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Assaying ATP synthesis in cultured cells: A valuable tool for the diagnosis of patients with mitochondrial disorders

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ABSTRACT

Mitochondrial ATP synthase plays a central role in cell function by synthesising most of the ATP in human tissues. In different cells, active regulation of mitochondrial ATP synthase in response to cellular energy demand has been demonstrated, as well as its alteration under several pathological conditions affecting oxidative phosphorylation (OXPHOS). Traditionally, detection of OXPHOS defects is based on the spectrophotometric measurement of respiratory chain complex activities in muscle biopsies. Considering the broad clinical spectrum of mitochondrial disorders, and the difficulty in arriving at a single diagnostic method, in this study we propose measurement of ATP synthesis in mitochondria from skin fibroblasts as an effective screening tool. In the light of our results this assessment emerges as a useful marker of impaired energy production in primary OXPHOS disorders of childhood and as a tool with the potential to drive further molecular genetic studies.

Introduction

The mitochondrial oxidative phosphorylation (OXPHOS) system comprises a group of four multimeric complexes (I–IV) plus two small electron carriers, coenzyme Q and cytochrome *c*. The energy generated by the reactions of the respiratory chain is used to pump protons from the mitochondrial matrix into the space between the inner and outer mitochondrial membranes. This creates an electrochemical proton gradient which is utilised by complex V (ATP synthase) to generate the ATP necessary for the many cellular functions of living organisms [1].

Since nuclear DNA (nDNA) and mitochondrial DNA (mtDNA) combine to encode the over 85 structural subunits of OXPHOS and the even greater number of proteins required for their correct functioning, the respiratory chain is under dual genetic control. For this reason, genetic mitochondrial diseases are mitochondrial DNA-related or related to mendelianly inherited mutations in nDNA [2].

Primary OXPHOS disorders have an estimated prevalence of about 1/10000 individuals [3] while an even higher impact in the general population (1/3100) emerges for mtDNA-related disorders [4], which suggests a heavy burden in at-risk families and raises a number of questions with regard to the need for better counselling and appropriate clinical management [5].

Mitochondrial disorders affect both children and adults and result in multisystem clinical manifestations. The tissues most affected are those (e.g., brain, heart and skeletal muscle) that require large amounts of energy for their cellular functions. Mutations in nDNA-encoded subunits of OXPHOS are relatively more frequent in infants and children, and tend to be clinically uniform. On the contrary, the spectrum and severity of the clinical manifestations associated with mutations in mtDNA-related disorders, resulting from the coexistence of mutant and wild-type genomes, are variable [6]. Careful analysis of morphological, biochemical and molecular findings, and of their combinations, crucial for obtaining the best possible diagnosis, can be carried out in highly specialised centres. However, in children, it is difficult to arrive at a definitive diagnosis of OXPHOS disorders, mainly because the tissue-specific manifestation of most syndromes limits the usefulness of morphological and biochemical data derived from often tiny muscle biopsy samples; furthermore, whole mtDNA studies are often negative.

We have already adopted a sensitive spectrophotometric method to assay ATP production in cells from patients with maternally-inherited Leigh syndrome associated with mutations in the *MT-ATP6* gene [7,8]. Considering that the end result of any OXPHOS

Abbreviations: OXPHOS, Oxidative phosphorylation; MDS, mitochondrial depletion syndrome.

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defect is a limited supply of ATP, we set out to establish the possible value of a similar assay in cultured skin fibroblasts from children presenting a wider array of biochemically confirmed OXPHOS defects.

Materials and methods

Patients. We studied cultured skin fibroblasts from 14 patients in whom clinical, morphological and biochemical data were consistent with a primary disorder of the mitochondrial OXPHOS system. Previous screening of respiratory chain enzyme activities had established that 6 patients had a complex I defect (Pts 1–6)—Pt 1 has already been reported in [9], one case had a complex II defect (Pt 7), whereas a complex IV deficiency was diagnosed in two children (Pts 8 and 9). We also analysed cells from 1 patient with a diagnosis of pyruvate dehydrogenase (PDH) deficiency (Pt 10), and two cases with a primary defect of CoQ_{10} biosynthesis (Pts 11 and 12)—these two cases have already been reported as Pts 2 and 1 in 10. In addition, we studied cells from two children (Pts 13 and 14) in whom previous molecular studies had established the presence of mutations in the *SUCLA2* gene associated with a reduced number of mtDNA copies, a condition which is suggestive of mitochondrial depletion syndrome (MDS) [Pts 1 and 2 in 11]. As controls, we used cultured skin fibroblasts from 30 age-matched children submitted to punch skin biopsy for the diagnosis of skin conditions.

This study was performed after receiving written informed consent from the parents of the children involved and in accordance with the guidelines of our institutions' ethics committees.

Biochemical and molecular studies. Respiratory chain complex activities were measured using a reported spectrophotometric method [12]. The whole mitochondrial genome as well as the coding exons and flanking intronic sequences of *SURF1*, *COQ2*, *PDHA1*, and *SUCLA2* were PCR amplified using methods and oligonucleotide primers described elsewhere [11,13–16]. The amplicons were gel purified and directly sequenced using BigDye 3.1 chemistry (Applied Biosystems, Foster City, CA).

Cell culturing studies. Human fibroblasts were obtained from skin biopsies and grown in DMEM medium supplemented with 10% foetal bovine serum, 4.5 g/L glucose and 50 μg/ml uridine.

ATP synthesis. ATP synthesis was assayed spectrophotometrically as reported elsewhere [7,17]. In brief, about 300 μg mitochondrial proteins were incubated in 20 mM Tris–HCl, pH 7.5, 150 mM sucrose, 1 mM ADP, 20 mM phosphate, 5 mM $MgCl_2$, 100 μM diadenosine pentaphosphate, 10 mM glucose, 30 U of hexokinase, and one of the following: 50 mM succinate, 6 mM malate, 6 mM α-ketoglutarate or 12 mM/1.2 mM pyruvate/malate, at 37 °C for 20 min in vials with vigorous stirring to ensure maximum oxygenation. The reaction was stopped with 25 mM EDTA + 2 μM carbonyl cyanide 3-chlorophenylhydrazone (CCCP), followed by transfer to ice-cold water. The synthesised glucose 6-phosphate was oxidised by NADP in the presence of 30 units of glucose 6-phosphate dehydrogenase. NADPH formation was monitored at 340 nm. Under these experimental conditions, a 1:1 ratio was found between the ATP synthesised and the NADPH formed.

Protein content was measured by BCA (Pierce, USA) and read at 562 nm.

Statistical analyses. Statistical analysis of the data was performed using Student's *t*-test. As a general rule, only *p* values of less than 0.01 were considered significant.

Results

A total of 14 patients, 10 boys and four girls, were studied. 12 patients presented a predominant neurological disorder; in seven children, Leigh syndrome was diagnosed on the basis of the presence of a bilateral hyperintense signal in the basal ganglia on T_2-weighted brain MRI. Primary renal involvement was observed in two cases [10], one (Pt 11) presenting rapidly developing end-stage renal disease complicated by progressive epileptic encephalopathy and leading to early death, and the other (Pt 12) infantile onset of steroid-resistant nephrotic syndrome.

Deficient complex I activity was observed in 6 patients (Pts 1–6, range of reduced activity: 47–90% of normal controls), and molecularly confirmed in only two of them (Pts 1 and 2). A defect of complex II activity was detected only in one case (Pt 7), while in patients 8 and 9 complex IV activity was reduced by 66% and 70%, respectively, the deficiency was molecularly confirmed in both of these patients. One child (Pt 10) had a marked reduction (67%) of PDH in association with a hemizygous mutation of *PDHA1*, encoding the E1 alpha subunit. Finally, four children displayed multiple OXPHOS defects, in two of them (Pts 11 and 12) characterised by a reduced CoQ_{10} level in muscle and kidney correlated with mutations in the *COQ2* gene [10], and in the other two (Pts 13 and 14) associated with MDS and mutations in *SUCLA2* [11]. Table 1 summarises the main clinical, biochemical and molecular features of the children studied in this work.

Using succinate, malate, α-ketoglutarate, and pyruvate/malate as substrates, the rates of ATP synthesis in mitochondria isolated

Table 1
Clinical, biochemical and genetic features of 14 patients with mitochondrial disease.

Patient	Sex	Age	Clinical phenotype	Muscle OXPHOS defect (% of controls)	Molecular defect
Pt 1	M	15 m	Leigh syndrome	CI = 75	*ND5*: p.D393N
Pt 2	M	6y	MELAS/LHON overlap syndrome	CI = 50	*ND 5*: p.A236T
Pt 3	M	3 m	Leigh syndrome	CI = 47	Unknown
Pt 4	M	7 m	Sideroblastic anaemia + Leigh syndrome	CI = 65	Unknown
Pt 5	M	4 d	Neonatal lactic acidosis	CI = 90	Unknown
Pt 6	M	3 m	Neonatal lactic acidosis	CI = 50	Unknown
Pt 7	F	14 m	Leukoencephalopathy + motor retardation	CII = 70	Unknown
Pt 8	F	18y	MELAS/MERRF overlap syndrome	CIV = 66	*COX3*: 9242 ins.A
Pt 9	F	14 m	Leigh syndrome	CIV = 70	*SURF1*: p.G257 fsX33
Pt 10	F	2 m	Leigh syndrome	Normal	*PDH-E1α:* p.R245G
Pt 11	M	21d	Encephalonephropathy	CI = 80; CIII = 21; CII + III = 78; CoQ = 92	*CoQ2*: p.S146N/p.S146N
Pt 12	M	1.8y	Myonephropathy	CI = 67 ; CII = 21; CIII = 16; CII + III = 68; CoQ = 84	*CoQ2*: p.R197H/p.N228S
Pt 13	M	7 m	Leigh syndrome	CI = 50; CIII = 44; CIV = 36	*SUCLA2*: p.R284C/p.R284C
Pt 14	M	1y	Leigh syndrome	CIV = 46	*SUCLA2*: p.R284C/p.G118R

Age at time of biopsy (d = days, m = months, y = years).
CI: complex I, NADH dehydrogenase; CII: complex II, succinate dehydrogenase; CIII: complex III, ubiquinol-cytochrome *c* oxidoreductase; CII + III, succinate cytochrome *c* oxidoreductase; CIV, complex IV, cytochrome *c* oxidase; CV, complex V, F1F0-ATP synthase; CoQ, coenzyme Q; PDH, pyruvate dehydrogenase.
All respiratory chain enzyme activities and the level of CoQ_{10} were measured in muscle except patient 12 in whom they were measured in the kidney. The values represent the % of reduction compared to control values.

from control cell lines were 29.8 ± 2.3, 10.0 ± 2.0, 10.0 ± 2.2 and 9.9 ± 1.4 nmol ATP/min/mg protein, respectively.

The rate of ATP production in patients with a complex I deficiency was heterogeneous. Two subgroups could be identified: the patients in the first (cases 1–4) showed a specific complex I defect, given that ATP production was reduced only in the presence of malate and α-ketoglutarate (the specific substrates for this complex), and was within normal range when succinate was used; in the second subgroup (Pts 5 and 6) the OXPHOS defect was less clear cut, ATP formation being found to be reduced in the presence of succinate (37%), malate (63%), and alpha-ketoglutarate (38%) (Fig. 1A). These patients had all been analysed for mutations in most of the complex I subunits, including the seven mtDNA-encoded proteins. In the first subgroup, a mutation was detected only in patients 1 and 2 (see Table 1), while in the other two (Pts 3 and 4), the results of the ATP synthesis assay strongly suggested that either a mutation in additional complex I genes or in assembly factors should be sought. Moreover, in the second subgroup patients (cases 5 and 6) the reduced complex V activity seemed to point to a more generalised disorder, involving additional complexes, either the ATP synthase complex itself or complex III, which may have been under-detected by the routine spectrophotometric method. It is intriguing that the method adopted seemed to be even more sensitive than the classical one in revealing the full extent of OXPHOS in patients 5 and 6.

The biochemical impairment highlighted in this study seems to allow a precise diagnosis to be reached in cases with complex II (Pt 7) or PDH (Pt 10) deficiencies. In patient 7, the biochemical defect was readily detectable in the presence of succinate (70% reduction), whereas with the remaining substrates production of ATP was normal (Fig. 1B). Mitochondrial succinate dehydrogenase (SDH), or complex II, consists of four nuclearly encoded subunits and mutations in the *SDHA-D* genes have been associated with strikingly diverse clinical presentations; moreover, further genetic heterogeneity is likely, given the large number of undiagnosed cases [18]. The sequence of subunits encoded by *SDHA* and *SDHB* in our patient was normal, suggesting that the molecular defect in this patient may reside in an as yet unidentified assembly gene. In patient 10, a specific defect was observed only when pyruvate + malate was used (62% in Fig. 2A), a finding later confirmed by the identification of a novel mutation in the *PDH1A* subunit. Pyruvate dehydrogenase (PDH) is a large multienzyme complex that plays a crucial role in aerobic energy metabolism. In view of the heterogeneity of the spectrum of human deficiencies, in terms of both clinical presentation and disease course [19], and the difficulties inherent in the enzymatic test for PDH due to the need for radioisotopes, it seems possible that assay of ATP production as a measure of PDH activity might overcome, in part, the limitations of this test in patients with mitochondrial encephalomyopathies.

Multiple partial defects were observed in cases presenting COX deficiencies, lack of the electron carrier (i.e., CoQ_{10}) in

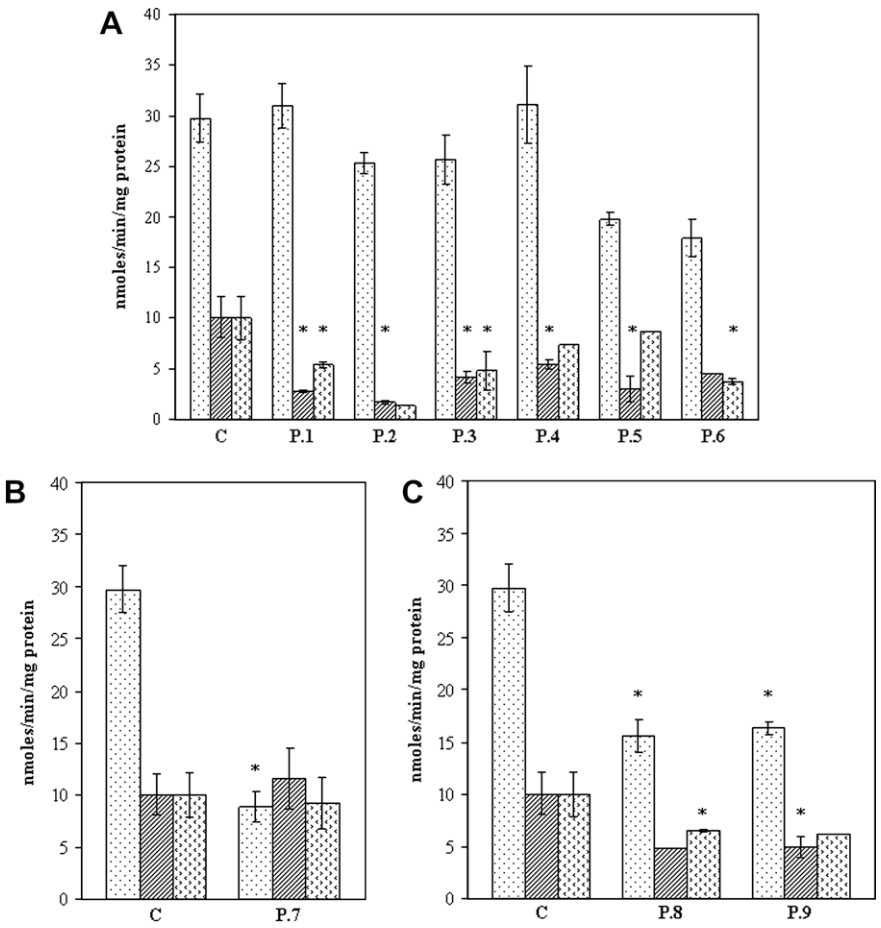

Fig. 1. ATP synthesis activity in 6 patients with complex I defect (A), in one case with complex II defect (B) and 2 patients with complex IV defect (C). (A) Patients 1–4 display a specific defect of complex I since the ATP production is reduced when using the specific substrates for complex I (that is, malate and alpha-ketoglutarate). Patients 5–6 show a more complicated OXPHOS defect with reduced ATP formation in the presence of three different substrates: succinate, malate, and alpha-ketoglutarate. (B) The biochemical defect in patient 7 is evidenced using succinate as substrate. (C) The amount of ATP production in patients 8 and 9 was similar with all substrates tested. C, controls; P, patients. The results are expressed as mean values ± SD. $^{*}p < 0.01$. ⬚, succinate; ▨, malate; ⬚, α-ketoglutarate.

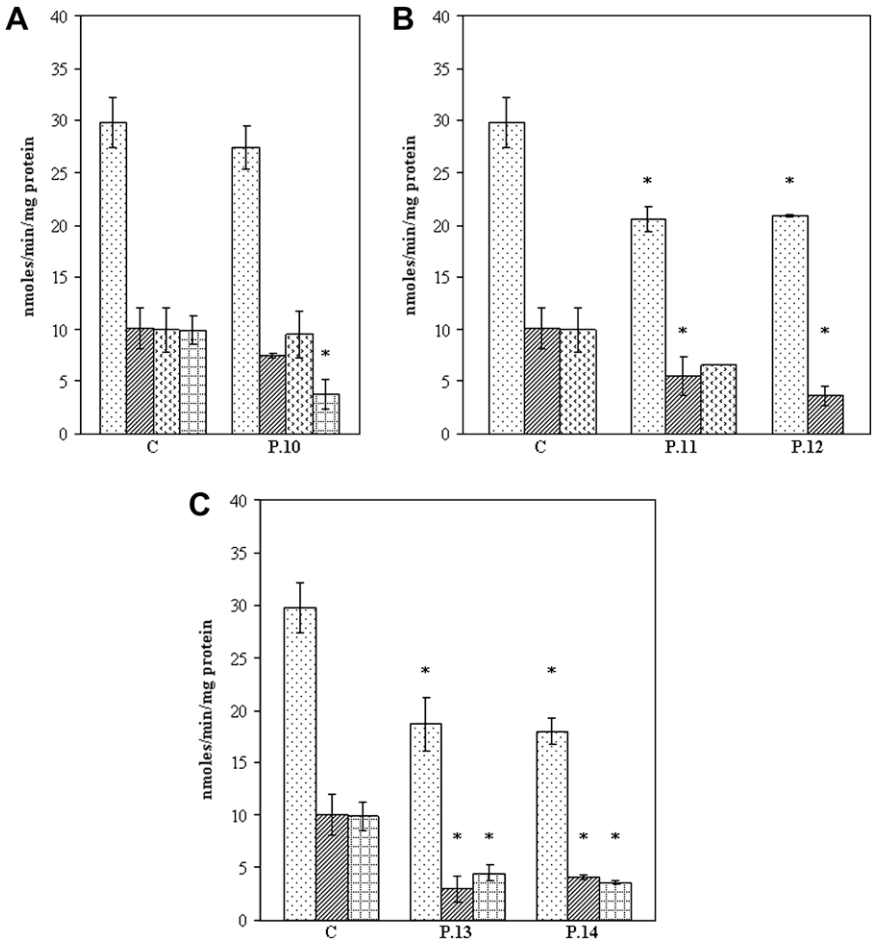

Fig. 2. ATP synthesis activity in 1 patient with PDH defect (A), two cases with CoQ_{10} defect (B), and 2 patients with mtDNA depletion (C). (A) ATP synthesis activity in patient 10 was highly reduced only in presence of pyruvate + malate; (B) The rate of ATP production in patients 11 and 12 was reduced by 30% and 55% in the presence of succinate or malate, respectively. (C) Patients 13–14 showed a reduced synthesis of ATP by around 39% in the presence of succinate, by 64% with malate, and 60% when pyruvate + malate were used as substrates. C, controls; P, patients. The results are expressed as mean values ± SD. $^{*}p < 0.01$. ⬚, succinate; ▨, malate; ⬚, α-ketoglutarate; ⬚, pyruvate + malate.

OXPHOS, or MDS. Complex IV (COX) is the terminal enzyme in the mitochondrial respiratory chain and is believed to be the rate-limiting enzyme [20]. Patients with COX deficiency show a diversity of clinical phenotypes including Leigh syndrome, cardiomyopathy, and myopathy [21]. Patients 8 and 9 showed the same rate of ATP production—in these patients ATP synthesis was reduced by 50% when either succinate or malate were used as substrates, and by around 40% in the presence of α-ketoglutarate (Fig. 1C)—regardless of their molecular defect (identified in an mtDNA-encoded subunit in patient 8 and in a nuclear protein required for the correct assembly of the holocomplex in patient 9). The two patients carrying mutations in *COQ2* (Pts 11 and 12), displayed a 30% reduction in ATP synthesis in the presence of succinate, rising to approximately 55% when malate was the substrate (Fig. 2B). Lack of CoQ_{10} in mitochondria will disrupt the flow of reducing equivalents to respiratory chain complex III, which in turn will lead to decreased ATP synthesis by oxidative phosphorylation. A combination of defects in the presence of multiple substrates was observed in two patients (cases 13 and 14) with mtDNA depletion and mutations in *SUCLA2*. MDS is usually characterised by a combination of defective OXPHOS enzymes, not including SDH [22]. As expected, the low proton flow through the respiratory chain reduced the synthesis of ATP by around 39% in the presence of succinate, by 64% and 60% when malate and pyruvate + malate were used as substrates (Fig. 2C).

Discussion

The mitochondrial OXPHOS defects probably constitute the most common group of neurometabolic disorders in childhood [23]. Their clinical features are sometimes characteristic but never specific for any genetic defect. Whereas mutations in mtDNA explain only around 20–25% of mitochondrial OXPHOS disorders in childhood [23], the list of nuclear genes involved in these disorders is growing all the time and includes assembly factors, translation factors as well as important proteins for the maintenance of mtDNA. Given the increasing number of genes and proteins involved, new screening techniques allowing more effective diagnostic routes are called for. In specialised laboratories, spectrophotometric determination of OXPHOS complexes in frozen skeletal muscle biopsy is the method of choice. However, it is not always possible in infants and children with suspected mitochondrial disorders in whom the opportuneness of surgical procedures must be carefully weighed up in view of the patient's general conditions; furthermore, in these subjects the amount of tissue accessible is generally too small to allow a comprehensive biochemical analysis. Finally, the traditional method is difficult and poorly reliable when measuring PDH or complex I or V in cultured skin fibroblasts. We believe that the spectrophotometric method proposed in this study, designed to measure ATP synthesis, might overcome difficulties in detecting mitochondrial respiratory chain defects and allow a clearer diagnosis even in cases in which direct measurement

of CoQ_{10} production is mandatory. We did not observe significant differences in residual activity between cases with confirmed (Pt 9) or presumed (Pts 5–7) mutations in nDNA genes and those harbouring mutations in mtDNA-encoded genes (Pts 1, 2 and 8). Although this means that the ATP synthesis assay does not select straightforwardly the gene to be screened first, it does suggest that the method is equally able to detect defects in any of the OXPHOS subunits, opening the way for precise diagnoses and the possibility of conducting more complete molecular studies. Finally, the use of substrates that enter the respiratory chain at different levels provides a means of measuring, indirectly, the function of the various complexes, and more important, the use of multiple substrates appeared to be helpful in the final biochemical diagnosis and could emerge as a tool with the potential to drive further molecular genetic studies. Several patterns of defective mitochondrial respiratory enzyme complexes can be identified. Low ATP production obtained in presence of malate, but not succinate, should alert the investigator to a possible complex I defect, whereas a defect of complex II can be predicted by a low rate of ATP production in the presence of succinate. A PDH defect should be hypothesised if the greatest reduction is observed when pyruvate + malate is the substrate. On the other hand, primary defects in COX, CoQ_{10} and MDS appear indistinguishable with low ATP production being observed with all the substrates, even though MDS patients show a relatively milder defect in the presence of succinate, probably due to the exclusively nuclear origin of complex II subunits (Fig. 2C). Nonetheless, these three groups can easily be distinguished subsequently, through the use of routine assays (e.g., cytochemical stain for COX and direct HPLC measurement of CoQ_{10}) [24].

In conclusion, we described the use of a biochemical test to depict respiratory chain function through direct measurement of ATP production (one of the most important parameters of mitochondrial metabolism) in 14 children with primary OXPHOS disorders. In addition to its use in primary defects of ATP synthase, this method opens up the prospect of further molecular studies in mitochondrial myopathies, and it seems particularly suitable for assaying the activities of respiratory chain complexes I and II and the PDH complex in primary cells. Its ability to detect complex I deficiency is especially important, as this defect is the most frequently encountered OXPHOS disorder. If used in combination with assays normally performed in specialised laboratories, the measurement of ATP synthesis activity in cultured skin fibroblasts may well prove to be an effective screening tool for detecting OXPHOS dysfunction and it has the potential to expand the list of patients, like those with low complex IV and primary CoQ_{10} deficiency, in whom a precise diagnosis can be reached. This is particularly important given that oral supplementation with CoQ_{10} improves clinical symptoms and can sometimes be life-saving [24].

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