

Cell Biological Consequences of Mitochondrial NADH: Ubiquinone Oxidoreductase Deficiency

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Abstract: Human complex I (NADH:ubiquinone oxidoreductase; EC 1.6.5.3) is the first and largest multi-protein assembly of the mitochondrial oxidative phosphorylation (OXPHOS) system; the final biochemical cascade of events leading to the production of ATP. The complex consists of 46 subunits, 7 encoded by the mitochondrial DNA and the remainder by the nuclear genome. In recent years, numerous gene mutations leading to an isolated complex I deficiency have been characterized in both genomes. Disorders associated with complex I deficiency (OMIM 252010) mostly lead to multi-system disorders affecting brain, skeletal muscle and the heart. Of these, Leigh syndrome, a progressive fatal encephalopathy symmetrically affecting specific areas of the brain, brainstem and myelin, is the most frequently observed phenotype. Here, we review the current understanding of the cell biological consequences of isolated complex I deficiencies and propose further directions the field needs to take in order to develop rational treatment strategies for these devastating disorders.

Key Words: Mitochondria, Cell biology, Oxidative Phosphorylation, Leigh disease, Complex I, Mitochondrial Medicine.

INTRODUCTION

Intracellular adenosine 5'-triphosphate (ATP), the cell's energy currency, is produced and regulated via a highly-complicated cascade of events involving cellular uptake of energy-rich substrates and their step-by-step enzymatic breakdown yielding NADH, transport of the various intermediates of this breakdown process between intracellular compartments, feeding of reducing equivalents of NADH into the electron transport chain (ETC) and consequent generation of a proton gradient (Proton Motive Force) across the mitochondrial inner membrane and, eventually, conversion of ADP and inorganic phosphate into ATP at the expense of the Proton Motive Force. The final biochemical system involved in the production of ATP, the OXPHOS-system, is embedded in the lipid-bilayer of the mitochondrial inner membrane and encompasses the multi-subunit complexes of the ETC and ATP synthase (Smeitink *et al.*, 2001a). The mitochondrial inner membrane is now best described as two contiguous but distinct membranes: the inner boundary membrane, which apposes the outer membrane, and the crystal membrane, which extends into the interior as tubules or lamellae (Gilkerson *et al.*, 2003). Recent results argue for restricted diffusion of OXPHOS-complexes, at least complex III and ATP synthase, between the crystal and inner boundary membrane thereby indicating that mitochondrial cristae comprise a regulated sub-mitochondrial compartment specialized for ATP production (Gilkerson *et al.*, 2003). The OXPHOS-system is composed of five multi-protein complexes (complex I-V) and two

mobile electron carriers (ubiquinone and cytochrome *c*). The five protein assemblies consist of 4 to 46 different subunits and with the sole exception of complex II, whose subunits are all encoded by the nuclear genome, the subunits of the other complexes are encoded by the mitochondrial as well as the nuclear genome (Smeitink *et al.*, 2001a). Following a brief summary concerning the genetics and pathology of complex I, this review will summarize our current understanding of the cell biological consequences of human complex I deficiencies and the state-of-the-art of complex I mitochondrial medicine.

Complex I (EC 1.6.5.3) consists of 46 proteins, seven of which encoded by the mtDNA and the remainder by the nDNA (Hirst *et al.*, 2003). The mtDNA, of which 2-10 copies are present per mitochondrion, is a 16,596 base-pair long circular double-stranded DNA originating from pro-bacteria (Gabaldon and Huynen, 2003). The mitochondrial "chromosome" contains the genetic information for 22 transfer (t) RNA's, 2 ribosomal (r) RNA's and 13 protein encoding genes (Anderson *et al.*, 1981; Smeitink *et al.*, 2001a). The 7 mtDNA-encoded (ND1-6, ND4L) and 39 nDNA-encoded proteins have to assemble into the holo-complex, a process also involving the incorporation of 8 (or 9) iron-sulphur clusters and one flavin mononucleotide (FMN). Knowledge about this assembly process is still in its infancy and so far, in sharp contrast with e.g. complex IV of the OXPHOS-system, only one complex I assembly gene has been described (Janssen *et al.*, 2002). This difference in knowledge about the assembly of the different OXPHOS-complexes is, among others, due to the fact that *Saccharomyces cerevisiae*, a model-system frequently used by mitochondrial biologists, does not contain complex I. Thanks to the work of John Walker on complex I of bovine heart and the information provided by the Human Genome Project, we were able to characterize all 39 nDNA-encoded

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structural subunits (Walker 1992; Smeitink *et al.*, 2001). The structural complex I genes are spread over 18 of the 23 chromosomes (Hirst *et al.*, 2003) and tissue distribution, based on Northern-blot hybridization, is mostly ubiquitous although expression is generally more increased in tissues and organs with a higher energy-demand like heart, skeletal muscle and brain (Triepels *et al.*, 2001).

Human complex I deficiency (OMIM 252010), first described in 1979 by Morgan Hughes and colleagues (Morgan-Hughes *et al.*, 1979), is the most frequently encountered mitochondrial disorder (Rahman *et al.*, 1996; Morris *et al.*, 1998; Loeffen *et al.*, 2000; Triepels *et al.*, 2001; Smeitink *et al.*, 2001). A deficient enzyme activity of this complex, for diagnostic purposes usually determined in skeletal muscle and fibroblasts, leads to multi-system disorders affecting predominantly organs and tissues with a high-energy demand like brain, heart, and skeletal muscle (Smeitink *et al.*, 2001 and 2001a). Different phenotypes are recognized of which Leigh syndrome is the most frequent being present in about half of the cases (Leigh 1951; Loeffen *et al.*, 2000). Leigh syndrome, first described in 1951, is a typical example of how mitochondrial diagnostics is carried out (Leigh, 1951; Rahman *et al.*, 1996; Smeitink, *et al.*, 2001). It is a progressive, sub-cortical encephalopathy. The first symptoms usually appear before two years of age and consist of optic atrophy, ophthalmoparesis, hypotonia, ataxia and dystonia. Neuropathy and myopathy are common and the specific neurological features help to distinguish Leigh syndrome from other brain diseases, such as encephalitis. The course is fluctuating, with exacerbations often triggered by mild inter-current illnesses. Most patients die a few years after the onset of the symptoms. Whenever the clinical suspicion of a mitochondrial disorder arises, laboratory, electrophysiological and neuroradiological investigations are needed to justify more invasive procedures, such as a muscle biopsy, which remains the mainstay of the diagnostic process (Sengers *et al.*, 1984; Munnich *et al.*, 1996; DiMauro *et al.*, 1999; Thorburn *et al.*, 2001; Wolf and Smeitink, 2002; Smeitink *et al.*, 2003). Light- and electronmicroscopy, histochemistry and measurement of enzyme activities of the individual OXPHOS complexes complete the diagnostic process (Sengers *et al.*, 1984; Thorburn *et al.*, 2001; Smeitink, 2003). Although Leigh syndrome has been associated with mitochondrial or nuclear mutations that affect virtually every step of the OXPHOS-system, isolated complex I or complex IV deficiencies are the most frequent deficiencies observed.

HUMAN COMPLEX I: STRUCTURE, FUNCTION AND PATHOLOGY

NADH:ubiquinone oxidoreductase is a multi-protein complex assembly of 980 kDa. The overall function of the complex is to pass electrons from NADH to ubiquinone while pumping hydrogen ions out of the mitochondrial matrix into the inter-membrane space. The electrochemical proton gradient caused by this process is used by complex V of the OXPHOS-system to synthesize ATP from ADP and inorganic phosphate.

Most knowledge regarding the spatial organization and function of human complex I has been obtained through

studies of this complex in *Escherichia coli*, *Neurospora crassa*, *Bos taurus* and, more recently, *Yarrowia lipolytica* (Weiss *et al.*, 1991; Walker, 1992; Weidner, *et al.*, 1993; Ahlers *et al.*, 2000; Carroll *et al.*, 2003). Although no electron microscopic studies of the human complex are available, its spatial structure most probably resembles that in *N. crassa* and *B. taurus*, the evolutionary closest species to man (Guenebaut *et al.*, 1997; Grigorieff, 1998). In these species complex I has an L-shape structure with a hydrophobic arm embedded in the mitochondrial inner membrane containing, among others, all mtDNA-encoded subunits, and a water-soluble peripheral arm protruding into the mitochondrial matrix (Galante and Hatefy, 1978 and 1979; Walker 1992; Smeitink *et al.*, 1998). The latter arm contains the FMN and the iron-sulphur clusters of the complex (Smeitink *et al.*, 1998). Detailed analysis of the subunit composition of complex I from bovine heart using a pleiad of techniques including one-dimensional SDS-PAGE, two-dimensional isoelectric focusing/SDS-PAGE, reverse phase high pressure liquid chromatography (HPLC), tryptic peptide mass fingerprinting and tandem mass spectrometry, has recently been completed and led to the discovery of 4 additional nDNA-encoded subunits (Carroll *et al.*, 2003). In addition, the mass of many of the intact subunits was determined providing valuable information about their post-translational modifications. The authors concluded that it is unlikely that any more subunits of bovine complex I remain undiscovered. Data from the human genome project indicate that this also holds true for human complex I (Table 1).

A small form of complex I, consisting of 14 subunits, has been found in *Escherichia coli* (Weidner *et al.*, 1993). This complex, which consists of the 7 mtDNA-encoded subunits and 7 nuclear-encoded subunits (NDUFS7, NDUFS8, NDUFS1-3, NDUFV1-2), is generally considered to be the minimal form of a proton-pumping NADH:ubiquinone oxidoreductase (Weidner *et al.*, 1993). The functional role of the remaining "accessory", also called "supernumerary" or "peripheral", subunits present in higher species is poorly understood. As will be discussed hereafter, the characterization of naturally occurring mutations may contribute to a better understanding of the functional properties of these accessory subunits.

Since the first description of human complex I deficiency thousands of patients with isolated complex I deficiency have been diagnosed. In general, complex I failure results in multi-system disorders with a fatal outcome. Leigh syndrome or Leigh-like disease, the latter similar to Leigh syndrome but without pathological manifestations in brain, are the most common phenotypes associated with isolated complex I deficiency, representing up to 50% of total cases (Rahman *et al.*, 1996; Morris *et al.*, 1998; Loeffen *et al.*, 2000; Triepels *et al.*, 2001). Other commonly observed phenotypes are encephalopathy and cardiomyopathy (~11%), fatal infantile lactic acidosis (~11%), macrocephaly with progressive leukodystrophy (~7%), and unspecified encephalopathy (~21%) (Loeffen *et al.*, 2000). Due to the bigenomic nature of complex I, genetic counselling and prenatal diagnostics in affected families are complicated. Both maternal, with a 100% recurrence risk, and Mendelian inheritance patterns (X-linked, autosomal recessive or dominant) can be observed. The need to elucidate the genetic

Table 1. Human Complex I Nuclear Genes: Present Knowledge

	cDNA sequence	nDNA sequence	Chromosomal localization	Leader sequence	Functional properties
Flavoprotein group					
<i>NDUFV1 (NuoF)</i>	+	+	11q13	+	NADH-binding, electron transfer
<i>NDUFV2 (NuoE)</i>	+	+	18p11.31-p11.21	+	Electron transfer
<i>NDUFV3</i>	+	+	21q22.3	+	
Iron-sulfur group					
<i>NDUFA5</i>	+	-	7q32		Electron transfer
<i>NDUFS1 (NuoG)</i>	+	-	2q33-q34	+	
<i>NDUFS2 (NuoD)</i>	+	-	1q23	+	
<i>NDUFS3 (NuoC)</i>	+	+	11p11.11	+	Phosphorylation
<i>NDUFS4</i>	+	-	5q11.1	+	
<i>NDUFS5</i>	+	-	1p34.2-p33		
<i>NDUFS6</i>	+	-	5pter-p15.33	+	
Hydrophobic group					
<i>NDUFA1</i>	+	+	Xq24		Q-binding?
<i>NDUFA2</i>	+	-	5q31.2		
<i>NDUFA3</i>	+	-	19q13.42		
<i>NDUFA4</i>	+	-	7p21.3		
<i>NDUFA6</i>	+	-	22q13.1		
<i>NDUFA7</i>	+	-	19p13.2		
<i>NDUFA8</i>	+	-	9q33.2-q34.11		
<i>NDUFA9</i>	+	-	12p13.3	+	Phosphorylation
<i>NDUFA10</i>	+	-	12p	+	Acylcarrier protein motif
<i>NDUFAB1</i>	+	-	16p12.3-p12.1		
<i>NDUFB1</i>	+	-	14q31.3		
<i>NDUFB2</i>	+	-	7q34-35	+	
<i>NDUFB3</i>	+	-	2q31.3		
<i>NDUFB4</i>	+	-	3q13.33		
<i>NDUFB5</i>	+	-	3q27.1	+	
<i>NDUFB6</i>	+	-	9p13.2		
<i>NDUFB7</i>	+	-	19p13.12-q13.11		
<i>NDUFB8</i>	+	-	10q23.2-p23.33	+	
<i>NDUFB9</i>	+	+	8q13.3		
<i>NDUFB10</i>	+	-	16p13.3		
<i>NDUFS7 (NuoB)</i>	+	-	19p13	+	Electron transfer
<i>NDUFS8 (NuoI)</i>	+	+	11q13.1-q13.3	+	Electron transfer
<i>NDUFC1</i>	+	-	4q28.2-q31.1		
<i>NDUFC2</i>	+	-	11q13.3		
<i>ESSS, NP17.3</i>			Xp11.23		
Unknown					
<i>B17.2, DAP13</i>	+		12q21.33		Cell death regulation
<i>B16.6 (GRIM-19)</i>			19p13.2		
<i>B14.7, NDUFA11</i>			19P13.3		

E. coli homologous of human complex I are set between parenthesis.

defect in affected families is high. Prenatal diagnostics is possible at the enzymatic level, but molecular prenatal diagnostics is more reliable (Niers *et al.*, 2001; Schuelke *et al.*, 2002).

In about 5-10% of our complex I deficient patients mtDNA mutations associated with isolated complex I deficiency are detected (Smeitink *et al.*, 2001). It should be stated, however, that our mutational analysis program only

included the search for deletions, marked rearrangements and frequently observed point mutations. Sequencing of the entire mtDNA might reveal a higher yield of pathogenic mtDNA mutations than found so far. Mutations associated with isolated complex I deficiency include mutations in both tRNAs, like the tRNA^{leu} mutation A3243G, as well as mutations in the hydrophobic ND proteins (Ugalde *et al.*, 2003 and references herein).

Characterization of the nuclear-encoded structural complex I subunits (Table 1) enabled us to perform mutational analysis in complex I deficient patients without a known mtDNA mutation (Smeitink *et al.*, 2001a). Thus far, this analysis yielded mutations in 7 nuclear complex I genes namely *NDUFS1*, *NDUFS2*, *NDUFS4*, *NDUFS7*, *NDUFS8*, *NDUFVI* and *NDUFV2* (van den Heuvel *et al.*, 1998; Schuelke *et al.*, 1999; Loeffen *et al.*, 1998, Triepels *et al.*, 1999; Loeffen *et al.*, 2000; Budde *et al.*, 2001; Petruzzella *et al.*, 2001; Triepels *et al.*, 2001; Smeitink *et al.*, 2001; Benit *et al.*, 2003a and b). Mutations in the *NDUFS4*, *S7* and *S8* genes all caused complex I deficient Leigh- or Leigh-like syndrome (van den Heuvel *et al.*, 1998; Loeffen *et al.*, 1998; Triepels *et al.*, 1999).

The first nuclear mutations in Leigh syndrome patients, missense mutations in the *NDUFS8* and *NDUFS7* genes, were described by Loeffen and Triepels (Loeffen *et al.*, 1998; Triepels *et al.*, 1999). Expression of these gene mutations in the obligate aerobic yeast *Yarrowia lipolytica* confirmed its deleterious effect (Ahlers *et al.*, 2000). Intriguingly, lactic acid concentrations in body fluids of these patients, till that time considered as the diagnostic hallmark of mitochondrial disease, were completely normal. A recent inventory revealed that about 20% of the complex I deficient patients known in our centre showed normal lactic acid concentrations; which in some cases leads to a real diagnostic dilemma (Smeitink 2003).

The clinical outcome of complex I deficient patients in general is very poor, all patients e.g. with mutations in the *NDUFS4* gene diagnosed so far died before the age of 16 months (Smeitink 2003). Although all kind of supplementations, like vitamins, and changes in the composition of the diet have been tried, the clinical outcome was only influenced by classical supportive care measures like anti-convulsive drugs, diuretics (in the case of cardiomyopathies), and physiotherapy. All this illustrates that there is an urgent need for the development of more rational treatment options to stabilize or even cure the affected patients. Obviously, this necessitates a detailed understanding of the pathophysiology of complex I disease.

Theoretically, the disease course in complex I deficiency is influenced by the relative importance of the mutated subunit and the degree to which its synthesis and/or functionality is disturbed by the mutation. For instance, the subunit can be involved in proper assembly of the holo-complex, maintenance of the cellular redox-state, prevention of the production of reactive oxygen species (ROS), promotion of mitochondrial fusion and/or fission and mitochondrial ATP production. Furthermore, the genetic background of the patient and environmental factors may have an impact on the disease course and outcome. Finally, the variable degree of energy dependency of the various tissues may explain some of the observed phenotypic differences but here detailed analysis awaits the generation of different complex I deficient animal models, which are, for the time being, not available. Thus far, most cell biological studies have been performed on complex I deficient cell lines, like human fibroblasts, harbouring mutations in structural complex I genes or human trans-mitochondrial hybrids (Bariantos *et al.*, 1998 and 1999).

GENETIC MUTATIONS IN THE COMPLEX I *NDUFS4* GENE AND COMPLEX I ACTIVITY REGULATION

The mechanisms regulating human complex I functioning as well as the cell biological consequences of complex I deficiency are only grossly understood. The characterization of the nuclear complex I genes, the detection of mutations in these genes in human complex I deficient cell lines and the results of studies in lower species have provided new opportunities for detailed physiological and cell biological studies of this complex in health and disease. This is *par example* illustrated by the results from studies of the *NDUFS4* gene. The first complex I deficient Leigh-like patient described with a nuclear gene mutation showed a 5 base-pair duplication in the *NDUFS4* gene, resulting in destruction of the consensus RVS phosphorylation site (van den Heuvel *et al.* 1998). Papa *et al.* have found that in bovine heart mitochondria the serine in the consensus site of this protein can be post-translationally phosphorylated by a cAMP-dependent protein kinase (Papa *et al.* 1996). In a collaborative study, we demonstrated that cAMP-dependent phosphorylation and activation of complex I was abolished in this particular cell line (Papa *et al.*, 2001). This finding provides substantial evidence showing that cAMP-mediated intracellular signal transduction, through serine-phosphorylation of the *NDUFS4* subunit of complex I, regulates the activity of the complex in cAMP-responsive mammalian tissues (Smeitink *et al.*, 2001). Complex I phosphorylation has also been implicated in the control of oxygen free radical formation, which will be discussed in more detail in one of the following paragraphs (Raha *et al.*, 2002).

Since the first description of a patient with a mutation in the *NDUFS4* gene about 10 new cases have been found, making the *NDUFS4* gene a "hotspot" for complex I deficient Leigh or Leigh-like syndrome. The severity of mutations in this gene is illustrated by the fact that all patients died within 16 months of age. Using two-dimensional gel electrophoresis, in collaboration with Sergio Papa's group, we recently showed a defect assembly of functional complex I in three different homozygous mutations in the *NDUFS4* gene (Scacco *et al.*, 2003). In all three mutations, in addition to destruction of the carboxyl-terminal segment of the 18 kDa subunit, the amino-terminal segment of the protein was also missing. These findings demonstrate that the expression of the *NDUFS4* gene is essential for the assembly of a functional complex I. Steady-state protein phosphorylation was recently analyzed using a novel fluorescent phosphosensor dye and revealed also phosphorylation of the *NDUFA10* (42 kDa) subunit (Schulenberg *et al.*, 2003). The functional consequences of this process await further studies.

TRANSCRIPTIONAL AND TRANSLATIONAL CONSEQUENCES OF COMPLEX I STRUCTURAL GENE MUTATIONS

To gain a broader perspective of the consequences of nuclear-derived complex I deficiency on the expression of genes involved in mitochondrial function, we investigated transcriptional responses in complex I deficient fibroblast cell lines. For this we used a tailor-made micro-array in a novel experimental approach comparing transcriptional

responses of complex I deficient cell lines cultured under glycolytic or oxidative conditions (Westhuizen, van der *et al.*, 2003). Briefly, a cDNA micro-array was constructed containing a selection of 615 human genes involved in mitochondrial structure or function, expression sequence tags identified as candidate genes involved in oxidative phosphorylation, and genes used for expression normalization. Genetically characterized complex I deficient fibroblasts were cultured with two different carbon sources, glucose and galactose. This approach allowed the use of the same cell line as control and test-sample and circumvents detecting differential expression that may be due to differences in genetic constitution, age or senescence. cDNAs obtained from these fibroblasts were labelled with the Cy3 and Cy5 dyes, respectively. Following hybridization the slides were scanned and analyzed. Using this experimental setup a vast number of genes were found to be markedly induced (Westhuizen van der *et al.*, 2003). In our opinion, one of the most important groups of induced genes encompassed the various isoforms of the metallothioneins (MTs)(1B, 1E, 1F, 1G, 1H, 1L, 1R). It is known that the expression of these relatively small, cysteine-rich proteins is induced by a variety of stimulants including ROS, and that they exhibit anti-apoptotic properties and act as potent antioxidants protecting DNA from oxidative damage. Knowing that ROS production is increased in complex I deficiency, it is fair to assume that induction of MTs transcription is ROS-related and may be protective against ROS-mediated damage in complex I deficient cells (Pitkanen *et al.*, 1996; Luo *et al.*, 1997). Before drawing definite conclusions about the importance of MTs and other observed transcriptional inductions in these cell lines further studies at the protein level should be performed simply because translational induction does not completely parallel transcriptional induction (Vasconcelos *et al.*, 2002). Radio-immuno assays to study the effect of complex I deficiency on expression of MTs at the protein level are in progress. Important in this context is the observation that overexpression or addition of MT-I, MT-II or MT-III has a neuroprotective effect under various experimental conditions including increased ROS production, axonal transection, cortical brain injury and interleukin-6 overexpression (Molinero *et al.*, 2003). The technological developments to study transcriptional and translational alterations are impressive and will have a major impact on our understanding of mitochondrial biology and complex I deficiency. This is, among others, illustrated by the recent (partially) characterization of the human heart mitochondrial proteome yielding a total of 615 distinct proteins the biochemical properties of about 19% of which are still unknown (Taylor *et al.*, 2003). Clearly, more tissues than fibroblasts have to be studied to judge whether induction of MT genes is a common cellular response in complex I deficiency.

ALTERATIONS IN NAD METABOLISM CAUSED BY COMPLEX I DEFICIENCY

Coenzyme nicotinamide adenine dinucleotide (NAD) is the major hydrogen donor or acceptor in numerous metabolic reactions; mainly those involved in the production of ATP. NAD⁺, the oxidized form of NAD, also plays a role in calcium homeostasis and longevity by serving as ADP-ribose

donor in the formation of cADP-ribose and covalent modification of proteins (Chambon *et al.*, 1963; Ziegler 2000; Rongvaux *et al.*, 2003).

The cellular NAD pool is determined by the balance between its biosynthesis out of three distinct precursors, L-tryptophan, nicotinic acid and nicotinamide (Rongvaux *et al.*, 2003) and its use by "NAD-consuming enzymes and proteins", like ADP-ribosyl transferases, NAD-glycohydrolases/ADP-ribosyl cyclases and members of the SIR2 family, which display NAD-dependent deacetylase activity. At least theoretically, a dysfunction of dehydrogenases, like complex I, which dehydrogenates NADH to NAD⁺ plus H⁺, may disturb the cellular NAD pool giving rise to an altered redox-state of the cell. Briefly, the aerobic oxidation of glucose by the glycolytic pathway, pyruvate dehydrogenase complex and citric acid cycle results in the reduction of NAD⁺ to NADH. To become reoxidized, the reduced coenzyme binds to the NDUFV1 subunit of complex I. Subsequent oxidation of NADH yields two electrons, which, following translocation through the prosthetic groups of the complex, are accepted by the electron carrier ubiquinone. In about 80% of the patients with isolated complex I deficiency high lactate concentrations and elevated lactate/pyruvate ratios are found, indicating a severely increased NADH/NAD⁺ ratio (Munnich *et al.*, 1996), which may have impact on NAD⁺-dependent processes. Thus, complex I deficiency may affect covalent protein modifications catalyzed by ADP-ribosyltransferases (ADPRTs; the best known being poly ADP-ribosyl polymerase or PARP1). These enzymes attach the ADP-ribose moiety of NAD⁺ to specific amino-acid residues of the acceptor proteins and, as a result of a relative lack of NAD⁺ in complex I deficiency, at least theoretically, essential protein modifications may not take place. Besides, NAD⁺ is used for the agonist-induced synthesis of cyclic ADP-ribose (cADPR), a signaling molecule involved in the initiation of calcium efflux from the endoplasmic reticulum. A decreased synthesis of cADPR may give rise to disturbances in intracellular calcium signaling. Further functions of NAD include its role as co-substrate in silent information regulator 2 (Sir2)-mediated histone deacetylation involved in gene silencing regulation and in increasing the lifespan of species ranging from yeast, to worm, to certain mammals (Guarente 2000; Moazed 2001; Zhang *et al.*, 2003). The above-mentioned considerations warrant further studies establishing the relationship between complex I deficiency and cellular NAD metabolism.

REACTIVE OXYGEN SPECIES AND HUMAN COMPLEX I DEFICIENCY

During the process of oxidative phosphorylation, i.e. the oxidation of fuel molecules by oxygen and the concomitant energy transduction into ATP, most oxygen is converted to water via tetravalent reduction (Scholte 1988). However, under normal physiological conditions, about 1-2% of oxygen metabolized during oxidative phosphorylation is converted into highly reactive substances, the reactive oxygen species (ROS) (Chance *et al.*, 1979). To counteract the attack of ROS to lipids, proteins and DNA evolution has provided cells with enzymatic and non-enzymatic antioxidant defence systems like superoxide dismutases, glutathione peroxidases and lipophilic antioxidants including

alpha-tocopherol and beta-carotene (Halliwell, 1991). Ex vivo experiments have clearly shown that in the presence of inhibitors of distinct parts of the OXPHOS-system ROS can increase several folds (Nohl *et al.*, 1989; Cleeter *et al.*, 1992). Conceivably, genetically determined dysfunction of the OXPHOS-system may give rise to imbalance between ROS production and ROS clearance thus leading to a sequence of harmful events. Univalent reduction of molecular oxygen results in the formation of the superoxide anion radical, which, by the action of dismutases is converted into hydrogen peroxide. Furthermore, superoxide can interact with its dismutation product i.e., hydrogen peroxide, to form the hydroxyl radical (Halliwell *et al.*, 1991). All oxygen species formed are highly reactive, the hydroxyl radical being the most potent, and react with all biological materials in their direct surrounding; e.g. for mitochondria being this the mtDNA, Fe-S clusters, proteins, trace elements and lipids (Richter, 1992).

Much information about the effects of excessive ROS formation in human complex I deficient fibroblasts has been generated by the group of Robinson. Southern blotting revealed that mtDNA, isolated from fibroblasts of complex I deficient patients with cataract and cardiomyopathy, contains many multiple deletions compared to control fibroblasts (Pitkanen *et al.*, 1996a), indicating free oxygen radical damage. Furthermore, they showed with western blot analysis and enzymatic assay that the mitochondrial Mn-SOD levels were elevated in patient fibroblasts. More recently, this group used the luminometric probe lucigenin to measure NADH-induced superoxide production in isolated mitochondrial membranes of complex I deficient patient fibroblasts. Their results revealed a broad spectrum with on one side elevated superoxide levels combined with reduced Mn-SOD values (phenotype of cataract and developmental delay) and on the other side reduced superoxide levels with increased values of Mn-SOD (phenotype of cataract and cardiomyopathy) (Pitkanen and Robinson, 1996b). The authors hypothesized that complex I deficiency increases the production of superoxide but that variable expression of Mn-SOD influences this process. Luo and co-workers demonstrated increased production of the hydroxyl radical in complex I deficient patients expressing cataract and cardiomyopathy by using the aromatic hydroxylation assay with salicylate as a probe (Luo *et al.*, 1997; Robinson 1998). Furthermore, they showed overproduction of aldehydes (indicative of lipid peroxidation) in fibroblasts of these patients. Barrientos and Moraes published studies in which they examined the pathophysiological aspects of complex I deficiency using a model of human:ape xenomitochondrial hybrids (HXC cells; 40% complex I deficiency) (Barrientos and Moraes, 1999). They used H₂DCF (converted to a fluorescent product after oxidation by H₂O₂) and found increased ROS formation in the HXC cells as well as the rotenone model. Using membranes of rat heart mitochondria, incubated with ATP to allow protein phosphorylation, Raha and co-workers recently showed that protein kinase A (PKA) and cAMP derivatives increased the phosphorylation of NDUFA1 (MWFE) and either NDUFS4 (AQDQ) or NDUF7 (B18), while decreasing NADH-driven superoxide production (Raha *et al.*, 2002). Conversely, PKA inhibitors along with the addition of a pyruvate dehydrogenase mix

(containing the pyruvate dehydrogenase kinase) decreased the phosphorylation of these subunits, while increasing the rate of NADH-driven superoxide production. Based on these findings, the authors concluded that electron flow through complex I and ROS production is under regulatory control of phosphorylation events. Future studies must be performed to entangle the influence of excessive ROS formation in the pathophysiology of complex I deficiency. Hydrogen peroxide for example, derived from superoxide catabolism, induces the expression of a variety of genes and, in mitochondrial diseases, may cause overexpression of antioxidant agents and transcription of death effector genes, as has already been demonstrated for other conditions. MnSOD, CuZnSOD and GSH (reduced glutathione) are expressed in muscle fibers of patients with chronic progressive external ophthalmoplegia (CPEO), MELAS and MERRF (Filosto *et al.*, 2002). Using immunohistochemistry and confocal microscopy it was found that these patients also displayed nuclear expression of c-Jun/AP-1 (activator protein-1), suggesting that AP-1 is involved in the oxidative stress response in muscle fibers from patients with mitochondrial disease (Filosto *et al.*, 2003).

STRUCTURAL COMPLEX I MUTATIONS AND THEIR EFFECTS ON COMPLEX I ASSEMBLY

The discovery in the past years of a variety of mutations affecting both the mtDNA- and nDNA-encoded subunits of complex I in man and other species like *Neurospora crassa*, *Escherichia coli*, *Yarrowia lipolytica*, *Chlamydomonas reinhardtii*, Chinese hamster and mouse has made it possible to study the effect of alterations in the structural complex I building-blocks in relation to complex I assembly (Chomyn *et al.*, 2001; Cardol *et al.*, 2002; Bai *et al.*, 1998; Yadava *et al.*, 2002; Scacco *et al.*, 2003; Ugalde *et al.*, 2003; Antonicka *et al.*, 2003). Results of these studies have revealed also additional insight in the functional properties of some of the complex I proteins with respect to electron and proton transport. As a prototype of a mtDNA complex I structural mutation we summarize some of the results obtained in studying the ND6 gene. Mutations in ND6 are associated with Leber's hereditary optic neuropathy or LHON disease with or without dystonia, but also with complex I deficient Leigh disease (Johns *et al.*, 1992; Oostra *et al.*, 1995; Ugalde *et al.*, 2003). In a complex I defective mouse cell line carrying a near-homoplasmic frameshift mutation in the mtDNA-encoded ND6 subunit, resulting in an almost complete absence of this polypeptide, a loss of assembly of the mtDNA-encoded subunits of the complex occurred. Moreover, disruption experiments of the ND6 gene in the green algae *Chlamydomonas* prevented the expression of the fully assembled 850 kDa complex (Cardol *et al.*, 2002). Recently, we investigated the molecular genetics in a complex I deficient Leigh syndrome patient who deceased at 7 months of age. Fibroblasts of this patient harboured an isolated complex I deficiency, which was not restored following fusion of enucleated patient fibroblasts (containing mtDNA) with rho-zero cells (containing nDNA). This result confirmed the existence of a mtDNA mutation as the disease causing mutation. Sequencing of the mtDNA revealed a novel mutation in the most conserved region of the structural mitochondrial complex I gene, ND6 (T14487) (Ugalde *et al.*,

2003). Two-dimensional blue native electrophoresis (Nijtmans *et al.*, 2002) showed a reduced amount of fully assembled complex I and the accumulation of lower molecular weight subcomplexes in both fibroblasts and cybrid clones. These results indicate the importance of ND6 in the assembly and/or stability of mitochondrial complex I. Further studies of patients with mutations in the various structural mtDNA complex I genes are necessary to unravel whether also these fulfil a role in the assembly of the complex.

Also mutations in the structural nDNA-encoded building blocks of the complex may prevent its normal assembly. This e.g. has nicely been demonstrated for three different mutations in the *NDUFS4* gene (Petruzella and Papa, 2002; Scacco *et al.*, 2003).

Little is known on how the 46 subunits of complex I, the 8 (or 9) Fe-S clusters and the single FMN are assembled into one active complex, which factors are involved in this assembly process and how this process is controlled. In *Neurospora crassa*, only two proteins, the complex I intermediate associated proteins CIA30 and CIA84, have been shown to function as assembly proteins, associating with intermediates of the assembly process but not with the mature complex (Kuffner *et al.*, 1998). In human, only the CIA30 homologue has been characterized (Janssen *et al.*, 2002). The protein is expressed ubiquitously with a slightly higher expression in various heart tissues, kidney, lung and liver. Thus far, however, no mutations in complex I deficient patients have been reported.

In a patient cohort of isolated complex I deficient patients in which the defect was present in at least two different tissues, among these, fibroblasts, mutations in structural nuclear complex I genes have been found in about 50% of the complex I deficient patients (Loeffen *et al.*, 2000). Based, among others, on sucrose gradient experiments and SDS-PAGE electrophoresis the remaining patients have been shown to have a disturbed complex I assembly (Triepels *et al.*, 2001). The numbers of affected subjects in these families are small, which makes classical genetic approaches like linkage-analysis to find the disease causing gene(s) in these patients virtually impossible. Other approaches like the chromosome transfer technique might be a helpful tool to discover affected complex I assembly proteins in these complex I deficient cell lines (Smeitink *et al.*, 2001).

Very recently, the first model for complex I assembly was presented by the group of Shoubridge (Antonicka *et al.* 2003). Analysis of complex I deficient patients, either with mtDNA or nDNA mutations, revealed similar patterns of partially assembled complexes. The authors proposed that the identified subcomplexes represent different assembly intermediates and they were able to construct an assembly pathway for human complex I. Crystallization of complex I and elucidation of its assembly process are among the most important challenges in the complex I research field.

ALTERATIONS IN MITOCHONDRIAL MORPHOLOGY ASSOCIATED WITH COMPLEX I DEFICIENCY

The classical textbook cartoon of a mitochondrion depicts a bone-shape organelle with an outer membrane and

a highly folded inner membrane encompassing two compartments, the inter-membrane space and the mitochondrial matrix. However, electron-microscopic analysis and more modern approaches like electron-microscopic tomography show different kinds of mitochondrial shape in the various tissues (Manella *et al.*, 2000). Moreover, these mitochondrial structures are not separated individuals but often form highly dynamic tubular networks spreading throughout the cell as can be nicely viewed by live-cell confocal microscopy. Mitochondrial morphology and distribution change in response to cellular activity, nutritional status and developmental programmes (Yaffe, 1999). Such alterations of mitochondrial morphology are sculpted by the fusion and fission of mitochondrial tubules and the balance between these two processes largely determines the form of the mitochondrial network (Sesaki *et al.*, 1999; Yaffe, 2003).

Morphological signs associated with a disturbed OXPHOS-system include the ragged red fibres (subsarcolemmal accumulation of mitochondria histochemically stained with Gomori-trichrome) and the sometimes bizarre shaped mitochondria as observed employing electron microscopy or tomography (Sengers *et al.*, 1984). The presence of ragged red fibres is associated with genetic mutations mainly involving the mtDNA tRNAs (Smeitink *et al.*, 2001). The occurrence of crystals in the intermembrane space is probably the most striking structural abnormality in patients with mitochondrial encephalo-myopathies (Farrants *et al.*, 1988). In 1992, we showed that these crystals contained mitochondrial creatine kinase (Mi-CK, EC 2.7.3.2). (Smeitink *et al.*, 1992). More recently, confocal studies performed by one of us (WK) revealed adaptive changes of the mitochondrial network in rotenone-treated human fibroblasts. Evidence was provided that these alterations were due to abnormal ROS production. Patient fibroblasts with different structural complex I subunits being mutated but also with different mutations in the same subunit displayed different degrees of mitochondrial network formation, suggesting that the adaptive changes of the mitochondrial network in complex I deficient patient fibroblasts are highly dependent on the subunit that is affected, the degree to which its function is disturbed and, last but not least, the patient's genetic background. Detailed knowledge about the mechanism(s) underlying these adaptive mitochondrial responses, involving changes in mitochondrial architecture, number and cellular distribution, may certainly contribute to a better understanding of complex I deficient pathobiology.

MITOCHONDRIA, COMPLEX I DEFICIENCIES AND ION HOMEOSTASIS

In relation to OXPHOS-system pathology the study of possible alterations in cellular and mitochondrial calcium homeostasis has thus far attracted most attention. Calcium ions are the most common second messengers of eukaryotic cells, decoding the information conveyed by a variety of extracellular molecules that do not cross the plasma membrane (hormones, neurotransmitters, growth factors) into widely diverse intracellular effects (for example muscle contraction, cell proliferation and cell death) (Rizutto and Pozzan, 2003). Mitochondria play an important role in maintaining the cytoplasmic calcium concentrations at strict

levels (Rizutto *et al.*, 1998). Given the strong dependence of mitochondrial calcium uptake on the inner membrane potential and the intracellular distribution of the organelle, both of which may be altered in mitochondrial diseases, Brini and co-workers, investigated the occurrence of defects in mitochondrial calcium handling in living cells with a mtDNA-inherited OXPHOS deficiency (Brini *et al.*, 1999). In MERRF (myoclonic epilepsy with ragged red fibres) cells, but not in NARP (neurogenic muscle weakness, ataxia and retinitis pigmentosa) cells, they found a derangement of agonist-induced mitochondrial calcium changes, whereas the cytosolic calcium response was normal in both cell types (Brini *et al.*, 99). Treatment of MERRF cells with drugs affecting organellar calcium transport mostly restored both the agonist-induced mitochondrial calcium uptake and ensuing ATP production. These results, as they stated, emphasize the differences in the cellular pathogenesis of the various mtDNA defects and indicate specific pharmacological approaches to the treatment of some mitochondrial diseases.

In a recent study, we questioned whether the use of transmitochondrial cell lines, cybrids of patient fibroblasts carrying a mtDNA mutation and human rho-zero osteosarcoma cells, rather than native cells, might have influenced the results obtained by Brini and co-workers. Furthermore, we questioned whether in complex I deficient patient fibroblasts with a defect in one of the nDNA-encoded structural subunits of this complex similar alterations were present. Preliminary measurements revealed, in contrast to the Brini study, significant alterations in agonist-induced cytosolic calcium raises in fibroblasts from 7 out of 8 complex I deficient patients (unpublished data). Subsequent detailed analysis of the most markedly affected cell line showed that these alterations in cytosolic calcium handling were accompanied by reduced mitochondrial calcium accumulation and consequent ATP synthesis. An acute rescue of all defects was achieved by preincubation of patient fibroblasts with a specific inhibitor of the mitochondrial sodium/calcium exchanger.

HUMAN COMPLEX I AND CELL DEATH

Mitochondria play an important role in (programmed) cell death (Mattson and Kroemer, 2003). The intrinsic pathway of apoptosis involves the permeabilization of mitochondrial membranes, which leads to the release of protease and nuclease activators, and to bioenergetic failure (Mattson and Kroemer, 2003). Mitochondrial permeabilization is induced by a variety of pathologically relevant second messengers, including reactive oxygen species, calcium, stress kinases and pro-apoptotic members of the Bcl-2 family. Despite the burst of interest in cell death-research, only limited information is available with respect to cell death and genetic defects of the OXPHOS-system including complex I deficiency. For several practical and theoretical reasons complex I deficient patient-cells might be more prone to cell death. In fact, one of the subunits of the complex, bovine B16.6 (GRIM-19 in man), is a cell death regulatory gene product (Fearnley *et al.*, 2001).

When forced by a reduced rate of glycolytic flux to utilize oxidative metabolism, LHON osteosarcoma-derived

cybrid cells, bearing mutations in the 3460/ND1 and 14484/ND6 subunits, showed chromatin condensation, nuclear DNA laddering and increased release of cytochrome *c* into the cytosol, established markers of apoptotic death (Gheli *et al.*, 2003). The fact that the only relevant difference between control and LHON cybrids is the presence of a mutated mtDNA-encoded structural subunit of complex I is in favour of a direct influence of complex I in apoptotic cell death triggered by metabolic stress. The further characterization of the exact steps of the apoptotic pathway in LHON will provide more details on the pathophysiology of this disease and the link between mitochondrial dysfunction induced by complex I mutations and cell death. (Danielson *et al.*, 2002 ; Gehli *et al.*, 2003).

HUMAN COMPLEX I THERAPY: CURRENT ATTEMPTS AND FUTURE PERSPECTIVES

Based on the above mentioned considerations concerning the cell biological consequences of human complex I disease several theoretical possibilities regarding treatment in order to stabilize or even cure these devastating disorders can be put forward including a) substrate by-passing of complex I, b) gene therapy with alternative dehydrogenases, c) radical scavenging, d) correction of abnormal mitochondrial calcium signaling, e) anti-apoptotic treatment, and f) controlling of negative environmental factors. To evaluate the effects of whatever treatment, besides the availability of genetically characterized cell lines, the generation of complex I deficient animal models is absolutely necessary.

High-Fat Infusion

High-fat, low-carbohydrate diets have been recommended for the treatment of patients with complex I deficiency because they may impose a metabolic challenge in these patients (Munnich *et al.*, 1996). The recommendation for a high-fat diet is based on the following reasons (Roef *et al.*, 2002a):

1. Mitochondrial oxidation of NADH is thought to be diminished in complex I deficient patients. FADH₂ may be an alternative carrier of reducing equivalents and may maintain oxidative phosphorylation because electrons from FADH₂ can enter the OXPHOS-system distal to complex I.
2. Supply of FADH₂ to the mitochondrion can be increased (relative to NADH) by increasing the amount of triacylglycerols and fatty acids in the diet. On the basis of the stoichiometry, it follows that oxidation of fatty acids yields a ratio of FADH₂ to NADH of 0.5, whereas glucose yields a much lower ratio of 0.2.

Roef *et al.* (Roef *et al.*, 2002 a-d) extensively studied 4 adult patients with a complex I deficient mitochondrial myopathy. Briefly, their results revealed that a) lactate disposal via gluconeogenesis was increased during exercise, b) resting oxygen consumption and *in vivo* ADP were increased, c) triacylglycerol infusion did not improve hyperlactemia in resting patients but d) improved exercise endurance as concluded from the leg exercise endurance time, which was clearly lower during glucose infusion compared to triacylglycerol infusion in 3 of the 4 patients. The

authors speculated that the differences observed between glucose and triacylglycerol infusions in complex I deficient patients may be caused by early depletion of muscle glycogen stores during exercise (Roef *et al.*, 2002a). Despite the beneficial effect of triacylglycerol on mitochondrial oxidative phosphorylation and muscle function during exercise, the investigators were not able to detect a change in the muscle's energy state measured by NMR spectroscopy (Roef *et al.*, 2002a). Whether or not this approach will be of benefit of complex I deficient patients warrants further double-blind studies.

Gene Therapy

In contrast to humans, mitochondria of *Saccharomyces cerevisiae* contain two different types of NADH dehydrogenases. One of them faces the matrix side and is responsible for the oxidation of NADH formed by the citric acid cycle (the internal dehydrogenase or NDI1). The NDI1 enzyme is composed of a single polypeptide of 53 kDa, contains a noncovalently bound FAD but no Fe-S clusters, does not pump protons, and is rotenone-insensitive (Seo *et al.*, 2002). In a series of elegant studies the Yagi group showed that this alternative dehydrogenase was able to restore the NADH oxidase activity in Chinese hamster cell lines, human embryonal kidney 293 cells (HEK 293), and finally human mutant complex I deficient cells lacking the essential mtDNA-encoded subunit ND4. (Yagi *et al.*, 2001; Bai *et al.*, 2001; Seo *et al.*, 2001).

Radical Scavenging

Lee and co-authors (Lee *et al.*, 2003), used a different genetic approach to rescue primary cortical neurons from rotenone-induced cell death. Their study was based on the observation that the antioxidant responsive element (ARE) plays an important role in the expression of genes encoding phase II detoxification enzymes and antioxidant proteins and that most of these genes are transcriptionally regulated by NF-E2-related factor 2 (Nrf2). They observed that Nrf2 knock-out neurons were more sensitive to rotenone-induced apoptosis and further elaboration on this finding by gene expression profiling using oligonucleotide microarrays revealed that Nrf2 positive neuronal cells had a higher expression of genes encoding detoxification enzymes, antioxidant proteins, calcium homeostasis proteins, growth factors, neuronal specific proteins, and signaling molecules than Nrf2 negative neuronal cultures. Subsequent adenoviral vector-mediated overexpression of Nrf2 recovered the ARE-driven gene expression in Nrf2 negative cultures and rescued the Nrf2 negative neurons from rotenone-, and also from ionomycin-, induced cell death. Ionomycin is a calcium ionophore that readily increases the intracellular calcium concentration and in accordance with the array data showing a decreased expression of calcium homeostasis proteins in Nrf2 negative neurons this ionophore was indeed found to induce cell death in Nrf2 negative neuronal cultures. This study indicates that Nrf2 plays an important role in protecting neurons from rotenone-induced toxic insult.

A completely different promising approach in counteracting radical toxicity is the delivery of bio-active molecules to mitochondria *in vivo*. Such bio-active

molecules have to accumulate at therapeutically effective concentrations within the mitochondria of the organs that are most affected i.e. the brain, heart and skeletal muscle. As a first step towards the successful implementation of such therapies, Michael Murphy and co-workers developed a lipophilic triphenylphosphonium cation which is able to covalently bind a biologically active molecule such as an antioxidant (Murphy 1997; Murphy and Smith, 2000; Kelso *et al.*, 2001). Orally feeding mice with mitochondrially-targeted antioxidants comprising the triphenylphosphonium cation coupled to a coenzyme Q or vitamin E derivative over long periods led to their steady-state distribution within brain, heart, liver and skeletal muscle. This finding opens the way to the testing of mitochondria-specific therapies in mouse models of human degenerative diseases like human complex I deficiency (Smith *et al.*, 2003). Very recently the first positive effect of such an approach was obtained in Friedreich ataxia fibroblasts (Jauslin *et al.*, 2003).

Correcting Calcium Homeostasis

Based on the initial observation made by Brini *et al.* (Brini *et al.*, 1999) in cybrids we have undertaken a detailed study of calcium homeostasis in our cohort of patient fibroblast cell lines with mutations in nuclear-encoded structural subunits of complex I (unpublished data). As outlined above, we observed that the cytosolic calcium response to hormonal stimulation was impaired in these complex I deficient cell lines. Experiments with the benzothiazepine CGP37157, an inhibitor of the mitochondrial sodium-calcium exchanger, completely restored the aberrant cytosolic calcium signal. This effect of the inhibitor was paralleled by a complete restoration of the hormone-induced increases in mitochondrial calcium concentration and ensuing ATP production. Further studies at the tissue and eventually the organismal level are necessary to evaluate the potential benefit of drugs interfering with cellular calcium homeostasis.

Anti-Apoptotic Treatment

Perhaps the best understood pathway of apoptosis is the Fas pathway, in which mitochondria play a signal amplification role in the cell death program (Wajant, 2002). Danielson and co-investigators could show that cells bearing mutations causing Leber's hereditary optic neuropathy (LHON) are sensitized to Fas-induced apoptosis (Danielson *et al.*, 2002). Further studies along this way are necessary to draw more definite conclusions about the applicability of anti-apoptotic treatment in complex I deficient patients.

Environmental Factors

The frequently encountered clinical observation that in complex I deficient patients, but also other OXPHOS-system affected children, the disease course is negatively influenced by common viral infections needs further attention. These patients are not more prone to viral infections but the effect of common infections leads often to much more severe deterioration than seen in healthy children. Besides, the recovery of e.g. a simple cold with fever takes much longer in complex I deficient patients compared to non-affected subjects. Attenuation of a disturbed energy metabolism and

its consequences during fever is the commonly given explanation for the observed negative effects. Based on these observations anti-pyretic drugs have a place in the treatment of complex I deficient patients.

EPILOGUE

It goes without saying that much has to be learned about complex I in health and disease. Based on the above summarized "state-of-the-art" information about complex I and human complex I deficiencies, it can be concluded that elucidation of the atomic complex I structure, characterization of the complex I assembly pathway, and fully understanding of the cellular consequences of complex I deficiency are absolutely necessary for the development of new treatment strategies to combat these devastating disorders. For the time being, the latter should focus on further elaboration of potential promising strategies including the use of selectively targeted bioactive molecules, drugs affecting mitochondrial calcium homeostasis, or gene therapy. Due to the complex and variable nature of cellular responses in multi-system disorders like human complex I deficiencies, we envisage that a combination of treatment strategies will probably be of most benefit. An important prerequisite for valuable therapeutic responses will be the targeting of drugs to the brain.

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