sensitive and specific assay for complex I that is suitable for diagnostic purposes.

Materials and Methods

Materials

Coenzyme Q_1, decylubiquinone, 2,6-dichloroindophenol (DCIP), rotenone and antimycin-A were obtained from Sigma. NADH and bovine serum albumin (BSA; fraction V, fatty acid free) were obtained from Roche. All other chemicals were of the highest purity commercially available. Spectrophotometric assays were performed on a Perkin-Elmer Lambda 25 UV/Vis spectrophotometer with an automatic water thermostattable 8-cell holder in disposable semi-micro 10-mm acryl-cuvettes from Sarstedt.

Preparation of muscle-mitochondria and mitochondrial enriched fractions from cultured skin fibroblasts

Human muscle tissue (musculus quadriceps or musculus semitendinosus) was homogenized as described[5]. Fractions of 200 to 300 µL of a frozen 600*g* supernatant were thawed at 2-4 ^{0}C and centrifuged (10 min at 14000*g* and 2 ^{0}C) in an Eppendorf 5402 centrifuge. We carefully removed the 14000*g* supernatant and the fluffy layer with an Eppendorf pipettor. The mitochondrial pellet was resuspended in 150 µL 10 mmol/L Tris, pH 7.6, frozen in 25-µL aliquots in liquid nitrogen and kept at -80 ^{0}C.

We cultured human skin fibroblasts in M199 medium (GIBCO) supplemented with 20% (vol/vol) fetal calf serum. Aliquots of 10 to15 x 10^6 cells were washed with ice-cold phosphate-buffered saline, frozen in liquid nitrogen and kept at -80 ^{0}C until use. For isolation of mitochondria-enriched fractions, the pellets were thawed at 2-4 ^{0}C and suspended in 2.9 mL ice-cold 10 mmol/L Tris, pH 7.6. We disrupted the cells mechanically with a 5 mL glass/Teflon Potter-Elvehjem homogenizer (clearance 0.025 mm), 8 strokes at 1800 rpm in melting ice. After homogenization, we added 0.6 mL ice-cold 1.5 mol/L sucrose and centrifuged the homogenate (10 min at 600*g* and 2 ^{0}C) in a Sorval-RC2B centrifuge. The 600*g* supernatant was centrifuged again (10 min at 14000*g* and 2 ^{0}C) and the resulting supernatant was carefully removed. The mitochondrial pellet was resuspended in 0.5 mL of 10 mmol/L Tris, pH 7.6, frozen in 50 µL aliquots in liquid nitrogen and kept at -80 ^{0}C.

COMPLEX I ASSAY

In the new complex I assay DCIP is used as a terminal electron-acceptor. Complex I oxidizes NADH and the electrons produced reduce the artificial substrate decylubiquinone that subsequently delivers the electrons to DCIP. The reduction of DCIP can be followed spectrophotometrically at 600 nm. As the electrons produced by other NADH-dehydrogenases are not accepted by decylubiquinone[3], reduction of DCIP is almost completely caused by complex I activity, resulting in a very high rotenone-sensitive activity.

We measured complex I spectrophotometrically at 600 nm in an incubation volume of 1.0 mL containing 25 mmol/L potassium phosphate, 3.5 g/L BSA, 60 µmol/L DCIP, 70 µmol/L decylubiquinone, 1.0 µmol/L antimycine-A, and 0.2 mmol/L NADH, pH 7.8. Decylubiquinone and antimycine-A were dissolved in dimethylsulfoxide (DMSO) (17.5 mmol/L and 1.0 mmol/L, respectively). We prepared a stock solution of 80 g/L BSA in 5 mmol/L potassium phosphate buffer, pH 7.4. Because BSA is a critical component of the complex I assay, we measured the concentration spectrophotometrically at 280 nm (A_{280} 1 g/L BSA=0.667). The stock solution was diluted to 70 g/L and stored in 1-mL aliquots at -30 °C. Of this solution, 50 µL was added to a final reaction volume of 1 mL. We preincubated an aliquot of 2.5 to10 µL mitochondrial suspension from muscle or 20 µL mitochondria-enriched fraction from fibroblasts at 37 ^{0}C in 960 µL incubation mixture without NADH. After 3 min, we added 20 µL 10 mmol/L NADH and measured the absorbance at 30-s intervals for 4 min at 37 ^{0}C. After 4 min, we added 1.0 µL rotenone (1 mmol/L in DMSO) and measured the absorbance again at 30-s intervals for 4 min.

Complex I activity was expressed as mU/U complex II, mU/U complex IV, or U/g protein, in which 1 U complex I activity equals 1 µmol DCIP reduced per min. Fibroblasts from 6 patients and muscle tissue samples from 3 patients with a diagnosed complex I deficiency were used to measure complex I to demonstrate the applicability of the assay to the diagnosis of complex I deficiency. The controls and patients have been described[5].

COMPLEX II ASSAY

During isolation of mitochondria from frozen samples of muscle or fibroblasts, part of the citrate synthase (CS) leaks out of the mitochondria and is lost, so, CS can not be used as a mitochondrial marker enzyme. For that reason, we used complex II and complex IV as

mitochondrial marker enzymes. We measured complex II spectrophotometrically at 600 nm, as described, with some modifications[6]. The 1.0 mL incubation volume contained 80 mmol/L potassium phosphate, 1 g/L BSA, 2 mmol/L EDTA, 0.2 mmol/L ATP, 10 mmol/L succinate, 0.3 mmol/L potassium cyanide (KCN), 80 µmol/L DCIP, 50 µmol/L decylubiquinone, 1 µmol/L antimycine-A and 3 µmol/L rotenone, pH 7.8. We preincubated an aliquot of 5 µL mitochondrial suspension from muscle or 10 µL mitochondria enriched fraction from fibroblasts at 37 ^{0}C in the incubation mixture without KCN and succinate. After 10 min, we added KCN and succinate to start the reaction and measured the absorbance at 1-min intervals for 5 min at 37 ^{0}C. Blanks were measured in the presence of 5 mmol/L malonate that was added before preincubation. Decylubiquinone (10 mmol/L) was dissolved in DMSO. Antimycine-A (1 mmol/L) and rotenone
(3 mmol/L) were dissolved in 1000 mL/L ethanol. For both assays, we used a molar absorptivity at 600 nm of 19.1 $(mmol/L)^{-1}$. cm^{-1} for DCIP.

COMPLEX IV AND PROTEIN ASSAYS

We measured complex IV activity as described[7] and protein according to Lowry et al[8].

Results

Lineweaver-Burk-plots revealed Km values of 0.04 mmol/L for NADH and 0.017 mmol/L for DCIP (data not shown). The pH-optimum was 7.8 (data not shown). In a concentration series experiment with decylubiquinone, the highest complex I activity was at 0.07 mmol/L decylubiquinone (data not shown). We tested coenzyme Q_1 as an alternative ubiquinone analogue, and found that in the presence of 0.07 mmol/L, the activity was approximately 80% of that measured with decylubiquinone, with similar rotenone sensitivities. Therefore we continued with decylubiquinone as electron acceptor for complex I in the reaction mixture. Although in most complex I assays Mg^{2+} and KCN are added, the latter to inhibit nonspecific NADH dehydrogenase activity[3], we observed no influence of Mg^{2+} and KCN in our assay (data not shown).

In most complex I assays, pretreatment of the mitochondria, such as repeated freezing and thawing or sonication, is necessary to disrupt the mitochondrial membrane. In our assay, a simple osmotic shock in 10 mmol/L Tris.Cl, pH 7.6, followed by a single freeze-thaw

cycle, was sufficient to measure optimal complex I activity. Repeated freezing and thawing did not improve this result, and sonication even decreased complex I activity (data not shown).

Complex I activity was linear for at least 4 min, with sample amounts varying between 0.25 µg protein (containing 0.9 mU complex IV) and 3 µg protein (11 mU complex IV) (Fig. 1).

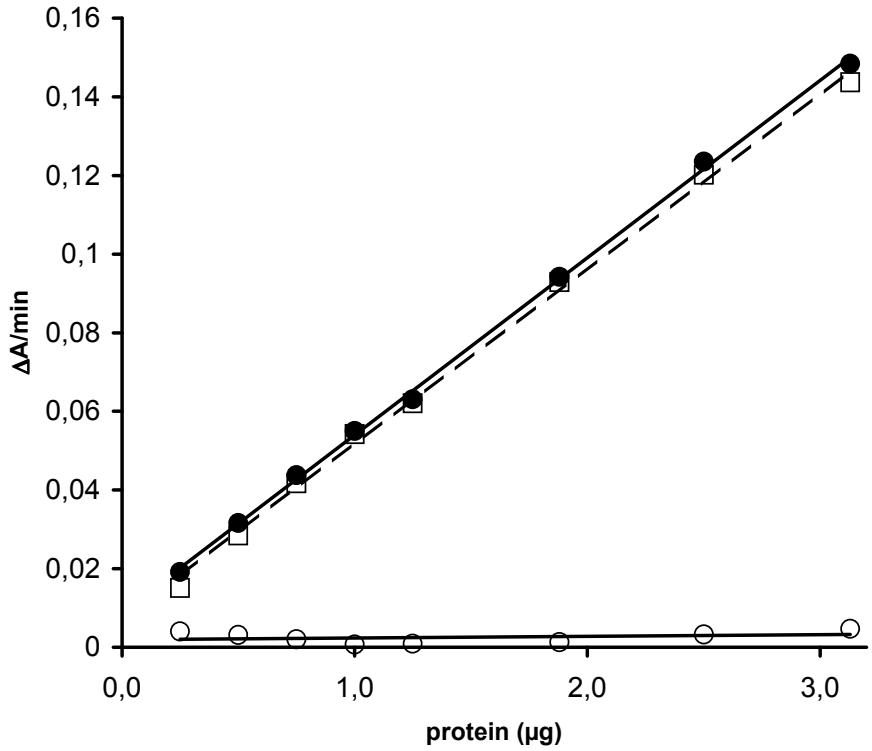

Fig. 1. Complex I activity versus the amount of muscle mitochondrial protein.

Complex I activity is expressed as ΔA/minute. Calculated linear regression line for the total activity (filled circles) was Y = 0.0451 X + 0.0089, R^2 = 0.999 and for the rotenone sensitive activity (squares) Y = 0.0446 X + 0.007, R^2 = 0.998. All data are mean values of incubations in duplicate. (open circles: Rotenone insensitive activity).

Figure 2. (A)

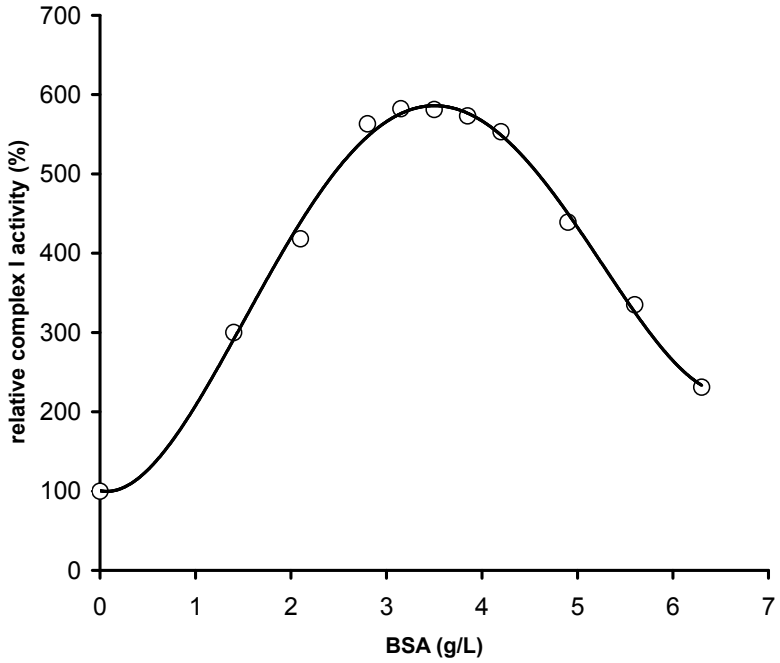

Figure 2. (B)

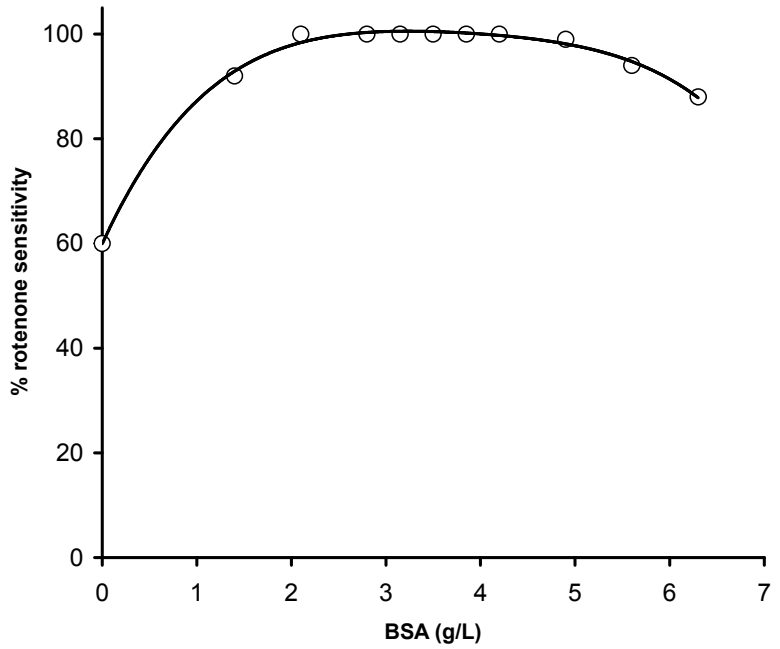

Fig. 2A+B. Effect of BSA on relative rotenone-sensitive complex I activity in muscle mitochondrial suspensions.

Complex I activity measured in the absence of BSA was set to 100%. Mitochondrial protein content in each incubations was 0.3 g/L. Data are the mean values of 2 experiments performed with different mitochondrial preparations, and all incubations were performed in duplicate. (B), effect of BSA on the percentage of rotenone-sensitive activity measured in the complex I assay. Complete inhibition by rotenone corresponds to 100% rotenone sensitivity. The percentages calculated were derived from the incubations described in (A).

Because BSA is essential for measuring optimal complex I activity (3), we studied the effect of BSA on the complex I activity in muscle mitochondria. We measured optimal complex I activity in the presence of BSA concentrations between 3.2 and 3.9 g/L, whereas the percentage rotenone sensitivity of complex I was near 100% at BSA concentrations between 2.1 and 4.9 g/L BSA (Fig. 2). Similar results were obtained when decylubiquinone was replaced by coenzyme Q_1 in the reaction mixture (data not shown).
The IC_{50} for rotenone in muscle mitochondria, which could only be determined in the presence of BSA, was 13.5 nmol/L (mean of 2 results, range 10-17 nmol/L) (Fig. 3). The presence of BSA in the reaction mixture is required not only for rotenone sensitivity of the assay (probably by solubilizing rotenone), but also for solubilization of decylubiquinone, as illustrated by the fact that in the absence of BSA, we observed an orange/yellow layer on the surface of the reaction mixture after centrifugation at 14000*g*. By spectrophotometric analysis at 278 nm, we found that approximately 70% of the amount of decylubiquinone added to the mixture was present in this layer.

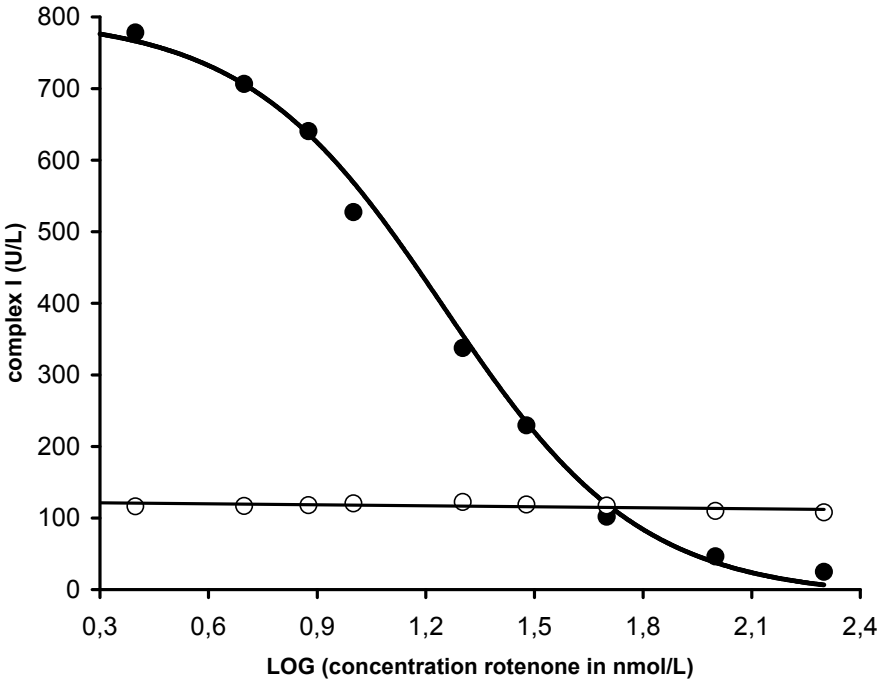

Fig. 3. Inhibition of complex I activity by rotenone measured in muscle mitochondria.

Complex I was measured in the absence (open circles) or presence (filled circles) of 3.5 g/L BSA, and with the rotenone concentration interval as indicated. Rotenone sensitive complex I activity (U/L mitochondrial suspension) is shown. Reaction mixtures contained 1.3 mg/L protein. Using nonlinear regression analysis, the mean (SD) IC_{50} for rotenone was 13.5 (3.5) nmol/L.

Intraassay imprecision (CV) was between 2% and 8%, and inter assay imprecision was between 2% and 11% (Table 1). To test the long-term reproducibility of the assay, complex I was measured repeatedly in 3 muscle mitochondrial samples over a period of 6 months. Mean (SD) complex I activity in these samples was 607 (47) U/L (n = 10; CV = 8%), 795 (30) U/L (n = 6; CV = 4%), and 888 (49) U/L (n = 6; CV = 5%).

To compare this method to the method described by Fischer et al.[3], we used the 2 methods to measure complex I in mitochondria from muscle and fibroblasts.

Table 1. **Intra- and interassay imprecision for complex I in mitochondrial fractions from muscle and cultured fibroblasts [a].**

Intraassay Imprecision		**complex I (U/L)**				
muscle mitochondria	**Exp. 1**	**Exp. 2**	**Exp. 3**	**mean**	**SD**	**CV%**
10 µL	240	267	262	256	14	6
10 µL 1:1 dilution	248	234	276	253	21	8
10 µL 1:2 dilution	252	249	241	247	6	2
Fibroblast mitochondria						
20 µL	117	131	124	124	7	6
20 µL 1:1 dilution	124	118	130	124	6	5
20 µL 1:2 dilution	132	129	123	128	5	4
Interassay Imprecision						
muscle mitochondria						
10 µL	251	256	263	257	6	2
10 µL 1:1 dilution	280	252	280	271	16	6
10 µL 1:2 dilution.	249	243	297	263	30	11
fibroblast mitochondria						
20 µL	124	131	110	122	11	9
20 µL 1:1 dilution	124	134	116	125	9	7
20 µL 1:2 dilution	129	147	123	133	13	9

[a] Complex I activities are expressed as U/L undiluted sample. Intra- and interassay imprecision were determined with undiluted, 1:1 diluted and 1:2 diluted mitochondrial fractions from muscle and cultured fibroblasts. The intraassay imprecision was determined by measuring complex I activities in 3-fold on the same day, and interassay imprecision was determined by measuring complex I activities on 3 different days. All incubations were performed in duplicate. The protein contents of the undiluted muscle and fibroblast mitochondrial fractions were 0.26 and 0.88 g/L, respectively.

The mean (SD) activity measured in muscle mitochondria using the method of Fischer et al. (3) was 31% (5%; n = 7; range: 26%-38 %; paired t-test p=0.0005) and in mitochondria from fibroblasts was 17% (3%; n = 46; range: 10%-26%; paired t-test $p=7x10^{-29}$) of the activities measured with our method, showing that the new method is 3-fold (for muscle) and more than 5-fold (for fibroblasts) more sensitive than the method of Fischer et al.[3].
We assessed the specificity of the complex I assay by measuring its rotenone sensitivity. The mean (SD) rotenone sensitivity in mitochondria from control muscle (n=17) was 95% (5%), and in mitochondria from control fibroblasts (n=46), it was 82% (9%).

Table 2. **Control values for complex I in mitochondrial fractions from muscle and cultured fibroblasts[a].**

	Complex I	
Muscle	**mU/U CII**	**mU/U C IV**
Mean (n = 17)	1140	343
SD	180	63
Observed range	783 - 1497	270 - 475
Mean (2 SD)	780 - 1500	217 - 469
Fibroblasts		
Mean (n = 46)	1161	1100
SD	237	245
Observed range	720 - 1708	678 - 1675
Mean (2 SD)	686 - 1636	610 - 1591

[a] All incubations were performed in duplicate.

We tested whether the new complex I assay could be applied to cruder muscle preparations than those used in the experiments described above. In 5 control muscle samples, the mean (SD) complex I activity in 600*g* supernatants was 46% (6%; range, 43% – 51%) of that in equivalent amounts of mitochondrial fractions, and the rotenone sensitivity was 71% (14%; range, 48% – 86%). Finally, we tested whether the new complex I assay could be applied to the diagnosis of complex I deficiencies in both muscle and fibroblasts. First, we measured control values for mitochondria from muscle and fibroblasts (Table 2). We examined fibroblasts from 6 patients carrying mutations in different complex I genes and suffering from a previously established complex I deficiency[5]. Using the new method, the enzyme deficiency could be confirmed in all 6 patients. In 3 of the patients, we measured complex I in muscle and also confirmed the deficiency in this tissue. The method showed lower results in all tested patients with complex I deficiencies than in any control subjects (Table 3).

***Table 3*. Complex I in muscle and fibroblasts of 6 patients with a previously established complex I deficiency.[a]**

Patient[a]	**Complex I by our method**[b]		**Complex I by the method of Fischer et al.** *(3)*
Muscle	**mU/U CII**	**mU/U C IV**	**mU/U CS**[c]
1	154	48	14
3	510	144	16
5	465	157	24
Control mean (2SD)	1140 (360)	343 (126)	85 (40)
Observed range	783 - 1497	270 - 475	53 - 163
n	17	17	43
Fibroblasts			**mU/U C IV**[c]
1 (NDUFS2)	255	179	29
2 (NDUFS4)	240	145	64
3 (NDUFS7)	484	459	65
5 (NDUFS7)	438	467	26
7 (NDUFV1)	426	416	85
8 (MT-ND2)	339	333	42
Control mean (2SD)	1161 (474)	1100 (490)	188 (104)
Observed range	720 - 1708	678 - 1675	110 - 260
n	46	46	14

[a] Patient-numbering is the same as in Janssen et al. *(5)*.

[b] Complex I was measured in mitochondrial fractions.

[c] Complex I was measured in 600*g* supernatants of muscle and mitochondrial fractions from fibroblasts.

Discussion

At present, the diagnosis of complex I deficiency is usually established using complex I assays that are based on the spectrophotometric measurement of rotenone-sensitive NADH oxidation in patient-derived tissue samples and cultured fibroblasts. In addition to complex I, muscle tissue and cultured fibroblasts contain several nonmitochondrial NADH-oxidizing dehydrogenases. Therefore, the use of tissue homogenates in complex I assays results in a relatively high rate of rotenone-insensitive NADH oxidation that interferes with the sensitivity of the complex I assay. Another disadvantage of measuring in muscle homogenate is the turbidity of the incubation-mixture which interferes with the spectrophotometric assay. For these reasons, Brooks and Krähenbühl[9] developed a radiochemical assay for complex I in muscle by measuring $^{3}H_2O$ production from

[4B-^{3}H]-NADH oxidation, based on the stereo-specificity of complex I for the 4B hydrogen atom of NADH.

Our new assay uses no radioactivity , is suitable for the diagnostic analysis of complex I in fibroblasts and muscle tissue, and uses DCIP as a final electron-acceptor. DCIP has a molar absorptivity that is approximately 3-times higher than that of NADH: the molar absorptivity at 600 nm of DCIP is 19.1 $(mmol/L)^{-1}$ $.cm^{-1}$ whereas the molar absorptivity at 340 nm of NADH is 6.2 $(mmol/L)^{-1}$ $.cm^{-1}$. Compared with the method described by Fischer et al.[3] that measures NADH oxidation, a 3- to 5-fold more complex I activity is measured using our method.

DCIP has been used by others in a complex I assay[10], but that assay was not suitable for diagnostic purposes because of nonlinearity of the absorbance with time. In our assay, the addition of an optimal concentration of BSA to the reaction mixture, combined with the use of isolated mitochondrial preparations instead of crude sample homogenates, resulted in a complex I activity that was linear in time and had high rotenone sensitivity. BSA is essential, as it facilitates the solubilization of both rotenone and decylubiquinone. Rotenone and decylubiquinone are both hydrophobic substances and practically insoluble in water; rotenone strongly and reversibly binds to BSA[11]. Direct binding to BSA probably also plays a role in the solubilization of decylubiquinone, as it is known that BSA reversibly binds molecules with long alkyl chains[10].

The mean (SD) complex I activities measured in fibroblasts and expressed on complex IV activity were similar to those measured by Kramer et al.[4]: 1100 (245; n=46) vs 1200 (170; n=15), respectively. The mean (SD) rotenone-sensitive activity in our assay was 82 (9%; n=46) compared with 30% (range, 15-50%) in the assay described by Kramer et al.[4]. The mean (SD) rotenone-sensitivity measured in digitonin-and Percoll-treated fibroblasts as described by Chrétien et al.[13], 86 (19%; n=22), was similar to our results. The radiochemical enzyme assay of Brooks and Krähenbühl[9] gave a slightly lower rotenone sensitivity of 60 – 80%[9]. As both Chrétien et al.[13] and Brooks and Krähenbühl[9] measured complex I in crude lysates and expressed activities on protein base, it was not possible to directly compare the complex I activities measured by these 2 methods with our results.

The method we developed is also suitable for measurements of complex I in 600*g* supernatants from muscle. The sensitivity and specificity appeared to be lower than observed with mitochondrial fractions.

This complex I assay has clear advantages over the commonly used assays because it is nonradioactive; shows high sensitivity, precision, and rotenone-sensitivity; and can be

performed on a simple spectrophotometer. The method is applicable to the analysis of muscle samples and is also suitable for measuring complex I in cultured fibroblasts.

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CHAPTER 7

Summery and suggestions for further studies

7.1. Summary

Chapter 1 describes the development of the currently practiced diagnostic methods to search for disturbances in the MEGS. A historical overview of the discovery of the biochemical pathways that comprise the MEGS is presented. Moreover, the present state of the molecular genetic studies concerning disturbances of the MEGS is presented.

Chapter 2 presents an overview of some practical aspects of diagnostics for mitochondrial disorders in muscle biopsies, cultured fibroblasts and chorionic villi. Before performing a muscle biopsy the patients should fulfill some prerequisites concerning clinical presentation and clinical chemical findings. A fresh muscle biopsy is preferred over a frozen biopsy because in fresh samples MEGS-capacity can be measured which provides optimal information, whereas in frozen samples only single enzyme activities can be measured. Investigations in cultured fibroblasts are important, but preferably start after a fresh muscle biopsy has been investigated or if no muscle tissue is available. Cultured fibroblasts are important for molecular genetic studies and for further biochemical studies, especially when there is a request for enzymatic prenatal diagnostics. Further studies on muscle tissue are mostly limited by lack of sufficient material.

Chapter 3 describes the method for measuring MEGS-capacity in fresh muscle tissue. This method has been used in our laboratory for more than twenty-five years and the reliability has been shown now. Many patients were diagnosed with disturbances in the MEGS in combination with deficiencies in RC-complexes I to IV, complex V or PDHc. In the majority of these patients the diagnosis was confirmed at the molecular genetic level. With the MEGS-capacity data obtained in these specific patients we evaluated the theoretical background behind all the incubations. The COV's for all the MEGS parameters (calculated from the intra-assay variations) showed that the method is reproducible.

Chapter 4 describes the results of a comprehensive study of 24 patients carrying the classic mtDNA 3243A>G MELAS mutation. MEGS-capacity and RC-enzyme activities were measured in muscle tissue together with extended molecular genetic studies on mtDNA: determination of the mutation load and sequencing of all the m.tRNAs if possible. In none of the patients additional mutations in the m.tRNAs were found. MEGS-capacity and RC-enzymes activities in the patient group were significantly decreased. In the patient group a

relevant correlation was proven between MEGS-capacity and mutation load, a phenomenon not described before. After dividing the patient-group in three subgroups with increasing percentages mutation load (12%-50%, 51%-80% and 81%-100%), MEGS parameters were significantly decreased in all three subgroups, while complex I, III and IV activities were only significantly decreased in the subgroup with high percentage mutation load (81%-100%). With help of quadratic regression analysis a significant correlation was found between complex I activity and the oxidation rate of [1-^{14}C]pyruvate + malate + ADP and an almost significant correlation between complex I activity and the ATP production rate. Sensitivity and specificity of the various assays with respect to MELAS syndrome were calculated. ”Sensitivity” indicates how well the presence of a pathologic test recognizes the presence of the disease and “specificity” reflects the probability of a healthy person to have a normal test value. The sensitivity for the MEGS parameters as well as for the complex I, III and IV activities was 100%. The specificity was clearly better for the MEGS parameters (65%-74%) than for the respiratory chain enzymes activities (29%-42%). Two patients showed much more decreased MEGS-capacity as predicted from their mutation load. In both patients the *ND* genes and the m.rRNAs were sequenced but no additional mutations were found in these genes. One patient with a high percentage mutation load showed normal MEGS-capacity and RC-enzyme activities. In all three patients the cause of the deviant biochemical phenotype remains unclear and might be associated with an unknown factor in their genetic background. Measurement of MEGS-capacity in parallel with RC-enzymes in patients with m.tRNA mutations, provides better information about mitochondrial function than only measuring RC-enzymes. Measuring MEGS parameters discriminates better because these parameters are decreased at a lower percentage mutation load than RC-enzymes activities. The MEGS parameters also showed better sensitivity than the RC-enzymes.

Chapter 5 presents an overview of 18 years experience with prenatal diagnostics for OXPHOS disorders. Enzymatic prenatal diagnostics for OXPHOS disorders can only be performed with strict precautions and after intensive deliberation between parents, physicians and laboratory staff. Prenatal diagnostics is most reliable when the genetic defect in the probandus is known but unfortunately in many cases of complex I and IV deficiency the genetic defect is unknown. Enzymatic prenatal diagnostics can only be performed when three important prerequisites are fulfilled: the deficiency in the probandus must be detectable in cultured fibroblasts and at least in one other tissue, by preference muscle tissue, maternal contamination of the embryonic tissue must have been excluded and mtDNA mutations must

have been excluded as a cause of the disease. Prenatal diagnostics should be performed by preference in native chorionic villi because this tissue can be taken in an early stage of the pregnancy and with less risks for the fetus.

Chapter 6 describes a new spectrophotometric assay for complex I. Because in this assay complex I activity is measured in isolated mitochondrial fractions from frozen material, complex IV and complex II were used as reference enzymes. Therefore a newly developed complex II assay is also described in this chapter. This complex II assay is based on a formerly published assay with some major modifications. Our formerly developed assays for complex I and II were reliable and sensitive, but were less appropriate for measuring large amounts of samples. The new complex I assay shows high sensitivity, precision and rotenone-sensitivity and can be performed on a simple spectrophotometer. The method was also very suitable for measuring complex I activity in cultured fibroblasts. The method was tested by measuring complex I activity in cultured fibroblasts and muscle tissue of patients with proven complex I deficiency. The complex I deficiencies were confirmed in all samples. In most complex I assays BSA is an essential constituent of the reaction mixture but the influence was not exactly known. We showed that BSA is essential as it facilitates the solubility of both rotenone and decylubiquinone.

7.2. Future perspectives

MEGS-capacity can be studied in a reliable and sensitive manner, by measuring oxidation rates of ^{14}C labeled substrates and ATP production rate from oxidation of pyruvate. For these assays, together with measurements of single enzyme assays of OXPHOS complexes and PDHc, at least 300 mg of muscle tissue are needed. This amount of muscle tissue can only be obtained by a surgical biopsy under total anesthesia, with certain risks for the patients. For most adults this amount of muscle tissue can also be obtained by taking several needle biopsies under local anesthesia but this is still an uncomfortable procedure for the patients. Miniaturizing the assays for measuring MEGS-capacity and automation of the assays of the OXPHOS complexes could lead to a six-fold reduction of the amount of muscle tissue needed to perform all these assays. The development of these miniaturized assays for measuring MEGS-capacity and automation of the assays for measuring OXPHOS-complexes have been started. Investigation whether a disturbed MEGS-capacity in muscle tissue of a patient is also

expressed in cultured fibroblasts or cultured myocytes, is also useful because using these cells much more experiments with intact mitochondria can be performed. These assays should also be miniaturized to reduce the minimal amount of cells needed for these experiments. The development of miniaturized assays for studying MEGS-capacity in digitonin-permeabilized cultured fibroblasts has been started. Digitonin partially disrupts the plasma membrane of the cells, making the cells freely accessible for mitochondrial substrates, but keeps the mitochondrial membranes fully intact.

An interesting group of enzymes to study with this miniaturized technique are the mitochondrial carriers (MCs) involved in the MEGS. These enzymes can be studied in cultured fibroblasts as well as in muscle tissue. The MCs have been studied for over three decades and much is known about their structures and functions (del Arco and Satrústegui, 2005; Wohlrab, 2005). Extensively studied MC_S involved in MEGS are the dicarboxylate carrier, the oxoglutarate carrier, the pyruvate carrier, ANT, the glutamate/aspartate carrier, the citrate carrier and the phosphate carrier. However, few studies have been published about disturbances in these MCs in patients suffering from a mitochondriopathy. So far, two mitochondrial carrier deficiencies have been described: one in the pyruvate carrier (Brivet et al., 2003) and one in the phosphate carrier (Mayer et al., 2007). The patients described in these studies were identified with techniques described in Chapter 3 of this thesis. At the moment we have a unique file of about 2500 patients computerized. In muscle tissue of these patients all incubations representing MEGS-capacity have been measured. Interpretation of the MEGS-capacity parameters and single enzyme activities revealed a number of patients that might suffer from a deficiency in one of these MCs. In future studies, assays must be developed to measure MEGS-capacity and MCs-activities in cultured fibroblasts of these patients. To measure specific MC-activities, also the number of substrates can be extended ([2-^{14}C] pyruvate, [1,5-^{14}C] citric acid, [1-^{14}C] ketoglutaric acid and [2,3-^{14}C] fumaric acid). Moreover, specific inhibitors are known for a number of the MCs mentioned above. Therefore, substrate oxidation rates can also be measured in the presence of these inhibitors to identify disturbances in the MCs (Brivet et al. 2003). Additionally, the amount of a MC can be quantified by using a specific inhibitor for (photoaffinity)labeling of the protein (Hildyard et al. 2005). Identification of MCs can also be proceeded by labeling with specific inhibitors and subsequently separation by 2D-electroforesis used in the Proteomics. These studies should be combined with molecular genetic studies, because for a number of MCs the gene sequences are known. These studies might elucidate defects in an important group of mitochondrial proteins that seem to be neglected in the diagnostics for MEGS-disorders.

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CHAPTER 8

Samenvatting

Samenvatting

Het in dit proefschrift beschreven onderzoek had als hoofddoel de biochemische technieken, die gebruikt worden bij het opsporen van patiënten met een mitochondriële (encephalo)myopathy te evalueren aan de hand van een aantal patiënten met een bewezen stoornis in het mitochondriële energie genererende systeem. Mitochondriën kunnen gezien worden als de energiecentrales van de cel. Het ziektebeeld van patiënten die lijden aan een mitochondriële (encephalo)myopathy wordt bepaald door een falende energieproductie in de mitochondriën van voornamelijk hersenen en spieren. De klinische symptomen bij deze patiënten variëren van spierzwakte, epilepsie en ontwikkelingsstoornissen bij jonge kinderen tot inspanningsgebonden klachten en diverse neurologische afwijkingen bij volwassenen. De belangrijkste enzymen die een rol spelen in de mitochondriële energieproductie zijn het pyruvaatdehydrogenase-complex (PDHc), de enzymen van de citroenzuurcyclus (CAC) en de vijf enzymcomplexen van de oxidatieve fosforylering (OXPHOS), complex I t/m V. Patiënten met een mitochondriële ziekte hebben meestal een deficiëntie in een of meer van deze enzymen. Een stoornis in de mitochondriële energieproductie in deze patiënten kan het beste worden opgespoord door het afnemen van een vers spierbiopt, d.w.z. het biopt wordt na afname niet ingevroren maar wordt vers gehomogeniseerd. De mitochondriën komen zo vrij uit de spiercellen maar blijven wel volledig intact. In dit homogenaat wordt vervolgens de totale mitochondriële energieproductie bepaald door het meten van de $^{14}CO_2$ productie uit oxidatie van ^{14}C gelabelde substraten en door meting van de ATP-productie uit oxidatie van pyruvaat.

Hoofdstuk 1 In deze inleiding wordt het verschil beschreven tussen het Mitochondriële Energie Genererende Systeem (MEGS) en de traditioneel gebruikte term OXPHOS. MEGS beslaat het geheel van de mitochondriële enzymatische reacties betrokken bij de productie van energie in de vorm van de energierijke verbinding ATP. MEGS omvat de oxidatie van pyruvaat door PDHc, de oxidatie van het door PDHc of door vetzuuroxidatie gevormde acetylcoenzym-A in de CAC, de oxidatie van de in de CAC gevormde reductie-equivalenten NADH en $FADH_2$ door de ademhalingsketen (AK), de vorming van een protonengradiënt over het mitochondriële membraan door drie complexen van de AK (complex I, III en IV), het herstel van het protonenevenwicht onder gelijktijdige productie van ATP door complex V, tot en met het transport van ATP over het mitochondriële membraan naar het cytoplasma door de adenine-nucleotide translocator (ANT). OXPHOS betreft slechts de vier complexen van de

ademhalingsketen en complex V. In een historisch overzicht wordt beschreven hoe vanaf het begin tot laat in de 20e eeuw de biochemische processen zijn ontdekt en bestudeerd die een rol spelen in het MEGS en wordt de ontwikkeling beschreven van de huidige methodes die gebruikt worden bij het opsporen van stoornissen in dit proces. Mitochondriën zijn van oorsprong α-proteobacteriën die tijdens de evolutie, door het toenemen van de zuurstofspanning in de aardse atmosfeer, zijn opgenomen in primitieve eukaryoten en in endosymbiose met deze cellen verder leefden. Vanwege deze bacteriële oorsprong zijn mitochondriën de enige organellen in dierlijke cellen met een eigen DNA, het mitochondriële DNA (mtDNA). Het mitochondriële genoom bestaat uit 16.569 bp en codeert voor 13 eiwitten van de 5 OXPHOS complexen (de subunits ND1 t/m ND6 en ND4L van complex I, cytochroom *b* van complex III, de subunits COI, II en III van complex IV en de subunits ATPase 6 en 8 van complex V), de *12S* en *16S* rRNA's en 22 tRNA's. Aangezien mitochondriën, wat betreft de cellen betrokken bij de voortplanting, alleen in de vrouwelijke eicel en niet in de kop van de mannelijke zaadcel vóórkomen, erft het mitochondriële DNA over via de moederlijke lijn.

In de laatste decennia heeft moleculair genetisch onderzoek bij mitochondriële ziekten een grote vlucht genomen en voor een groot aantal deficiënties van enzymen van het MEGS zijn nu ook mutaties bekend, zowel in het mtDNA als in het nucleaire DNA (nDNA). Momenteel zijn er meer dan 250 pathogene mutaties bekend in het mtDNA. Als laatste worden in dit hoofdstuk de momenteel bekende mutaties in de nDNA genen, coderend voor enzymen betrokken bij het MEGS, beschreven.

Hoofdstuk 2 geeft een overzicht van een aantal praktische aspecten betreffende de diagnostiek van mitochondriële stoornissen in spierbiopten, gekweekte fibroblasten en chorion villi. Alvorens besloten wordt om bij een patiënt een spierbiopt af te nemen, moet deze voldoen aan een aantal voorwaarden betreffende klinische presentatie en klinisch chemische bevindingen. Bij voorkeur wordt er een vers spierbiopt genomen, omdat hierin naast de afzonderlijke OXPHOS complexen en PDHc, ook het MEGS kan worden gemeten, waardoor de meest optimale informatie kan worden verkregen uit het onderzoek. In ingevroren biopten kunnen alleen de afzonderlijke OXPHOS complexen en PDHc worden gemeten. Onderzoek aan gekweekte fibroblasten is ook belangrijk, maar wordt bij voorkeur uitgevoerd na onderzoek aan een vers spierbiopt, of indien er geen spierweefsel beschikbaar is. Gekweekte fibroblasten zijn belangrijk voor moleculair genetisch onderzoek en voor verdere biochemische studies, omdat deze studies meestal niet meer aan spierweefsel

uitgevoerd kunnen worden vanwege de beperkte beschikbare hoeveelheid van dit weefsel. Indien er vraag is naar enzymatische prenatale diagnostiek voor een van de enzymen van het MEGS, is het noodzakelijk dat de enzymdeficiëntie ook is aangetoond in gekweekte fibroblasten van de patiënt.

Hoofdstuk 3 beschrijft de methode voor het meten van het MEGS in intacte mitochondriën uit vers spierweefsel. De methode wordt al meer dan vijfentwintig jaar toegepast in ons laboratorium. Veel patiënten met stoornissen in het MEGS zijn met deze methode gediagnostiseerd, waarna deficiënties werden aangetoond in onder andere de AK-complexen I t/m IV, complex V of PDHc. Bij de meeste patiënten werd de diagnose bevestigd, doordat er mutaties werden gevonden in structurele genen, in assemblage genen of in het mtDNA. Aan de hand van de gemeten waarden van de MEGS capaciteit in deze patiënten werden de theoretische achtergronden van alle incubaties geëvalueerd en bevestigd. Voor alle MEGS parameters werd de intra-assay variatie bepaald. De berekende variatie coëfficiënten voor alle parameters toonde de reproduceerbaarheid van de methode aan.

Hoofdstuk 4 beschrijft een uitgebreide studie van 24 patiënten met de klassieke mtDNA 3243A>G MELAS mutatie. In spierbiopten van deze patiënten werden de MEGS capaciteit en de AK-complexen gemeten, in combinatie met uitgebreide moleculair genetische studies aan het mtDNA. Van alle patiënten werd het percentage heteroplasmie voor de mutatie bepaald en indien mogelijk werden alle m.tRNA's gesequenced. Buiten de 3243A>G mutatie werd in geen van de patiënten een additionele mutatie gevonden in de m.tRNA's. MEGS capaciteit en de activiteiten van de AK-complexen waren in de patiëntengroep significant verlaagd. In de patiëntengroep werd een relevante correlatie aangetoond tussen percentage heteroplasmie en de MEGS capaciteit, een fenomeen nog niet eerder beschreven. Na opdeling van de patiëntengroep in drie subgroepen met oplopend percentage heteroplasmie (12%-50%, 51%-80% en 81%-100%), bleken de MEGS parameters in alle drie de groepen significant verlaagd ten opzichte van de controles, terwijl de activiteiten van complex I, III en IV alleen in de groep met een hoog percentage heteroplasmie (81%-100%) significant verlaagd waren. Met behulp van kwadratische regressieanalyse werd er een significante correlatie aangetoond tussen complex I en de oxidatie van [1-14C]pyruvaat + malaat en een bijna significante correlatie tussen complex I en de ATP productie. De "sensitivity" en de "specificity" van het MELAS syndroom ten opzichte van de controles werd berekend voor zowel de MEGS parameters als de AK-complexen. Sensitivity geeft aan hoe nauwkeurig een pathologische test

de aanwezigheid van het ziektebeeld (in dit geval MELAS syndroom) herkent. Specificity geeft de kans aan dat een gezond persoon een normaal testresultaat heeft. De sensitivity was zowel voor de MEGS parameters als de AK-complexen 100%. De specificity varieerde voor de AK-complexen tussen 29% voor complex IV en 42% voor complex I en voor de MEGS parameters tussen 65% voor de oxidatie van pyruvaat + malaat + ADP en 74% voor de ATP productie. Twee patiënten vertoonden een veel sterker verlaagde MEGS capaciteit dan kon worden voorspeld uit het percentage heteroplasmie. In deze patiënten werden ook alle ND genen en de m.rRNA's gesequenced, maar ook in deze genen werden geen additionele mutaties gevonden. In één patiënt met een hoog percentage heteroplasmie, werden normale activiteiten voor de MEGS parameters en de AK-complexen gemeten. In deze drie patiënten blijft de oorzaak van het afwijkende biochemische phenotype vooralsnog onduidelijk maar kan geassocieerd zijn met een tot nu toe onbekende factor in hun genetische achtergrond. In patiënten met mutaties in de m.tRNA's geeft het meten van de MEGS capaciteit in combinatie met het meten van de AK-complexen betere informatie over het functioneren van de mitochondriën dan alleen meting van de AK-complexen. Het meten van de MEGS parameters discrimineert beter omdat deze parameters reeds verlaagd zijn bij een lager percentage heteroplasmie dan de activiteiten van de AK-complexen. Bovendien vertoonden de MEGS parameters een betere sensitivity dan de AK-complexen.

Hoofdstuk 5 geeft een overzicht van de ervaringen opgedaan in achttien jaar studie naar prenatale diagnostiek voor OXPHOS stoornissen. Enzymatische prenatale diagnostiek voor OXPHOS stoornissen kan alleen uitgevoerd worden onder strikte voorwaarden en na intensief overleg tussen ouders, artsen en laboratorium-medewerkers. Prenatale diagnostiek is het meest betrouwbaar als het genetisch defect in de probandus bekend is maar helaas is dit in veel gevallen van een complex I of IV deficiëntie niet bekend. Enzymatische prenatale diagnostiek naar OXPHOS stoornissen kan slechts uitgevoerd worden als aan drie belangrijke voorwaarden is voldaan. De deficiëntie in de probandus moet aangetoond zijn in gekweekte fibroblasten en in minstens één ander weefsel, bij voorkeur spier, maternale contaminatie van het embryonale weefsel moet zijn uitgesloten en de deficiëntie mag niet veroorzaakt worden door een mutatie in het mtDNA. Prenatale diagnostiek moet bij voorkeur uitgevoerd worden op natieve chorion villi omdat dit weefsel reeds in een vroeg stadium van de zwangerschap afgenomen kan worden met minder risico's voor de foetus.

Hoofdstuk 6 beschrijft een nieuw ontwikkelde spectrofotometrische bepaling voor complex I. Omdat in deze methode complex I bepaald wordt in geïsoleerde mitochondriën afkomstig van ingevroren materiaal, werden complex IV en complex II gebruikt als referentie-enzymen. Om deze reden werd ook een nieuw ontwikkelde bepaling voor complex II in dit hoofdstuk opgenomen. Deze complex II bepaling is gebaseerd op een vroeger reeds gepubliceerde methode maar met een aantal belangrijke modificaties. Onze vroeger ontwikkelde methodes voor het meten van complex I en II waren gevoelig en betrouwbaar, maar minder geschikt voor het meten van grote aantallen monsters omdat ze alleen gemeten konden worden op een geavanceerde research-spectrofotometer. De nieuwe complex I methode vertoond een hoge gevoeligheid, precisie en rotenon gevoeligheid en kan uitgevoerd worden op een eenvoudige spectrofotometer. De methode is ook zeer geschikt voor het meten van complex I in gekweekte fibroblasten. De methode werd getest door complex I te meten in gekweekte fibroblasten en spierweefsel van een aantal patiënten met een bewezen complex I deficiëntie. Met de nieuwe complex I methode werden de deficiënties bevestigd in alle monsters. In de meeste complex I bepalingen is BSA een essentieel bestanddeel van het reactiemengsel, maar de invloed van BSA op de reactie was niet precies bekend. Wij toonden aan dat toevoeging van BSA essentieel is omdat het de oplosbaarheid van zowel rotenon als decylubiquinone bevordert.

Dankwoord

Aan een project, begonnen in het voorjaar van 2003, is nu een einde gekomen. Op dat moment waren twee publicaties van mijn hand over biochemische diagnostiek naar mitochondriële afwijkingen verschenen en dit aantal moest nog worden uitgebreid met minimaal twee om tot een proefschrift te komen. Voor een en ander was een periode van ongeveer twee jaar gepland. Zoals zo vaak echter, was de praktijk weerbarstiger dan de theorie en twee jaren liepen uit naar vier jaren. Het resultaat is weergegeven in dit proefschrift. Zoals gewoonlijk bij een promotieonderzoek, is dit voor een deel geen solowerk geweest, maar teamwork met enthousiaste hulp van velen. Zonder iemand te kort willen doen, wil ik er hier een aantal persoonlijk noemen.

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Mijn promotor, prof. dr. ir. J.M.F. Trijbels. Beste Frans, jij hebt mij in 1974 van de afdeling Antropogenetica naar het voormalige Laboratorium Kindergeneeskunde en Chirurgie gehaald. Vanaf het begin heb je mij alle vrijheid gegund in de persoonlijke uitvoering van mijn werkzaamheden en het uitwerken en testen van ideeën. Jij bent in al die jaren steeds een stimulerende factor geweest met altijd een luisterend oor. Samen met wijlen dr. Piet van Munster heb je er vaak bij mij op aangedrongen om te gaan promoveren, maar je weet waarom ik dit steeds heb afgehouden. Toen ik in 2003 alsnog bij jou kwam met het plan om bij jou te gaan promoveren, zat je gelukkig stevig op je stoel, zodat je er nog net niet vanaf viel. Vervolgens nam je meteen vol enthousiasme de taak van promotor op je. Ik ben daarom blij dat je dit nu, bijna vijf jaar na je emeritaat, toch nog mag meemaken. Frans, bedankt voor alles en ik hoop dat onze vriendschap nog vele jaren mag voortduren.

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Curriculum vitae

Antonius Jacobus Maria Janssen werd geboren op 23 augustus 1947 te Venlo. Na het behalen van het M.U.L.O. diploma in 1965 begon hij met de studie voor medisch analist aan de Stichting Analistenschool Noord-Limburg, waar hij in 1968 afstudeerde als bacteriologisch analist. Van 1968 tot 1971 werkte hij op het microbiologisch laboratorium van Organon B.V. in Oss. In 1971 begon hij zijn werkzaamheden aan de Medische Faculteit van de Katholieke Universiteit Nijmegen op het laboratorium van de afdeling Antropogenetica (hoofd destijds prof. dr. S. Geerts). In 1974 haalde prof. dr. ir J.M.F.Trijbels hem naar, destijds, het Laboratorium Kindergeneeskunde en Chirurgie en vanaf dat jaar tot heden is hij werkzaam op het Laboratorium Kindergeneeskunde en Neurologie van het UMC St Radboud (hoofd, achtereenvolgens: dr. P. van Munster, prof. dr. ir. J.M.F. Trijbels en prof. dr. R. Wevers). In 1974 begon hij met de avondstudie H.B.O.-B, richting biochemie, aan de Hogere en Middelbare Laboratoriumschool Oss (tegenwoordig Hoge School Arnhem-Nijmegen). In 1976 werd deze studie afgerond met het behalen van het diploma H.B.O.-B richting Biochemie. Hij volgde een cursus hogere wiskunde bij het V.O.R.N.A. schriftelijk studiecentrum (Stichting tot Vorming van Middelbaar en Hoger Natuurwetenschappelijk Personeel), en cursussen vloeistofscintillatietelling, immunologie en immunochemie. Vanaf 1976 tot heden is hij betrokken bij het onderzoek naar het mitochondriële energiemetabolisme bij patiënten lijdende aan een mitochondriële stoornis, onder leiding van achtereenvolgens dr. W. Ruitenbeek, prof. dr. ir. J.M.F. Trijbels en dr. R. Rodenburg. Vanaf juni 2003 tot maart 2007 werden de in dit proefschrift beschreven studies uitgevoerd onder leiding van prof. dr. ir. J.M.F. Trijbels, de helaas veel te vroeg overleden prof. dr. R.C.A. Sengers, prof. dr. J.A.M. Smeitink, dr. L van den Heuvel en dr. R. Rodenburg. Hij is getrouwd met Lies Meerts en zij hebben twee zonen en een schoondochter, Michiel, Mark en Rosalie.

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