Mitochondrial respiratory chain dysfunction causes a wide range of primary diseases in adults and children, with highly variable organ involvement. Diagnosis involves weighing evidence from a number of sources, including the clinical presentation, metabolic measurements in vivo, imaging studies, analysis of respiratory chain function or enzyme activities in vitro, studies of mitochondrial morphology after biopsy, and mitochondrial (mt) DNA mutation analysis. Irrespective of the category of the information, it can be difficult to determine whether abnormal results are due to primary defects of the respiratory chain or to practical problems that complicate the diagnostic methodology. This review describes six sources of such problems: genetic complexity, tissue and temporal variation, methodological limitations, secondary effects, logistical issues, and questions of interpretation. When these issues are all addressed, a reliable categorization of the diagnosis as definite, probable, or possible respiratory chain defect becomes possible.

Key words: diagnosis/mitochondrial disease/mitochondrial DNA/mitochondrial function/respiratory chain

Introduction

The mitochondrial respiratory chain is the central energy generating pathway in humans and all other eukaryotic species; oxidation of fuels such as sugar, fat, and protein feeds electrons into it. These electrons are transferred through respiratory chain complexes I–IV to generate an electrochemical proton gradient (the protons, or hydrogen ions, accumulate in the intermembranal space) and the gradient is then utilized by respiratory chain complex V to drive ATP synthesis. In virtually all tissues, this process of oxidative phosphorylation (OXPHOS) is responsible for the bulk of ATP synthesis. Thus it is not surprising that respiratory chain dysfunction can affect any organ in the body. Severe respiratory chain defects cause a wide range of primary diseases in children and adults; these are described elsewhere in this issue (Christodoulou, 2000). Their clinical heterogeneity means that selecting patients for mitochondrial evaluation is a significant conundrum (Chinnery and Turnbull, 1997).

Mitochondrial respiratory chain defects together comprise one of the more common inborn errors of metabolism, with an estimated incidence of 1/10 000 births (Schuelke et al., 1999). Genetically based mitochondrial respiratory chain dysfunction also contributes to the
pathogenesis of common degenerative diseases (Wallace, 1999), so clearly it would be desirable to have simple and robust methods for detecting abnormal mitochondrial respiratory chain function and the underlying defects in mitochondrial (mt) DNA. Presently it is likely that many symptomatic patients who have a respiratory chain defect are undiagnosed; furthermore, there are other patients who have been diagnosed with a respiratory chain defect but who in fact do not have one. The reason for this unsatisfactory state of affairs is the subject of this review.

Clinical suspicion of a respiratory chain defect is typically based on the presence of suggestive clinical features, family history, and the results of imaging or metabolic studies, particularly the finding of elevated lactic acid in cerebrospinal fluid or in blood. Since my laboratory is located in a children’s hospital and is presently referred tissues from most children in Australia and New Zealand suspected of a respiratory chain defect, our referral pattern makes our diagnostic experience different from that of laboratories focused on adult neurology patients. In most children we have investigated for a respiratory chain disorder, the family history is not suggestive of a particular mode of inheritance, except that consanguinity is present in ~20% of our diagnosed cases, implying autosomal recessive inheritance in these families.

Patients suspected of a respiratory chain defect are usually investigated by one or more of three main methods: (i) respiratory chain enzyme and functional studies on mitochondria or homogenates prepared from biopsied tissues and/or cultured cells; (ii) histology, enzyme histochemistry and ultrastructural studies of tissue sections; and (iii) mtDNA mutation analysis. In some cases one or more of these investigations will give a definitive diagnosis, but in others the results might just be supportive of a respiratory chain defect (Figure 1).

![Figure 1](image_url)

**Figure 1.** Diagnostic yield of the three major methods used to diagnose respiratory chain defects in children. Between 1992 and 1998, 843 patients were studied at the Murdoch Institute Mitochondrial Laboratory, of whom 199 had a definite diagnosis of respiratory chain disease made according to the criteria of Walker and colleagues (Walker et al., 1996), as modified by Thorburn and co-workers (Thorburn et al., 1998). The pie chart shows data for 165 patients who had a severe defect detected by one or more of the three main classes of investigation, namely respiratory chain enzymes, mitochondrial morphology, and mtDNA mutation analysis. Of our definite diagnoses, >80% had all three classes of testing, but in 86% of the definite diagnoses only one class of testing gave a definitively abnormal result, with the other methods giving normal or non-specific results. Not shown are data for 34 patients who were classified as having a definite defect because of moderate defects detected by two or more methods.

**Confounding aspects of mitochondrial diagnosis**

This review describes six classes of problems that complicate diagnosis of respiratory chain dysfunction. Many of these problems are relevant not only to making a diagnosis in a particular patient but to investigational studies generally of the respiratory chain in specific cell types or lineages, such as the female germ line.

**Genetic complexity**

The mitochondrial genome comprises 37 genes, including genes for 13 protein subunits of the respiratory chain. The majority of respiratory chain enzyme subunits are encoded by nuclear genes, of which there are at least 70. A large number of other nuclear gene products, probably in excess of 100, are required for normal respiratory chain function. Table I gives examples of respiratory chain disease genes, showing that these disorders can exhibit virtually any mode of inheritance, including
**Table I.** Mechanisms, genes, and inheritance of mitochondrial respiratory chain defects

<table>
<thead>
<tr>
<th>Inheritance</th>
<th>Gene category</th>
<th>Examples</th>
<th>OMIM reference</th>
<th>Gene or locus</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maternal</td>
<td>1. mtDNA tRNA</td>
<td>MELAS, MERRF/Leigh disease</td>
<td>590050</td>
<td>MTTL1, MTTK</td>
</tr>
<tr>
<td></td>
<td>2. mtDNA rRNA</td>
<td>Non-syndromic deafness</td>
<td>590060</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3. mtDNA RC subunit</td>
<td>NARP/Leigh disease</td>
<td>561000</td>
<td>MTRNA1</td>
</tr>
<tr>
<td>Sporadic</td>
<td>Single deletions encompassing multiple mtDNA genes</td>
<td>CPEO/Kearns–Sayre syndrome/Pearson syndrome</td>
<td>530000</td>
<td>Multiple mtDNA genes deleted</td>
</tr>
<tr>
<td>Autosomal recessive</td>
<td>RC complex I subunit</td>
<td>Leigh disease, mitochondrial cytopathy</td>
<td>602141</td>
<td>NDUFS8b</td>
</tr>
<tr>
<td></td>
<td>RC complex II subunit</td>
<td>Leigh disease</td>
<td>600857</td>
<td>SDHA</td>
</tr>
<tr>
<td></td>
<td>Protein import/assembly</td>
<td>Leish disease</td>
<td>185620</td>
<td>SURF1</td>
</tr>
<tr>
<td></td>
<td>mtDNA/nuclear DNA interaction</td>
<td>MNGIE</td>
<td>550900</td>
<td>ECGF1</td>
</tr>
<tr>
<td></td>
<td>Regulation of iron transport (?)</td>
<td>Friedreich ataxia</td>
<td>229300</td>
<td>FRDA</td>
</tr>
<tr>
<td></td>
<td>Metalloprotease/chaperonin (?)</td>
<td>Hereditary spastic paraplegia</td>
<td>602783</td>
<td>SPG7</td>
</tr>
<tr>
<td></td>
<td>mtDNA transcription/replication</td>
<td>(ΔTfam knockout mouse)</td>
<td>600438</td>
<td>Tfam</td>
</tr>
<tr>
<td></td>
<td>Free radical scavenging</td>
<td>(ΔSod2 knockout mouse)</td>
<td>147640</td>
<td>Sod2</td>
</tr>
<tr>
<td></td>
<td>ATP/ADP exchange</td>
<td>(ΔAnt1 knockout mouse)</td>
<td>103220</td>
<td>Ant1</td>
</tr>
<tr>
<td>Autosomal dominant</td>
<td>mtDNA/nuclear DNA interaction</td>
<td>CPEO</td>
<td>157640</td>
<td>10q23.3-q24.3</td>
</tr>
<tr>
<td>X-linked</td>
<td>(multiple mtDNA deletions)</td>
<td>601226</td>
<td>3p14.1-p21.2</td>
<td></td>
</tr>
<tr>
<td>Complex</td>
<td>Mitochondrial protein import</td>
<td>Deafness dystonia syndrome</td>
<td>304700</td>
<td>DDP1</td>
</tr>
<tr>
<td></td>
<td>Mitochondrial iron export (?)</td>
<td>Sideroblastic anaemia/ataxia</td>
<td>300135</td>
<td>ABC7</td>
</tr>
<tr>
<td></td>
<td>Unknown role</td>
<td>Barth syndrome</td>
<td>302060</td>
<td>BTHS</td>
</tr>
<tr>
<td></td>
<td>mtDNA point mutation plus gender and environment</td>
<td>LHON</td>
<td>516000</td>
<td>MTND1</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>516003</td>
<td>MTND4</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>516006</td>
<td>MTND6</td>
</tr>
</tbody>
</table>

*References to these disorders refer to the Online Mendelian Inheritance in Man (OMIM) catalogue, 1999.

*b Other complex I subunit mutations have been reported in the NDUFS1 (OMIM 161015) and NDUFS4 (OMIM 602694) genes.

CPEO = chronic progressive external ophthalmoplegia; LHON = Leber hereditary optic neuropathy; MELAS = mitochondrial myopathy, encephalopathy, lactic acidosis, stroke-like episodes; MERRF = myoclonic epilepsy, ragged red fibres; MNGIE = myoneurogastrointestinal encephalopathy; NARP = neuropathy, ataxia, retinitis pigmentosa; RC = respiratory chain.

Autosomal dominant, autosomal recessive, X-linked recessive as well as maternal (‘cytoplasmic’ or mitochondrial), complex and sporadic patterns. The non-Mendelian, dosage (or ‘population’) effects of mtDNA genetics (including heteroplasmy, tissue variation, the threshold effect, and the mtDNA bottleneck) all confer complexity of a unique kind to mitochondrial inheritance.

A large number of mtDNA point mutations and deletions have been reported (DiMauro *et al.*, 1998; MITOMAP database, 1999), with a relatively small number of ‘classical’ mtDNA mutations identified now in multiple families. These families have well-characterized (if uncommon) mitochondrial disease syndromes that go by such acronyms and abbreviations as MELAS, MERRF, LHON, CPEO, KSS and NARP (Wallace, 1999; Christodoulou, 2000). In adult patients with mainly neuromuscular symptoms suspected of these disorders, a routine molecular analysis for these classical mutations can result in up to 80% of patients having a mutation identified. The high diagnostic yield in these (relatively rare) conditions can result in the misperception that mtDNA mutations are the most common cause of respiratory chain dysfunction. However, such molecular findings are relatively uncommon in children suspected of mitochondrial disease. Unlike adults, typically <5% of children investigated for a respiratory chain disorder will have a known mtDNA mutation identified, and most dia-
Diagnoses are achieved by finding an enzyme defect in a tissue biopsy or cell line (Figure 1) (Shoffner, 1996; Lamont et al., 1998).

Although some children undoubtedly have rare mtDNA mutations that have not been tested, it is likely that most children with respiratory chain defects have mutations in the nuclear rather than mitochondrial genome. To date, mutations of only a handful of nuclear genes (these include paraplegin, Surf-1, and thymidine phosphorylase; see Table I) have been shown to cause respiratory chain dysfunction in more than one or two families. As more genes are identified in the next few years, it ought to be possible to identify a genetic basis in more cases, but the large number of genes involved, and the blurred correlation between genotype and phenotype, will still make it difficult to decide which gene(s) to target for investigation in particular cases.

**Tissue and temporal variation**

When investigating a patient with a suspected respiratory chain defect, the first choices to be made are the methods to use and the tissue or tissues to study. Ideally, the tissue chosen would be able to be obtained in a minimally intrusive manner; the tissue of first choice for mtDNA testing could thus be blood, hair follicles, mouth squames, or skin fibroblasts. However, some mtDNA mutations, particularly deletions, can be absent from these tissues, despite being present in high amounts in post-mitotic tissues such as muscle or brain (Holt et al., 1989). Patients with the syndrome of mtDNA depletion often show marked tissue specificity, e.g. showing normal mtDNA:nuclear DNA ratios in muscle, but gross mtDNA depletion in liver or kidney (DiMauro et al., 1998).

Inter-tissue variation can particularly affect enzyme studies and be vividly evident morphologically. Dramatic abnormality of mitochondrial morphology in one tissue but a completely normal appearance in another tissue is relatively common and has been described in detail elsewhere in this issue (Chow and Thorburn, 2000). In our experience, ~50% of the children with a respiratory chain defect in skeletal muscle have normal enzyme activities in cultured skin fibroblasts or liver. Tissue specificity is even more marked for patients with a diagnosis based on a liver respiratory chain enzyme defect; of 36 such patients whom we have diagnosed where skeletal muscle was also available for enzyme analysis, only six had muscle respiratory chain enzymes that were diagnostic of respiratory chain dysfunction (a typical example is shown in Figure 2a,b).

Respiratory chain defects can also show marked within-tissue variation; classic examples are mtDNA deletions and tRNA mitochondrial gene mutations in skeletal muscle. Enzyme histochemical staining of muscle sections for cytochrome c oxidase activity (COX, or respiratory chain complex IV) in such patients usually shows a mosaic of COX-negative and COX-positive fibres (in contrast to patients with nuclear-encoded COX defects, who have uniformly decreased staining). Single-fibre polymerase chain reaction (PCR) analysis typically reveals that the COX-negative fibres contain very high levels of mutant mtDNA, whereas adjacent COX-positive fibres show a higher proportion of wild-type mtDNA (Moraes et al., 1993). Within-tissue variation is not restricted to skeletal muscle; it is observed in blood, skin fibroblasts, and other tissues. Within-tissue variation can be particularly pronounced in the ovaries; a striking difference in the mutant loads of individual oocytes has been reported from a carrier of the mtDNA T8993G mutation (Blok et al., 1997). This is presumably a germ cell segregation effect related to the mitochondrial bottleneck phenomenon.

Some tissues can show dramatic changes in
Detecting mitochondrial abnormalities

Figure 2. Respiratory chain enzyme profiles in two patients with mitochondrial disease. Data are shown for respiratory chain complexes I, II, III and IV, and for the mitochondrial marker enzyme citrate synthase (CS). Residual enzyme activities are expressed as the percentage of normal control mean value for (A, C) skeletal muscle or (B) liver. The vertical bars represent the observed range for normal control samples. Patient 1 (mitochondrial encephalopathy with chronic intestinal pseudo-obstruction) had normal respiratory chain enzyme activities in muscle, but severe defects of complexes I, III and IV in liver. Patient 2 (Kearns–Sayre syndrome) had normal (average) enzyme activities in skeletal muscle despite having a mtDNA deletion present in 30% of mtDNA molecules (see Figure 3 in Blok et al., 1995) and substantial numbers of ragged red fibres on histology.

the amount of mtDNA mutations over time. It has been reported (Poulton and Morten, 1993) that blood and muscle mutant loads of the MELAS A3243G mutation appear to be the same at birth, but diverge with increasing age. This appears to be mostly due to selection against the mutation in blood (a tissue undergoing active mitosis), in which the mutation load can decrease with age, whereas in muscle (a mostly post-mitotic tissue) the mutation load can increase with age (Weber et al., 1997). Temporal variation is not confined to measurements of mtDNA mutation load, but can apply to morphological changes and to respiratory chain enzyme activity estimation, as in the patient with Alpers syndrome described by Chow and Thorburn (2000).

Tissue and temporal variation of respiratory chain defects should be borne in mind in choosing which tissue to study and in deciding whether to biopsy a new tissue or to re-biopsy a tissue studied at an earlier stage of the patient’s disease. The absence of a mutation, enzyme defect or abnormal morphological finding in one tissue clearly will not exclude its presence in a tissue more closely implicated by the patient’s symptoms. As a general rule, if there is a clinically-apparent myopathy, then skeletal muscle is the tissue most likely to yield a diagnosis and a child with abnormal conventional liver function tests should have a liver biopsy studied.

**Methodological limitations**

A limitation of most respiratory chain enzyme and functional assays is that they represent the activity averaged over all the mitochondria or cells in the sample. Enzymology typically loses information because it relies on homogenized samples. Enzyme histochemical staining, on the other hand, retains spatial information about the distribution of enzyme activity among muscle fibres. This difference is exemplified by Figure 2c, which shows a normal muscle respiratory chain enzyme profile in a patient who had a mtDNA deletion present at ~30% mutant load and had demonstrable numbers of COX-negative and ragged red fibres on histochemical study (Blok et al., 1995). The lack of spatial information in biochemical respiratory chain enzyme analysis is balanced by the fact that it is more quantitative than histochemical staining.

Enzyme assays are usually performed in a dilute aqueous system that differs from circumstances in vivo in several ways (Table II). Any of these differences could result in
Table II. Differences between the in-vitro assay system for respiratory chain complex I and the in-vivo situation as possible explanations for why some complex I defects do not affect the measured enzyme activity

<table>
<thead>
<tr>
<th></th>
<th>In-vitro assay</th>
<th>In-vivo system</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mitochondria</td>
<td>Disrupted</td>
<td>Intact</td>
</tr>
<tr>
<td>Protein concentration</td>
<td>1 mg/ml</td>
<td>500 mg/ml</td>
</tr>
<tr>
<td>Substrate concentration</td>
<td>Saturating (i.e. &gt;&gt;K_m)</td>
<td>Low (i.e. &lt;K_m)</td>
</tr>
<tr>
<td>Quinone substrate</td>
<td>5-carbon saturated chain</td>
<td>50-carbon isoprenoid chain</td>
</tr>
<tr>
<td>Function</td>
<td>Electron transport</td>
<td>Electron transport and proton pumping</td>
</tr>
</tbody>
</table>

an abnormal kinetic variant appearing to have normal enzyme activity in the in-vitro assay system. An example of this is the mtDNA G11778A mutation in the NADH dehydrogenase (ND)4 subunit gene of complex I, which has little effect on assayed complex I activity, although it strongly inhibits oxidation of complex I substrates (Hofhaus et al., 1996). Studies of respiratory chain function require intact mitochondria, and so this problem can in principle be avoided; but respiratory chain function studies also suffer from limitations (e.g. the need for fresh tissue) and are less robust than enzyme assays because of artefacts (e.g. inhibition or uncoupling by anaesthetics, variation in the efficiency of isolating well-coupled mitochondria, and other secondary effects discussed below).

Mitochondrial morphology can be normal or might show only non-specific changes in patient biopsies (Chow and Thorburn, 2000). Although enzyme histochemical studies retain spatial information, they are unable to detect respiratory chain complex I defects, which are the most common respiratory chain enzyme defects in children and which typically show unremarkable histochemical or morphological changes (Kirby et al., 1999).

Methods of detecting mtDNA point mutations are also subject to specific limitations related to sensitivity, specificity, expense, and ease of use. Sequencing of mtDNA is a highly specific approach to mutation detection, but is not a ‘front line’ method in most centres due to the work-load and expense of sequencing the entire 16.5 kb mtDNA genome, together with the relatively low sensitivity, which means that mutations present at heteroplasmic mutant loads of <20% may not be distinguished from background signals. PCR with restriction fragment-length polymorphism analysis is one of the most common direct tests used to detect a specific mutation. This test detects mtDNA mutations that either introduce or remove a restriction site by digesting patient and control DNA with the appropriate restriction enzyme, followed by separation of the fragments using gel electrophoresis. If the mutation introduces a restriction site, then the mutant DNA fragment will be smaller than the control fragment and will migrate faster. If the mutation abolishes the site, the mutant DNA fragment will be larger and slower to migrate. These tests are relatively sensitive and can detect mtDNA mutations present in only a few percent of total mtDNA. However, they can give false positive or false negative results if a mtDNA polymorphism is present within the restriction enzyme site. It has been estimated for example that such polymorphisms gave a false-positive rate of 2–7% for mtDNA mutations associated with LHON (Johns and Neufeld, 1993). Polymorphisms further away from the mtDNA mutation can also potentially cause false positive results for mtDNA mutations associated with MELAS (Kirby et al., 1998) and Leigh syndrome (White et al., 1998). These false results are more likely with mtDNA than with nuclear DNA tests, because polymorphisms are more
common in the mitochondrial genome than in the nuclear genome.

Methodological limitations affecting respiratory chain enzyme, morphological and mtDNA analyses mean that none of these methods on their own can provide a benchmark for the detection of all patients with respiratory chain defects. In practice, most patients require an integrated approach to diagnosis, utilizing each of these methods either simultaneously or sequentially (Chinnery and Turnbull, 1997).

**Secondary effects**

Mitochondrial numbers and function are influenced by the intracellular milieu and by external environmental factors impinging on the cell and organ in which they are located. This can blur the distinction between changes due to a primary respiratory chain defect and those due to secondary effects (reviewed by Trijbeis et al., 1993). Skeletal muscle respiratory chain enzyme levels, for example, can be influenced by a number of factors, e.g. inactivity of age, fitness, immobility, and variation in fibre type composition. In our experience, skeletal muscle homogenates from neonates tend to have only about one-third of the level of respiratory chain enzymes compared with older children (presumably reflecting the relatively anaerobic environment of the preceding fetus). There are numerous reports of declining skeletal muscle respiratory chain enzyme levels in old age, and this is at least partly due to the effects of detraining rather than of mitochondrial mutations or ageing per se (Brierley et al., 1997). To distinguish primary deficiencies in respiratory chain enzymes from secondary deficiencies, activity measurements are made relative to a marker enzyme such as citrate synthase (Kirby et al., 1999) or as internal ratios (Chretien et al., 1998), rather than relative to protein. Interestingly, our experience suggests that liver and cardiac muscle have less age-related secondary variation, perhaps because as organs they are used more constantly than skeletal muscle.

Tissue pathology and other inborn errors of metabolism can also secondarily affect respiratory chain enzyme levels and mitochondrial morphology. Our experience is that healthy livers have quite narrow observed ranges for each of the respiratory chain enzymes (lowest activities are typically >70% of the mean value). Livers from patients who have a known inborn error of metabolism (e.g. pyruvate dehydrogenase deficiency, fatty acid oxidation defects, or organic acidemias) or liver disease (e.g. chronic biliary cirrhosis or massive hepatic necrosis) can have much more variable respiratory chain enzymes. Different respiratory chain enzymes show different extents of secondary variation. In our experience, complexes II, III, and the combined II+III and I+III assays show most variability (lowest activities are down to ~30% of the mean value), followed by complex I and then complex IV, with citrate synthase being the most robust assay. This pattern of lability tends to hold for secondary effects generally, whether caused by other inborn errors of metabolism, liver pathology, post-mortem delay in sample collection, or exposure to free radical damage, as in Friedreich's ataxia (Rotig et al., 1997) or, experimentally, in the Sod-2 knockout mouse (Melov et al., 1999).

Another potential cause of secondary effects on respiratory chain enzymes and function are drugs [particularly antiviral nucleoside analogues such as zidovudine (AZT) (Lewis and Dalakas 1995) and fialuridine (Lewis et al., 1996)], poisons (e.g. carbon monoxide or cyanide) and anaesthetic agents (particularly barbiturates, which inhibit complex I, and bupivacaine).

A number of authors have suggested that purified mitochondria from frozen tissue samples should not be used for respiratory chain enzyme assays, because of potential...
D.R. Thorburn

artefacts (as examples: Zheng et al., 1990; Scholte and Trijbels 1995). Our experience is that assays for respiratory chain complex V and combined assays relying on endogenous ubiquinone (complex I+III and II+III) are not reliable in frozen tissue homogenates. However, in our laboratory, the activities of complexes I, II, III, IV and citrate synthase in homogenates prepared from frozen muscle or liver appear to be stable for over a decade if the tissue samples have been frozen rapidly and stored at −70°C.

Mitochondrial morphology and mtDNA studies are also prone to secondary effects. Markedly abnormal mitochondrial morphology has been reported in conditions as diverse as fat oxidation defects (Tyni et al., 1996), alcoholic liver disease (Inagaki et al., 1992), cyclosporin therapy (Larner et al., 1994) and, of course, anoxic tissue damage of any cause. In an inexperienced laboratory, the increased mitochondrial numbers in skeletal muscle of an elite athlete could be mistaken for abnormal mitochondrial proliferation. False positive and false negative results for mtDNA point mutation tests due to polymorphisms have been described above. Another example of a secondary effect on mtDNA mutation analysis lies behind a recent claim that multiple heteroplasmic mtDNA mutations were a major risk factor for late-onset Alzheimer’s disease (Davis et al., 1997). It was subsequently shown that this finding was an artefact of co-amplifying nuclear-embedded mtDNA pseudogenes rather than an identification of authentic mtDNA mutations (Hirano et al., 1997; Wallace et al., 1997).

A substantial number of cases of mtDNA depletion have been reported in recent years. While there is no doubt that this is a real syndrome, a number of secondary factors can affect the mtDNA:nuclear DNA ratio. It has been reported (Berger et al., 1998) that 19 of 20 patients with spinal muscular atrophy had mtDNA:nuclear DNA ratios in skeletal muscle of <20% of the mean normal control value. This is probably due to muscle disuse rather than primary mitochondrial involvement in the disease process. Similarly, we have studied mtDNA:nuclear DNA ratios in post-mortem liver samples from patients with liver failure (e.g. chronic biliary cirrhosis, massive hepatic necrosis). Some samples had values less than 20% of the mean normal control value, and could be mistaken for primary mtDNA depletion.

Since secondary effects on respiratory chain enzymes, mitochondrial morphology, and mtDNA can potentially result in misdiagnosis, it is particularly important to consider whether a normal range for respiratory chain enzymes that has been established on healthy control tissues is appropriate for comparison with tissues from patients in other circumstances and with primary tissue pathology of other cause.

Logistical issues

Logistical issues can influence what tests may be performed on a patient suspected of respiratory chain dysfunction. Common mtDNA mutation tests and routine histology and morphology tests are widely available. More extensive mtDNA mutation analysis can be obtained at only a limited number of centres. Similarly, the type of respiratory chain enzyme and functional assays available and the tissues that can be studied vary widely between different centres.

The patient’s location and, in some rapidly progressive cases, the time of his or her death can govern whether fresh tissue can be transported to the laboratory for functional studies or if only frozen tissue can be furnished. The large muscle biopsies (> 0.5 g) needed for traditional enzyme and polarographic studies cannot be obtained from patients presenting in the neonatal period. While it is preferable to study ‘symptomatic’
tissues, this is often not practical (for example a brain biopsy would be impractical for a patient whose only symptoms related to encephalopathy). Finally, economic issues can dictate what tests are available. Full testing of respiratory chain enzymes and function in multiple tissues, mitochondrial morphology and detailed mtDNA analysis may cost thousands of dollars for a patient, and the local centre might have only the resources to devote a certain amount of time and effort to each sample. In such situations, it may be necessary to ration the availability of testing to high-suspicion patients, or to focus on using only the methods that are likely to give the highest number of diagnoses per annum.

**Interpretation**

The last difficulty in the diagnosis of respiratory chain defects lies with the interpretation of the data obtained. The secondary effects discussed above mean that many patients will have respiratory chain enzyme levels or morphological changes that are moderately below the ‘normal’ range and which overlap with levels or changes caused by authentic, primary respiratory chain disorders. It is difficult or impossible to define absolute cut-offs for normal ranges that are at once realistic and yet can reliably distinguish primary from secondary changes. While some respiratory chain enzyme defects or morphological changes are sufficiently marked to be diagnostic, others should therefore be interpreted as only suggestive of respiratory chain dysfunction. It is frustrating to patients, their families, clinicians and the laboratory to label patients as having a probable rather than a definite defect. However, I believe it is better to err on the conservative side in making such diagnoses. Once a patient is regarded as having a primary defect of the mitochondria, the clinician’s focus may shift to patient management and away from considering alternative, potentially treatable diagnoses.

Problems in interpretation also extend to mtDNA studies. If a patient has a novel mtDNA nucleotide change identified, a substantial amount of work is required to confirm that the change is pathogenic (Moraes et al., 1993; Walker et al., 1996). A classic example of interpreting the role of mtDNA mutations in pathology relates to the role of mtDNA mutations that accumulate in somatic tissues with age. Many of these studies have focused on the accumulation of the common 4977 bp deletion (ΔmtDNA4977), which is typically undetectable in muscle or other tissues from normal young individuals but becomes increasingly detectable in different post-mitotic tissues with advancing age. Even in old individuals, the amount of this mutant mtDNA species is typically <1% of total mtDNA, and debate continues as to whether this mutation (and others like it) is a major contributor to the phenotypic expression of ageing and common degenerative diseases or simply a clinically unimportant epiphenomenon. Two recent reviews have argued both the case for (Nagley and Wei, 1998), and against (Lightowlers et al., 1999), a causal relationship.

**Conclusions**

I do not intend to convey the impression that practical problems always provide an insurmountable barrier to accurate diagnosis of respiratory chain dysfunction. My own laboratory has been involved in diagnosing ~200 children with definite respiratory chain defects, and other large centres would have the experience of having made a similar number of reliable diagnoses. It is clear that issues such as genetic complexity, tissue and temporal variation, methodological limitations, secondary effects, logistical issues and interpretation need to be addressed in the process of investi-
gating suspected cases. The final process involves weighing evidence from sources which include clinical findings, metabolic and imaging investigations, respiratory chain enzymes and function, mitochondrial morphology, and DNA studies in order to achieve a diagnosis of definite, probable or possible respiratory chain dysfunction. A recent review of this process has been published (Walker et al., 1996). In many cases a definite diagnosis can be achieved, but it is sensible to retain a cautious view of any individual case as to whether such a diagnosis has been confirmed or excluded.

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Detecting mitochondrial abnormalities


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