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Clinical presentations and laboratory investigations in respiratory chain deficiency

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Abstract Respiratory chain deficiencies have long been regarded as neuromuscular diseases. In fact, oxidative phosphorylation, i.e., ATP synthesis by the respiratory chain not only occurs in the neuromuscular system, indeed, a number of non-neuromuscular organs and tissues are dependent upon mitochondrial energy supply. For this reason, a respiratory chain deficiency can theoretically give rise to any symptom, in any organ or tissue, at any age with any mode of inheritance, due to the twofold genetic origin of respiratory enzymes (nuclear DNA and mitochondrial DNA).

Key words Oxidative phosphorylation · Respiratory chain deficiency · Mitochondrial disorders · Metabolic diseases

Abbreviations *CoQ* co-enzyme Q · *COX* cytochrome c oxidase · *ETF* electron transfer flavoprotein · *FAD* flavine adenine dinucleotide · *KSS* Kearns-Sayre syndrome · *LHON* Leber's hereditary optic neuroretinopathy · *L/P* lactate/pyruvate molar ratio · *MtDNA* mitochondrial DNA · *MELAS* mitochondrial encephalomyopathy with lactic acidosis and stroke-like episodes · *MERRF* myoclonus epilepsy, ragged red fibres · *MNGIE* mitochondrial myopathy, peripheral neuropathy, encephalopathy and gastro-intestinal disease · *MRS* magnetic resonance spectroscopy · *NADH* reduced nicotinamide adenine dinucleotide · *NARP* neurogenic muscle weakness, ataxia, retinitis pigmentosa · *PDH* pyruvate dehydrogenase · *PEO* progressive external ophthalmoplegia · *RRF* ragged red fibre

Introduction

In the last few years, it has become increasingly clear that genetic defects of oxidative phosphorylation account for a large variety of clinical symptoms in childhood. Among 100 respiratory chain deficiencies identified in our centre, 56% presented with an extra neuromuscular symptom and 44% were referred because of neuromuscular problems. It appears that the diagnosis of respiratory chain deficiency is difficult to consider early when the presenting symptom is solely present. By contrast, this diagnosis is easier to consider when two seemingly unrelated symptoms are observed.

The clinical presentation

Due to the ubiquitous nature of oxidative phosphorylation, a defect in the mitochondrial respiratory chain should be considered in patients presenting (1) an unexplained association of neuromuscular and/or non neuromuscular symptoms, (2) with a rapidly progressive course, (3) involving seemingly unrelated organs or tissues.

Although the disease may begin virtually at any age, it is worth noting that onset before 1 month concerned 36% of cases in our series of 100 respiratory enzyme deficient children; between 1 month and 2 years: 44% of cases; after 2 years: 20% of cases. Table 1 summarizes the pre-

Table 1 The most frequently observed symptoms in respiratory chain deficiencies

<i>A. Neonatal period (0–1 month)</i>	
Central nervous system	– Chronic interstitial pseudo-obstruction
Iterative apnoea, lethargy, drowsiness, near-miss	Endocrine
Limb and trunk hypotonia	– Short stature, retarded skeletal maturation
Congenital lactic acidosis	– Growth hormone deficiency
Ketoacidotic coma	– Recurrent hypoglycaemia
Muscle	– Insulin dependent diabetes mellitus (IDDM)
Myopathic presentation	– Hypothyroidism
Muscular atrophy, hypotonia	– Hypoparathyroidism
Stiffness, hypertonia	– Adrenocorticotrophin deficiency
Recurrent myoglobinuria	Bone marrow
Poor head control, poor spontaneous movements	– Sideroblastic anaemia
Liver	– Neutropenia, thrombopenia
Hepatic failure, liver enlargement	– Myelodysplastic syndrome, dyserythropoiesis
Heart	Ear
Hypertrophic cardiomyopathy (concentric ++)	– Hearing loss
Kidney	– Sensorineural deafness (brainstem or cochlear origin)
Proximal tubulopathy (De Toni Debré Fanconi syndrome)	Eye
<i>B. Infancy (1 month–2 years)</i>	– Optic atrophy
Central nervous system	– Diplopia
– Recurrent apnoeas, near-miss	– PEO
– Recurrent ketoacidotic comas	– Limitation of eye movements (all directions, upgaze ++)
– Poor head control, limb spasticity	– “Salt and pepper” retinopathy, pigmentary retinal degeneration
– Psychomotor regression, mental retardation	– Lid ptosis
– Cerebellar ataxia	– Cataract
– “Stroke-like” episodes	Skin
– Myoclonus, generalized seizures	– Mottled pigmentation of photo-exposed areas
– Subacute necrotizing encephalomyopathy (Leigh syndrome)	– Trichothiodystrophy
– Progressive infantile poliodystrophy (Alpers syndrome)	– Dry, thick and brittle hair
Muscle	<i>C. Childhood (> 2 years) and adulthood</i>
– Myopathic features	Central nervous system
– Muscular atrophy	– Myoclonus
– Limb weakness, hypotonia	– Seizures (generalized, focal, drop attacks, photosensitive, tonicoclonic)
– Myalgia, exercise intolerance	– Cerebellar ataxia
– Recurrent myoglobinuria	– Spasticity
Liver	– Psychomotor regression, dementia, mental retardation
– Progressive liver enlargement	– “Stroke-like” episodes
– Hepatocellular dysfunction	– Hemicranial headache, migraine
– Valproate-induced hepatic failure	– Recurrent hemiparesis, cortical blindness or hemianopsia
Heart	– Leukodystrophy, cortical atrophy
– Hypertrophic cardiomyopathy (concentric)	– Peripheral neuropathy
Kidney	Muscle
– Proximal tubulopathy (De Toni Debré Fanconi syndrome)	– Progressive myopathy
– Tubulo interstitial nephritis (mimicking nephronophtosis)	– Limb weakness (proximal)
– Nephrotic syndrome	– Myalgia, exercise intolerance
– Renal failure	– Recurrent myoglobinuria
– Haemolytic uraemic syndrome	Heart
Gut	– Concentric hypertrophic or dilated cardiomyopathy
– Recurrent vomiting	– Complete heart block
– Chronic diarrhoea, villous atrophy	– Intraventricular conduction block
– Exocrine pancreatic dysfunction	– Right bundle branch block
– Failure to thrive	

(continued on next page)

Table 1 (continued)

Endocrine

- Diabetes mellitus (insulin- and non-insulin dependent)
- Growth hormone deficiency
- Hypoparathyroidism
- Hypothyroidism
- Adrenocorticotropic deficiency
- Hyperaldosteronism
- Infertility (ovarian failure or hypothalamic dysfunction)

Eye

- Lid ptosis
- Diplopia
- PEO
- Limitation of eye movements (all directions, upgaze ++)
- Pigmentary retinal degeneration
- Cataract, corneal opacities
- LHON

Ear

- Sensorineural deafness
- Aminoglycoside-induced ototoxicity (maternally inherited)

senting symptoms most frequently observed in the neonatal period, infancy, childhood and adulthood respectively. Whatever the age of onset and initial symptom, the major feature is the increasing number of tissues affected in the course of the disease. This progressive organ involvement is constant and the CNS is almost consistently involved in the late stage of the disease.

While initial symptoms usually persist and gradually worsen, they may occasionally improve or even disappear as other organs become involved. This is particularly true for bone marrow and gut. Indeed, remarkable remissions of pancytopenia or watery diarrhoea have been reported in infants who later developed other organ involvement. Moreover, several patients whose disease apparently started in childhood or adulthood were retrospectively shown to have experienced transient sideroblastic anaemia, neutropenia, chronic watery diarrhoea or failure to thrive of unexplained origin in early infancy. Similarly, a "benign" reversible infantile myopathy, with hypotonia, weakness, macroglossia, respiratory distress and spontaneous remission within 1–2 years has been described.

Certain clinical features or associations are more frequent at certain ages and have been occasionally identified as distinct entities, suggesting that these associations are not fortuitous. Yet, considerable overlap in clinical features leads to difficulty in classification of many patients and this should question the usefulness of subclassifications. In fact, attempts to ascribe clinical profiles to particular syndromes or to delineate hermetic boundaries between syndromes is useless and unavailing, especially as the nature, clinical course and severity of recruited symptoms vary among (and even within) affected individuals. In our opinion, it is more helpful to bear in mind that

respiratory chain deficiency should be considered when dealing with an unexplained association of signs with a progressive course involving seemingly unrelated organs or tissues, regardless the age of onset and the nature of the presenting symptom. The non exhaustive list of clinical profiles listed below illustrates the diversity of presentations.

In the neonate (< 1 month):

1. Ketoacidotic coma with recurrent apnoeas, seizures, severe hypotonia, liver enlargement and proximal tubulopathy in the neonatal period, with or without symptom-free period [7].
2. Severe neonatal sideroblastic anaemia (\pm hydrops fetalis) with neutropenia, thrombopenia and exocrine pancreatic dysfunction of unexplained origin (Pearson marrow pancreas syndrome) [15].
3. Concentric hypertrophic cardiomyopathy and muscle weakness with an early onset and a rapidly progressive course (dilated cardiomyopathies are exceptional) [20].
4. Concentric hypertrophic cardiomyopathy with profound central neutropenia and myopathic features in a male patient (Barth syndrome) mapped to chromosome Xq28 [1].
5. Hepatic failure with lethargy, hypotonia and proximal tubulopathy of unknown origin with neonatal onset [7].

In infancy (1–2 years):

1. Failure to thrive with or without chronic watery diarrhoea and villous atrophy, unresponsive to gluten-free and cow milk protein-free diet [8].
2. Recurrent episodes of acute myoglobinuria, hypertonia, muscle stiffness and elevated plasma levels of sarcoplasmic enzymes unexplained by an inborn error of glycolysis, glycogenolysis, fatty acid oxidation or muscular dystrophy [22].
3. Proximal tubulopathy (de Toni Debré Franconi syndrome) with recurrent episodes of watery diarrhoea, rickets and mottled pigmentation of photo-exposed areas; or a tubulo interstitial nephritis mimicking nephronophthisis and subsequently developing renal failure and encephalomyopathy with leukodystrophy [16].
4. Severe trunk and limb dwarfism, unresponsive to growth hormone administration with subsequent hypertrophic cardiomyopathy, sensorineural deafness and retinitis pigmentosa.
5. Early-onset insulin dependent diabetes mellitus with diabetes insipidus, optic atrophy and deafness (Wolfram syndrome) [17].
6. Rapidly progressive encephalomyopathy with hypotonia, poor sucking, weak crying, poor head control, cere-

bellar ataxia, pyramidal syndrome, psychomotor regression, developmental delay, muscle weakness, respiratory insufficiency, occasionally associated with proximal tubulopathy and/or hypertrophic cardiomyopathy.

7. Subacute necrotizing encephalomyopathy (Leigh disease): a devastating encephalopathy characterized by recurrent attacks of psychomotor regression with pyramidal and extra pyramidal symptoms, leukodystrophy and brainstem dysfunction (respiratory abnormalities). The pathological hallmark consists of focal, symmetrical and necrotic lesions in the thalamus, brainstem and the posterior columns of the spinal cord. Microscopically, these spongiform lesions show demyelination, vascular proliferation and astrocytosis [11].

In childhood (> 2 years) and adulthood, the neuromuscular presentation is the most frequent:

1. Myopathy, muscle weakness, myalgia, exercise intolerance with or without progressive external ophthalmoplegia [11].

2. Progressive sclerosing poliomyopathy (Alpers disease) associated with hepatic failure (occasionally triggered by valproate administration) [5].

3. Encephalomyopathy with myoclonus, ataxia, hearing loss, muscle weakness and generalized seizures (myoclonus epilepsy, ragged red fibres; MERRF) [11].

4. Progressive external ophthalmoplegia (PEO) ranging in severity from pure ocular myopathy to Kearns-Sayre syndrome (KSS), a multisystem disorder characterized by the unvariant triad: onset before age 20, PEO, pigmentary retinal degeneration plus at least one of the following: complete heart block, CSF protein above 100 mg/dl, cerebellar ataxia [11].

5. Mitochondrial encephalomyopathy with lactic acidosis and stroke-like episodes (MELAS). This syndrome is characterized by onset in childhood with intermittent hemispheric headache, vomiting, proximal limb weakness, and recurrent neurological deficit resembling strokes (hemiparesis, cortical blindness, hemianopsia), lactic acidosis and ragged red fibres in the muscle biopsy. CT brain scan shows low density areas (usually posterior) which may affect both white and grey matter but does not always correlate to clinical symptoms or vascular territories. The pathogenesis of stroke like episodes in MELAS has been ascribed to either cerebral blood flow disruption or acute metabolic decompensation in biochemically deficient areas of the brain [11].

6. Leber's hereditary optic neuroretinopathy (LHON). This disease is associated with rapid bilateral central vision loss due to optic nerve death. Cardiac dysrhythmia is frequently associated with the disease but no evidence of skeletal muscle pathology or gross structural mitochon-

drial abnormality has been documented. The median age of vision loss is 20–24 years but it can occur at any age between adolescence and late adulthood. Expression among maternally related individuals is variable and there is a bias toward males being affected [11].

7. Neurogenic muscle weakness, ataxia, retinitis pigmentosa and variable sensory neuropathy, (NARP) seizures and mental retardation or dementia [13].

8. Mitochondrial myopathy, peripheral neuropathy, encephalopathy and gastro intestinal disease manifested as intermittent diarrhoea and intestinal pseudo obstruction (myoneuro-gastro-intestinal encephalopathy (MNGIE)) [11].

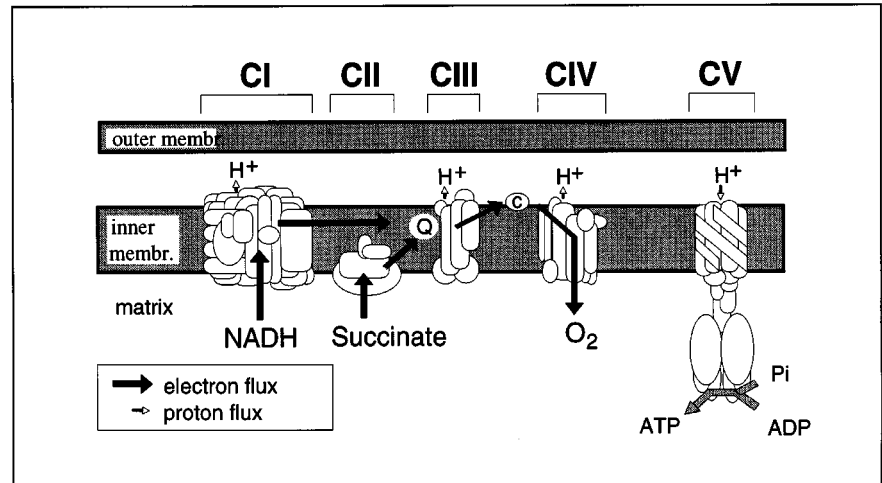
The metabolic derangement

The respiratory chain is divided into five functional units or complexes, embedded in the inner mitochondrial membrane (Fig. 1). Complex I (reduced nicotinamide adenine dinucleotide (NADH)-coenzyme Q reductase) carries reducing equivalents from NADH to coenzyme Q (CoQ) and consists of 25–28 different polypeptides, 7 of which are encoded by mitochondrial DNA (mtDNA). Complex II (succinate-CoQ reductase) carries reducing equivalents from flavin adenine dinucleotide (FADH₂) to CoQ and contains 5 polypeptides, including the FAD-dependent succinate dehydrogenase, and a few nonhaem iron sulphur centres. This is the only complex that does not contain any mtDNA-encoded protein. Complex III (reduced CoQ-cytochrome c reductase) carries electrons from CoQ to cytochrome c. It contains 11 subunits, one of which (the apoprotein of cytochrome b) is encoded by mtDNA. Complex IV (cytochrome c oxidase, COX), the last component of the respiratory chain, catalyses the transfer of reducing equivalents from cytochrome c to molecular oxygen. It is composed of 2 cytochromes (a and a₃), 2 copper atoms, and 13 different protein subunits, 3 of which are encoded by mtDNA [12].

The mitochondrial respiratory chain catalyses the oxidation of fuel molecules by oxygen and the concomitant energy transduction into ATP. During the oxidation process, electrons are transferred to oxygen via the energy-transducing complexes of the respiratory chain: complexes I, III and IV for NADH-producing substrates; complexes II, III and IV for succinate; complexes III and IV for FADH₂ derived from the β -oxidation pathway, via the electron transfer flavoprotein (ETF) and the ETF-CoQ oxidoreductase system. CoQ (a lipoidal quinone) and cytochrome c (a low molecular weight haemoprotein) act as "shuttles" between complexes.

The free energy generated from the redox reactions is converted into a transmembrane proton gradient. Protons are pumped through the mitochondrial inner membrane at three coupling sites (represented by complexes I, III and IV) which creates a charge differential. Complex V (ATP synthase)

Fig. 1 The mitochondrial respiratory chain. *CI* complex I (NADH-CoQ reductase), *CII* complex II (succinate-CoQ reductase), *CIII* complex III (ubiquinol-cytochrome c reductase), *CIV* complex IV (cytochrome c oxidase), *CV* complex V (ATPase)



allows protons to flow back into the mitochondrial matrix and uses the released energy to synthesize ATP. Three ATP molecules are made for each NADH oxidized.

Since the respiratory chain transfers NADH to oxygen, a disorder of oxidative phosphorylation should result in an increase of reducing equivalents in both mitochondria and cytoplasm and in the functional impairment of the citric acid cycle, due to the excess of NADH and the lack of NAD. Therefore, an increase of ketone body (β -OH butyrate/acetoacetate) and lactate/pyruvate (L/P) molar ratios with a secondary elevation of blood lactate might be found in the plasma of affected individuals. This is particularly true in the post absorptive period, when more NAD is required for adequately oxidizing glycolytic substrates.

Similarly, as a consequence of the functional impairment of the citric acid cycle, ketone body synthesis increases after meals due to the channelling of acetyl CoA toward ketogenesis. The elevation of total ketone bodies in a fed individual is paradoxical, as it should normally decrease after meals, due to insulin release (paradoxical hyperketonaemia). For this reason, in patients likely to suffer from respiratory chain deficiency, our current screening includes the determination of plasma lactate, pyruvate, ketone bodies and their molar ratios in both fasted and fed individuals.

Yet, the level of the block might differentially alter the metabolic profile of the patients. A block at the level of complex I impairs the oxidation of the 3 mol of NADH formed in the citric acid cycle. In theory, at least, oxidation of FADH_2 derived from the succinate dehydrogenase reaction and succinate-producing substrates (methionine, threonine, valine, isoleucine and odd numbered fatty acids) should not be altered, because it is mediated by complex II. Similarly, oxidation of FADH_2 derived from the first reaction of the β -oxidation pathway should occur normally because it is mediated by the ETF-CoQ reductase system. On the other hand, complex II deficiency should not markedly alter the redox status of affected individuals fed a carbohydrate-rich diet. A block at the level

of complex III would impair oxidation of both NAD-linked and FAD-linked substrates. Finally, given the crucial role of complex IV in the respiratory chain, it is not surprising that severe defects of COX activity would cause severe lactic acidosis and markedly altered redox status in plasma.

Diagnostic tests

Screening tests

These include the determination of lactate, pyruvate, ketone bodies and their molar ratios in plasma, as indices of oxidation reduction status in cytoplasm and mitochondria respectively (Table 2). Determinations should be made in the fasted and 1-h fed individuals and repeated during the day. In order to avoid artefactual elevation of lactic acid, blood samples should be taken from a patient at rest through an heparinized venous catheter and immediately deproteinized by perchloric acid. Samples should be either forwarded in ice to the laboratory or immediately frozen (-20°C or below). Blood glucose and non esterified fatty acids should be simultaneously monitored.

The observation of a persistent hyperlactataemia (> 2.5 mM) with elevated L/P and ketone body molar ratios (particularly in the post-absorptive period) is highly suggestive of a respiratory chain deficiency. In addition, investigation of the redox status in plasma can help discriminate between the different mechanisms of congenital lactic acidosis, based on L/P and ketone body molar ratios in vivo. Indeed, an impairment of oxidative phosphorylation usually results in L/P ratios > 20 and ketone body ratios > 2 , whereas a defect of the pyruvate dehydrogenase (PDH) complex results in low L/P ratios (< 10). Although little is known regarding tricarboxylic acid cycle disorders, it appears that these diseases also result in high L/P ratios but ketone body molar ratios are lower in these conditions

Table 2 Screening procedures

Standard screening tests (at least 4 determinations daily in fasted and 1-h fed individuals)

1. Plasma lactate
2. L/P molar ratio = redox status in cytoplasm
3. Ketonaemia ("paradoxical" elevation in fed individuals)
4. β -OH butyrate/acetoacetate molar ratio = redox status in the mitochondria
5. Blood glucose and free fatty acids
6. Urinary organic acids = lactate, ketone bodies, citric acid cycle intermediates

Provocative tests (when standard tests are inconclusive)

1. Glucose test (2 g/kg, orally) in fasted individuals with determination of blood glucose, lactate, pyruvate, ketone bodies and their molar ratios at t 15 min, 30 min, 45 min, 60 min, 90 min
2. L/P molar ratios in the CSF (only when no elevation of plasma lactate is observed)
3. Redox status in plasma following exercise

Screening for multiple organ involvement

- Liver = hepatocellular dysfunction?
- Kidney = proximal tubulopathy, distal tubulopathy, proteinuria, renal failure?
- Heart = hypertrophic cardiomyopathy, heart block? (ultrasounds, ECG)
- Muscle = myopathic features? (CK, ALAT, ASAT, histological anomalies, RRF?)
- Brain = leukodystrophy, poliodystrophy, hypodensity of the cerebrum, cerebellum and the brainstem, multifocal areas of hyperintense signal (MELAS), bilateral symmetrical lesions of the basal ganglia and brainstem (Leigh) ? (EEG, NMR, CT scan)
- Peripheral nerve: distal sensory loss, hypo- or areflexia, distal muscle wasting (usually subclinical), reduced motor nerve conduction velocity and denervation features? (nerve conduction velocity, EMG, peripheral nerve biopsy showing axonal degeneration and myelinated fibre loss)
- Pancreas = exocrine pancreatic dysfunction?
- Gut = villous atrophy?
- Endocrine = hypoglycaemia, hypocalcaemia, hypoparathyroidism, growth hormone deficiency? (stimulation tests)
- Bone marrow = anaemia, neutropenia, thrombopenia, pancytopenia, vacuolization of marrow precursors?
- Eye = PEO, ptosis, optic atrophy, retinal degeneration? (fundus, electroretinogram, visual evoked potentials)
- Ear = sensorineural deafness? (auditory evoked potentials, brainstem evoked response)
- Skin = trichothiodystrophy, mottled pigmentation of photo-exposed areas?

(< 1) that in respiratory chain defects (as also observed in pyruvate carboxylase deficiency) [2, 21].

Provocative tests

These should be carried out when basal screening tests are inconclusive (Table 2):

1. Glucose loading test (2 g/kg orally) so as to unmask latent hyperlactataemia and/or paradoxical hyperketonaemia.

2. Screening for urinary lactate and citric acid cycle intermediates, using gas chromatography-mass spectrometry.

3. Determination of the redox status in the CSF, so as to detect elective increase of lactatorrachia and/or elevated L/P ratios (this determination is useless when the redox status is altered in plasma).

Yet, the above diagnostic tests may fail to detect any disturbance of the redox status in plasma, as pitfalls in the metabolic screening are numerous. For example:

1. Proximal tubulopathy may lower blood lactate and increase urinary lactate.
2. Diabetes mellitus may hamper entry of pyruvate into the citric acid cycle
3. Tissue-specific isoforms may be selectively impaired, barely altering the redox status in plasma (this is particularly true for hypertrophic cardiomyopathies).
4. The defect may be generalized but partial: the more those tissues with higher dependence on oxidative metabolism suffer (such as brain and muscle), the more the oxidation reduction status in plasma is impaired.
5. The defect may be confined to complex II, barely altering (in principle) the redox status in plasma.

When diagnostic tests are negative, respiratory chain deficiency may be misdiagnosed, especially when the onset symptom is solely present. By contrast, this diagnosis is easier to consider when seemingly unrelated symptoms are observed. For this reason, the investigation of patients at risk for respiratory chain deficiency (whatever the onset symptom) includes the systematic screening of all target organs and tissues for possible clinical/biological derangement, as multiple organ involvement is an important clue to the diagnosis of this condition (Table 2).

Enzyme assays

The observation of an abnormal redox status in plasma and/or the evidence of multiple organ involvement prompts one to carry out further enzyme investigations. These investigations include two entirely distinct procedures which provide independent clues to the diagnosis of respiratory chain disorders: polarographic studies and spectrophotometric studies.

Polarographic studies consist in the measurement of oxygen consumption by mitochondria-enriched fractions in a Clarke electrode in the presence of various oxidative substrates (malate + pyruvate, malate + glutamate, succinate, palmitate, etc.). In case of complex I deficiency, polarographic studies show impaired respiration with NADH-producing substrates, while respiration and phosphorylation are normal with FADH-producing substrates (succinate).

nate). The opposite is observed in case of complex II deficiency, whereas a block at the level of complexes III or IV impairs oxidation of both NADH- and FADH-producing substrates. In complex V deficiency, there is an impaired respiration with various substrates, but adding the uncoupling agent 2,4-dinitrophenol, or calcium ions, returns the respiratory rate to normal, suggesting that the limiting step involves phosphorylation rather than the respiratory chain [9].

It is worth remembering that polarographic studies not only detect disorders of oxidative phosphorylation but also PDH deficiencies, citric acid cycle enzyme deficiencies and genetic defects of carriers, shuttles and substrates (including cytochrome c, cations and adenylate), as these conditions also impair production of reduced equivalents in the mitochondrion. In these cases, however, respiratory enzyme activities are expected to be normal (see below).

While previous techniques required gram amounts of muscle tissue, the scaled-down procedures available now allow the rapid recovery of mitochondria-enriched fractions (400–500 µg protein) from small skeletal muscle biopsies (100–200 mg obtained under local anaesthesia), thus making polarography feasible in infants and children [19]. Polarographic studies on intact circulating lymphocytes (isolated from 10 ml of blood on a Percoll cushion) or detergent-permeabilized cultured cells (lymphoblastoid cell lines, skin fibroblasts) are also feasible and represent a non invasive and easily reproducible diagnostic test [3]. The only limitation of these techniques is the absolute requirement of fresh material: no polarographic studies are possible on frozen material.

Spectrophotometric studies consist of the measurement of respiratory enzyme activities separately or by groups, using specific electron acceptors and donors. They do not require isolation of mitochondrial fractions and can be carried out on tissue homogenates. For this reason, the amount of material required for enzyme assays (1–20 mg) is very small and can be easily obtained by needle biopsies of liver, kidney and even by endomyocardial biopsies [20]. Similarly, a 25 ml flask of cultured skin fibroblasts or a lymphocyte pellet derived from a 10 ml blood sample are sufficient for extensive spectrophotometric studies. Samples should be immediately frozen and kept dry in liquid nitrogen (or at -80°C).

Since conclusive diagnostic evidence of respiratory chain deficiency is provided by enzyme assays, the question of what tissue should be investigated deserves particular attention. In principle, the relevant tissue is that which actually expresses the disease. When the skeletal muscle clinically expresses the disease, the appropriate working material is a micro biopsy of the deltoid. When the haematopoietic system expresses the disease (i.e. Pearson syndrome), tests should be carried out on circulating lymphocytes, polymorphonuclears or bone marrow. However, when the disease is mainly expressed in liver or heart, gaining access to the target organ is far less conve-

nient, but a needle biopsy of the liver or an endomyocardial biopsy are usually feasible. If not, or when the disease is mainly expressed in a barely accessible organ (brain, retina, endocrine, smooth muscle), peripheral tissues should be extensively tested (including skeletal muscle, cultured skin fibroblasts, circulating lymphocytes).

Whatever the expressing organ, it is therefore mandatory to take skin biopsies from those patients (even post mortem) for subsequent investigations on cultured fibroblasts.

It should be born in mind, however, that the *in vitro* investigation of oxidative phosphorylation remains difficult regardless the tissue tested. Several pitfalls should be mentioned.

1. Normal respiratory enzyme activities do not preclude mitochondrial dysfunction even when the tissue tested clinically expresses the disease. One might be dealing with a kinetic mutant, tissue heterogeneity or a cellular mosaicism (heteroplasmy, see below). In this case, one should carry out extensive molecular genetic analyses, test other tissues and possibly repeat investigations later.
2. The scattering of control values occasionally hampers the recognition of enzyme deficiencies as normal values frequently overlap those found in the patients. It is helpful to express results as ratios, especially as the normal functioning of the respiratory chain requires a constant ratio of enzyme activities [18]. Under these conditions, patients whose absolute activities are in the low normal range can be unambiguously diagnosed as enzyme deficient (this expression of results may fail to recognize generalized defects of oxidative phosphorylation).
3. No reliable method for assessment of complex I activity in circulating or cultured cells is presently available, because oxidation of NADH-generating substrates by detergent-treated or freeze-thawed control cells is variable and the rotenone-resistant NADH-cytochrome c reductase activity is very high in this tissue.
4. The phenotypic expression of respiratory enzyme deficiencies in cultured cells is unstable and activities return to normal values when cells are grown in standard medium [10]. Adding uridine (200 µM) to the culture medium avoids counterselection of respiratory enzyme deficient cells and allow them to grow normally, thereby stabilizing the mutant phenotype (uridine which is required for nucleic acid synthesis is probably limited by the secondary deficiency of the respiratory chain-dependent dehydroorotate dehydrogenase activity) [4].
5. Discrepancies between control values may indicate faulty experimental conditions. Activities dependent on a single substrate should be consistent when tested under non-rate limiting conditions.
6. Incorrect freezing may result in a rapid loss of quinone-dependent activities, probably due to peroxidation

of membrane lipids. Tissue samples fixed for morphological studies are inadequate for subsequent respiratory enzyme assays.

Histopathological studies

The muscle specimen taken under local anaesthesia must be immediately frozen in liquid nitrogen-cooled isopentane. The histological hallmark of mitochondrial myopathy is the ragged red fibre (RRF) demonstrated with the modified Gomori trichrome stain, containing peripheral and inter myofibrillar accumulations of abnormal mitochondria. Although the diagnostic importance of pathological studies is undisputed, the presence of RRF as the unique criterion has been challenged by recent advances. It is now clear that absence of RRF does not rule out the diagnosis of mitochondrial disorder [11]. Different histochemical stains for oxidative enzymes are used to analyse the distribution of mitochondria in the individual fibres and to evaluate the presence or absence of the enzymatic activities. Histochemical staining allows an estimation of the severity and heterogeneity of enzyme deficiency in the same muscle section. Myofibrillar integrity, muscle type fibre predominance and distribution can be evaluated with the myofibrillar ATPase stain. Studies using polyclonal and monoclonal antibodies directed against COX subunits are carried out in specialized centres.

Magnetic resonance spectroscopy (MRS) of muscle and brain

Phosphorus MRS allows study of muscle and brain energy metabolism *in vivo*. Inorganic phosphate, phosphocreatine, adenosine mono-, di- or tri-phosphate (AMP, ADP, ATP) and intracellular pH may be measured. The inorganic phosphate/phosphocreatine ratio is the most useful parameter and may be mentioned at rest, during exercise and recovery. An increased ratio is found in most patients and MRS is becoming a useful tool in diagnosis of mitochondrial diseases and also to monitor therapeutic trials. Yet, the observed anomalies are not specific to respiratory enzyme deficiencies and no correlation between MRS findings and the site of the respiratory enzyme defect can be made [11].

Treatment and prognosis

No satisfactory therapy is presently available for respiratory chain deficiency. Treatment remains largely symptomatic and does not significantly alter the course of the disease. It includes avoidance of drugs and procedures known to have a detrimental effect, symptomatic treatments, supplementation with cofactors, prevention of oxygen radical

damage to mitochondrial membranes and dietary recommendations.

It is advisable to avoid sodium valproate and barbiturates which inhibit the respiratory chain and have occasionally been shown to precipitate hepatic failure in respiratory enzyme-deficient children [5]. Tetracyclines and chloramphenicol should be avoided as well, as they inhibit mitochondrial protein synthesis. Due to the increasing number of tissues affected in the course of the disease, it is recommended that organ transplantation (bone marrow, liver, heart) be avoided.

Symptomatic treatments include slow infusion of sodium bicarbonate during acute exacerbation of lactic acidosis, pancreatic extract administration in the case of exocrine pancreatic dysfunction and repeated transfusions in cases of anaemia or thrombopenia.

Sustained improvement has been reported in cases of complex III deficiency given menadione (vit K₃, 40–160 mg/day) or coenzyme Q10 (80–300 mg/day). Treatment with riboflavin (100 mg/day) has been associated with improvement in a few patients with complex II deficiency myopathy. A combination of menadione (or coenzyme Q10) and riboflavin is usually given to the patients. Carnitine is suggested in patients with secondary carnitine deficiency. The prevention of oxygen radical damage is the rationale for ascorbate administration (2–4 g/day). Dichloroacetate or 2-chloropropionate administration have been proposed to stimulate PDH activity and have occasionally reduced the level of lactic acid [23] but detrimental effects of dichloroacetate have been recently reported (reversible peripheral neuropathy).

Dietary recommendations are a high lipid-low carbohydrate diet in patients with complex I deficiency. Indeed, a high glucose diet is a metabolic challenge for patients with impaired oxidative phosphorylation, especially as glucose oxidation is largely aerobic in the liver. Based on our experience, we suggest avoiding a hypercaloric diet and parenteral nutrition and recommend a low carbohydrate diet in addition to the symptomatic treatment. Succinate (16 g/day), succinate-producing aminoacids or propionyl carnitine have occasionally been given to patients with complex I deficiency, as these substrates enter the respiratory chain via complex II.

Genetics

Any mode of inheritance can be observed in mitochondrial diseases: sporadic, autosomal recessive, dominant, X-linked or maternal inheritance. Indeed, among the 70 genes encoding the respiratory chain proteins, most are located in the nucleus and undergo classical mendelian inheritance. On the other hand, each human cell contains thousands of molecules of mitochondrial DNA (mtDNA) which is a maternally inherited intronless 16569 bp circular genome encoding 13 genes: a large and a small rRNA,

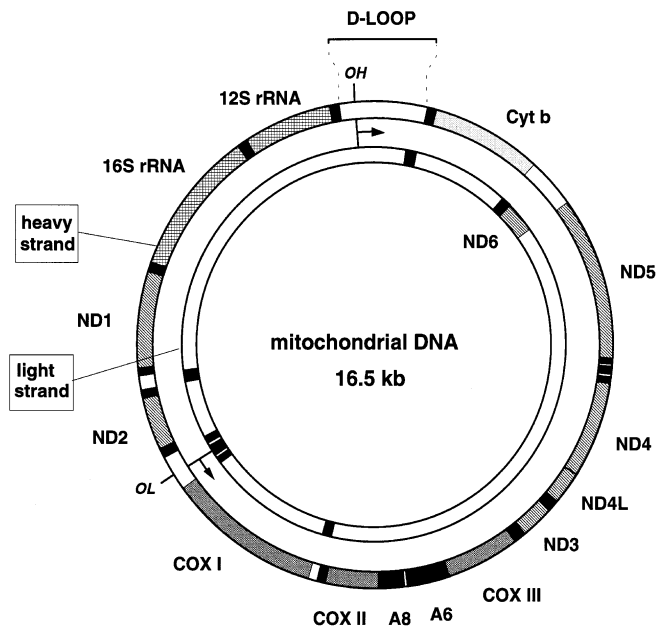


Fig. 2 Map of the mitochondrial genome. Regions encoding cytochrome b (*Cyt b*), various subunits of NADH-coenzyme Q reductase (*ND* cytochrome oxidase (*COX*), and ATPase (*A*), and ribosomal RNAs (*rRNA*) are indicated. Replication of the heavy strand origin (*OH*), and that of the light stand at *OL*

22 tRNAs and 13 keys subunits of the respiratory enzymes including seven subunits of complex I, one subunit of complex III, three subunits of complex IV and two subunits of complex V. Replication of the heavy (H) strand is initiated at the displacement loop (D-loop), which encompasses the two strand-specific promoters and the H-strand origin of replication (*OH*). Replication of the light (L) strand begins at the L-strand origin (*OL*), two-thirds of the way around the molecule (Fig. 2). The mt genome is transcribed from the specific promoters on both strands into polycistronic RNAs which are further processed into tRNAs and mRNAs. Mitochondrial mRNAs are translated within the mitochondrial matrix using a nuclear-encoded enzymatic machinery, but following a different genetic code (the stop codon being often encoded post-transcriptionally). The mt DNA has a number of unique genetic features [6]:

1. mtDNA is maternally inherited, as it is predominantly transmitted through the egg cytoplasm. The mother transmits her mtDNA to all her progeny and her daughters transmit their mtDNA to the next generation. Males never transmit their mtDNA. This feature accounts for the maternal inheritance of mtDNA mutations.
2. mtDNA has a very high mutation rate, involving both nucleotide substitutions and insertion-deletion mutations.
3. mtDNA can switch genotypes completely into two or three generations, as the number of mtDNA molecules is

greatly reduced at some points in oogenesis and there is an uneven transmission to progeny. This switch appears not to occur so rapidly when mtDNA mutations are harmful, presumably because of selection in favour of wild-type mtDNA.

4. In cells with a mixture of mutant and wild-type mtDNAs (heteroplasmy), the mtDNA genotype can shift during cellular replication (replicative segregation). This feature results from the random partitioning of mitochondria into daughter cells during cell division. Consequently, some lineages drift toward pure mutant mtDNAs (homoplasmy), others toward pure wild-type mtDNAs while still others remain heteroplasmic. In cells harbouring mutant and wild-type molecules, the phenotype is a reflection of the proportion of mutant mtDNA molecules and the extent to which the cell type relies on mitochondrial function.

Mitochondrial DNA diseases

Pathological alterations of mtDNA fall into three major classes: point mutations, deletion-insertions and copy number mutations (depletions).

Point mutations include amino acid substitutions and protein synthesis mutations (tRNA, rRNA, Table 3). Most of these are maternally inherited and heteroplasmic but they are associated with a striking variety of clinical phenotypes [24] depending on the proportion of mutant mtDNAs inherited by the different maternal relatives. Indeed, within one particular pedigree, clinical presentations may range from migraines and attention deficit disorders to the full MELAS syndrome. Maternal relatives of patients are generally healthy as long as they have no more than 85% mutant mtDNA. Once the percentage of mutant mtDNA rises above this level, there are increasingly serious consequences on the clinical phenotype, illustrating the sharp threshold of protein synthesis mutants.

Table 3 summarizes the clinical phenotypes currently associated with base substitutions. It also indicates the mutations to be first tested according to the clinical presentation of the patient. It is worth noting, however, that there is no strict genotype-phenotype correlation in mtDNA mutations as a given base substitution can be associated to markedly different clinical profiles (Table 3). While MERRF, MELAS, NARP and Leigh syndrome mutations are frequently heteroplasmic, LHON mutations are usually homoplasmic, at least in circulating leukocytes. Moreover, several LHON families harbour distinct mtDNA base substitutions that may act synergistically to increase the probability of blindness (likelihood of blindness might be increased in individuals who have more severe mutations or combinations of base substitutions).

The second class of mtDNA diseases are deletions-duplications of the mt genome. Although the size and the

Table 3 Base substitution mutations in mitochondrial diseases

Clinical disorder	Locus symbol	Gene mutated	Position of mutation (bp)	Amino acid change	
Leber hereditary optic neuroretinopathy	LHON	ND1	3394	Y→H	
			3460	A→T	
			4160	L→P	
		ND2	4917	D→N	
			COI	7444	Term→K
			ND4	11778	R→H
			ND5	13708	A→T
ND6	14484	M→V			
	<i>cytb</i>	15257	D→N		
Subacute necrotizing encephalopathy, Leigh syndrome	SNE	ATPase6	8993	L→R	
Neurogenic muscle weakness, ataxia and retinitis pigmentosa	NARP or MTRP1	ATPase6	8993	L→R	
Non insulin dependent diabetes mellitus and deafness	NIDDMD	tRNA ^{Leu}	2343	tRNA	
Mitochondrial myopathy	MEM	tRNA ^{Leu}	3250	tRNA	
Fatal infantile cardiomyopathy	MTCMH1	tRNA ^{Ileu}	4269	tRNA	
		tRNA ^{Ileu}	4317	tRNA	
		tRNA ^{Leu}	3303	tRNA	
Cardiomyopathy and adult onset myopathy	MTCMH2	tRNA ^{Leu}	3260	tRNA	
Mitochondrial encephalopathy, lactic acidosis, stroke like episodes	MELAS	tRNA ^{Leu}	3271	tRNA	
		tRNA ^{Leu}	3243	tRNA	
Calcification, hearing loss, hypogonadism, encephalopathy, retinitis pigmentosa	CHERP	tRNA ^{Leu}	3271	tRNA	
Myoclonus epilepsy and ragged red fibres	MERRF	tRNA ^{Lys}	8344	tRNA	
	MERRF/MELAS	tRNA ^{Lys}	8356	tRNA	
Chronic intestinal pseudo-obstruction with myopathy and ophthalmoplegia	CIPO	tRNA ^{Gly}	10006	tRNA	
		tRNA ^{Ser}	12246	tRNA	
		tRNA ^{Leu}	12308	tRNA	

position of the deletion differ markedly among patients, they usually encompass several coding genes, tRNA genes and occasionally remove the origin of replication of the light strand. They are usually sporadic, heteroplasmic, unique and frequently occur between directly-repeated sequences, suggesting that they are caused by de novo rearrangements which arose during oogenesis or early development [24]. Several mechanisms have been proposed for the origin of these rearrangements: (1) homologous recombination between repeated sequences; (2) topoisomerase cleavage and (3) slip-replication, especially as DNA breaks occasionally occur a few nucleotides aside from the direct repeats. Table 4 summarizes the clinical presentation of hitherto reported mtDNA deletions-duplications. It is worth noting that the most common deletion (4997 bp) found in 30% of patients harbouring a unique deletion and flanked by a 13 bp direct repeat has been simultaneously described in Pearson syndrome and KSS,

and subsequently reported in PEO. Similarly, identical mtDNA duplications have been reported in strikingly different conditions such as Pearson syndrome and proximal tubulopathy. Thus, no correlation is found between clinical presentation and the nature or extent of rearrangements. The observation of a progressive organ involvement should prompt one to consider the diagnosis of mtDNA rearrangement and carry out Southern blot analysis of total DNA. Indeed, unlike mtDNA point mutations, the proportion of deletions-duplications increases in the course of the disease, suggesting that they have a replicative advantage over normal molecules.

While the vast majority of mtDNA rearrangements are sporadic, occasional pedigrees have been reported in which mtDNA deletions-duplications are present in close maternal relatives. This indicates that maternal transmission of rearranged molecules may occur, although germ line transmission is limited. Rare cases are associated with

Table 4 Deletions and duplications in mitochondrial disorders

Clinical disorder	Locus symbol	Type of rearrangement	Mode of inheritance
Progressive external ophthalmoplegia	PEO	– Single deletion – Single deletion – Multiple deletions	– Sporadic – Maternal – Autosomal dominant
Kearns-Sayre syndrome	KSS	– Single deletion – Multiple deletions	– Sporadic, maternal (?) – Autosomal dominant
Recurrent myoglobinuria		– Multiple deletions	– Autosomal recessive?
Pearson marrow pancreas syndrome	PMPS	– Single deletion – Tandem duplication	– Sporadic
Diabetes insipidus, diabetes mellitus, optic atrophy and deafness	DIDMOAD	– Single deletion	– Sporadic
Non insulin dependent diabetes mellitus and deafness	NIDDM	– Single deletion – Tandem duplication	– Maternal
Diabetes mellitus and cerebellar ataxia		– Single deletion – Tandem duplication	– Sporadic – Maternal
Oculo cerebro renal syndrome	OCRL	– Single deletion	– Sporadic

autosomal dominant multiple mtDNA deletions that are flanked by direct repeats [25]. This feature suggests that a mutation occurred in a nuclear gene essential for replication or maintenance of the mt genome.

The last class of mtDNA diseases are mtDNA depletions due to copy number mutations. Rare cases of lethal infantile respiratory, muscle, liver or kidney failure have been ascribed to mtDNA depletions [14], and are consistent with autosomal recessive inheritance. In these patients, there is a marked (sometimes tissue specific) deficiency in mtDNA level but not in nuclear gene levels.

Nuclear DNA mutations

While pathological alterations of mtDNA have been extensively investigated, little is known regarding the molecular bases of nuclear-inherited diseases. Linkage analyses have resulted in the genetic mapping of Barth disease to chromosome Xq28 [1] and family studies in dominantly inherited mtDNA deletions have led to the mapping of a disease gene to chromosome 10 [25, 26]. It is interesting to note that there may be an X-linked gene which determines susceptibility to visual loss as a bias toward males being affected in LHON [27]. On the other hand, the chromosomal localisation and cDNA sequence of several nuclear genes are known. Up till now, however, only one mutation in a nuclear encoded gene has been reported in a respiratory enzyme deficiency [28] and the systematic sequence analysis of the nuclear encoded subunits of complex IV has failed to detect any mutation in their coding sequences.

The genetic investigation of respiratory chain deficiency

The genetic investigation of a mitochondrial disorder requires an extensive pedigree reporting on minor signs in relatives. This information is of paramount importance for deciding what molecular studies should be carried out first. For example, maternal inheritance points toward mtDNA mutations (especially those reported to cause the relevant presentation, Table 3); autosomal dominant inheritance points toward multiple mtDNA deletions; sporadic cases and cases consistent with autosomal recessive inheritance (consanguineous parents) should be tested for mtDNA deletion-duplications and depletions respectively.

It should be born in mind, however, that the molecular genetic investigation of mtDNA is not a routine procedure and several pitfalls should be mentioned:

1. A twofold population of mtDNA molecules not always corresponds to deletions: it may be accounted for by mtDNA polymorphism. (One has to repeat mtDNA digestions using various restriction enzymes and use a full-length mtDNA probe for Southern blot hybridization).
2. The distribution of mutated mtDNA molecules may differ widely among tissues, possibly accounting for variable clinical expression. For this reason, the tissue to be investigated is the one which actually expresses the disease (mtDNA deletion is frequently absent in circulating lymphocytes of KSS patients).
3. mtDNA deletion may be occasionally associated with duplication. Due to the symmetry of several rearrangements, detection of duplication requires enzymatic cleavage of the mtDNA at sites located within the deletion.

4. mtDNA rearrangements are unstable and gradually disappear in cultured cells unless uridine is included in the culture medium. No conclusion can be drawn from the molecular study on cells grown under standard conditions [10].

5. The detection of mtDNA depletion requires systematic re-hybridization of Southern blots using a control nuclear DNA probe for densitometric determination of the mtDNA/nuclear DNA ratio.

6. Finally, while a positive test supports the mitochondrial nature of the disease, a negative result does not rule out a mtDNA mutation nor does it represent a clue that a nuclear mutation is involved.

Prenatal diagnosis and genetic counselling

Knowledge concerning the mutant genotypes currently associated with clinical presentations sometimes helps predicting the heritability of mitochondrial disorders: maternal transmission of base substitutions in LHON, MERRF, MELAS or NARP; sporadic occurrence of deletions-duplications in Pearson syndrome and KSS (unless germline mosaicism is involved); autosomal recessive transmission of depletions in multiorgan failure; autosomal dominant transmission of multiple deletions in PEO. In case of maternal inheritance of a mtDNA mutation (or deletion), there is no risk for the progeny of an affected male. The risk is high, however, for the progeny of carrier female. In this case, prenatal diagnosis in chorion villi or

amniotic cells represents a rational approach to the prevention of these severe diseases. In fact, it is currently hampered by our incomplete knowledge regarding the actual proportion of mutant mtDNA, its relationship to disease severity, its random tissue distribution, and selection against the mutant population during development, possibly related to variable metabolic activities. A percentage of mutant mtDNA below 30% or above 80% should predict a reasonable chance of good or bad prognosis respectively. Intermediate results would have an even less certain predictive value. Whatever the results, studies aimed to deliver prenatal diagnosis or predictive genetic advice require careful validation, as proportions of mutant mtDNA may change both between fetal life and adult life and also during adult life.

In the majority of cases, however, the heritability of a mtDNA rearrangement remains unknown and no reliable genetic counselling can be given. Indeed, dealing with an isolated mtDNA deletion, it is impossible to predict whether a de novo event occurred or if a heritable mutation is involved (germline mosaicism). Future systematic screening for disease-causing mtDNA mutations will hopefully contribute to improve genetic counselling, especially as only one nuclear encoded mutation has been hitherto identified in respiratory chain deficiency.

When no mtDNA alteration is detected, measurement of respiratory enzyme activities in cultured amniocytes or chorionocytes represents the unique possibility of prenatal diagnosis. Unfortunately, only 40–50% of enzyme deficiencies are expressed in cultured fibroblasts of the proband (when grown in the presence of uridine).

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