Clinical, biochemical, and histopathological diagnosis of Egyptian paediatric patients with suspected mitochondrial diseases: A hospital-based study

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Background. Mitochondrial respiratory chain (RC) disorders are a growing group of disorders with a large variety of clinical presentations ranging from well-defined clinical syndromes to nonspecific manifestations, such as failure to thrive, exercise intolerance and seizures.
Objective. To describe the clinical, biochemical, and histochemical spectrum of 38 Egyptian patients clinically suspected of having mitochondrial RC disorders.
Methods. A total of 38 patients (female, *n*=18 (47.4%); male, *n*=20 (52.6%)) clinically suspected of having mitochondrial diseases had been referred to the Inherited Metabolic Diseases Laboratory at Cairo University Children's Hospital. Laboratory investigations and analyses included histochemical staining of cytochrome *c* oxidase and succinate dehydrogenase in muscle biopsies, as well as spectrophotometric assays of RC complexes in muscle homogenates.
Results. Twenty-three patients (60.5%) were diagnosed with different RC enzyme deficiencies. Fifteen patients (65%) had complex I deficiency and all of them also had lactic acidosis (mean (standard deviation)) plasma lactate concentration of 4 (1.65) mmol/L). Two patients (9%) with marked complex IV deficiency both showed COX-negative ragged red fibers (RRFs) on histochemical staining. Combined complex I and complex II deficiency with scattered COX-stain deficiency and RRFs was diagnosed in 2 patients (5.25%), while a further 2 patients (5.26%) had combined (complex I, II+III, complex IV) deficiencies. Isolated complex II deficiency was diagnosed in 2 patients (5.26%) and 15 (39.5%) patients had normal RC enzyme activities.
Conclusion. Biochemical assay of RC complexes is considered the cornerstone for diagnosis of RC complex mitochondrial disorders. These disorders are common among Egyptian paediatric patients.

Mitochondrial disorders (MDs) are an expanding heterogeneous group of disorders caused by defects in intracellular energy production characterised by morphological and biochemical abnormalities of mitochondria that are usually associated with mitochondrial (mtDNA) mutations or nuclear gene defects.[1] They are one of the most common inborn errors of metabolism, with a conservative estimated prevalence of ~1: 5 000.[2]

The respiratory chain complexes (RCCs) consist of four enzymatic complexes (complexes I - IV) embedded in the inner mitochondrial membrane. These complexes catalyse the transfer of reducing equivalents from high-energy compounds (produced during the Krebs cycle) to oxygen, with the ultimate production of an electrochemical gradient through the inner mitochondrial membranes to drive the synthesis of adenosine triphosphate (ATP) by ATP synthase.[3]

Mitochondrial-associated myopathies are clinical features commonly affected in patients with a multisystem phenotype which includes chronic progressive external ophthalmoplegia, rhabdomyolysis, muscle fatigue and severe proximal weakness.[4]

Unfortunately, most paediatric patients present with nonspecific symptoms including developmental delay, failure to thrive, hypotonia, seizures and encephalopathy. This presents a diagnostic challenge to neuro-paediatricians and accounts for the considerable diagnostic delay.[5]

The diagnosis of MDs is usually based on diagnostic criteria that include clinical data, biochemical alterations (e.g. decreased RCC enzyme activity, and lactate acidosis), histological features, and genetic results (mtDNA or nuclear mitochondrial gene variants).[4]

Despite their invasiveness, biochemical assays measure the actual activity of individual oxidative phosphorylation (OXPHOS) enzyme (complexes I - V) in homogenised muscle tissues, and the results are normalised to the activity of a mitochondrial matrix enzyme, i.e. citrate synthase (CS).[1] The histochemical assessment of cytochrome *c* oxidase (complex IV, COX) and succinate dehydrogenase (complex II, succinate dehydrogenase (SDH)) activities assess RCC enzyme function in tissue biopsies, interrogating the activities of these complexes in the individual cell.[5] Complete molecular diagnosis requires several different methods for the detection and quantification of mtDNA gene variants and large deletions.[6]

Over the last 50 years, next-generation sequencing (NGS) technologies have accelerated our ability to detect molecular

aetiologies of the disease, while whole-exome sequencing (WES) approaches have significantly improved the diagnosis of monogenic mitochondrial mimics and advanced our understanding of mitochondrial biology.[7]

A limited number of studies have focused on the diagnosis of MDs among Egyptian patients;[8-11] however, the role of the histochemical and the biochemical RCC assays for proper diagnosis of these disorders has not been clarified.

The current study focused on biochemical assays of RCCs and histochemical staining of muscle biopsies to elucidate their respective roles in the diagnosis of mitochondrial-related disorders in a sample of Egyptian paediatric patients.

Methods

The present study was conducted at the Inherited Metabolic Diseases Laboratory at Cairo University Children's Hospital (CUCH). Thirty-eight patients were included in the study after clinical recruitment based on diagnostic scoring for MDs[12] (Fig. 1). Thirty-eight age- and sex-matched controls were recruited. Controls were attending the hospital for orthopaedic surgeries. Ethical approval was obtained from the Cairo University Research Ethical Committee (ref. no. N-15-2014). Parents of patients and controls gave written informed consent for all the procedures performed in the study. Written informed consent was obtained from each participant after a full explanation of the study protocol. All procedures included individual data were treated with confidentiality.

Routine laboratory investigations including liver function tests, kidney function tests, blood glucose, arterial blood gases, and plasma lactate were performed as first-line investigations to detect highly suspected cases. Amino acid and acyl carnitine profiles were done using liquid chromatography/mass spectrometry (LC/MS-MS) to exclude other inborn errors of metabolism that present with similar manifestations.

All patients were subjected to imaging studies, including brain magnetic resonance imaging (MRI), neurophysiological investigations, including ectroencephalogram (EEG) and electromyogram (EMG). Nerve conduction studies (NCS) and visual-evoked potentials (VEPs) were done for all patients. Some patients were subjected to a fundus examination.

Muscle biopsies from quadriceps muscles were performed under general anaesthesia for selected patients. For histochemical examinations, fresh transverse sections of muscle fibers from muscle biopsies were stained with haematoxylin and eosin, Gömöri stain, cytochrome c oxidase (COX) and succinate dehydrogenase (SDH), using staining kits (Bio-Optica S.p.A, Italy). Some muscle fibers were fixed in glutaraldehyde and routinely processed for electron microscopic examination. The rest of the biopsy was immediately preserved at −80° C for biochemical assays. All these procedures were performed for the control group.

For the biochemical RCC assay, biopsies were homogenised on ice using a conical tissue grinder in a 1:9 (w/v) ratio in potassium
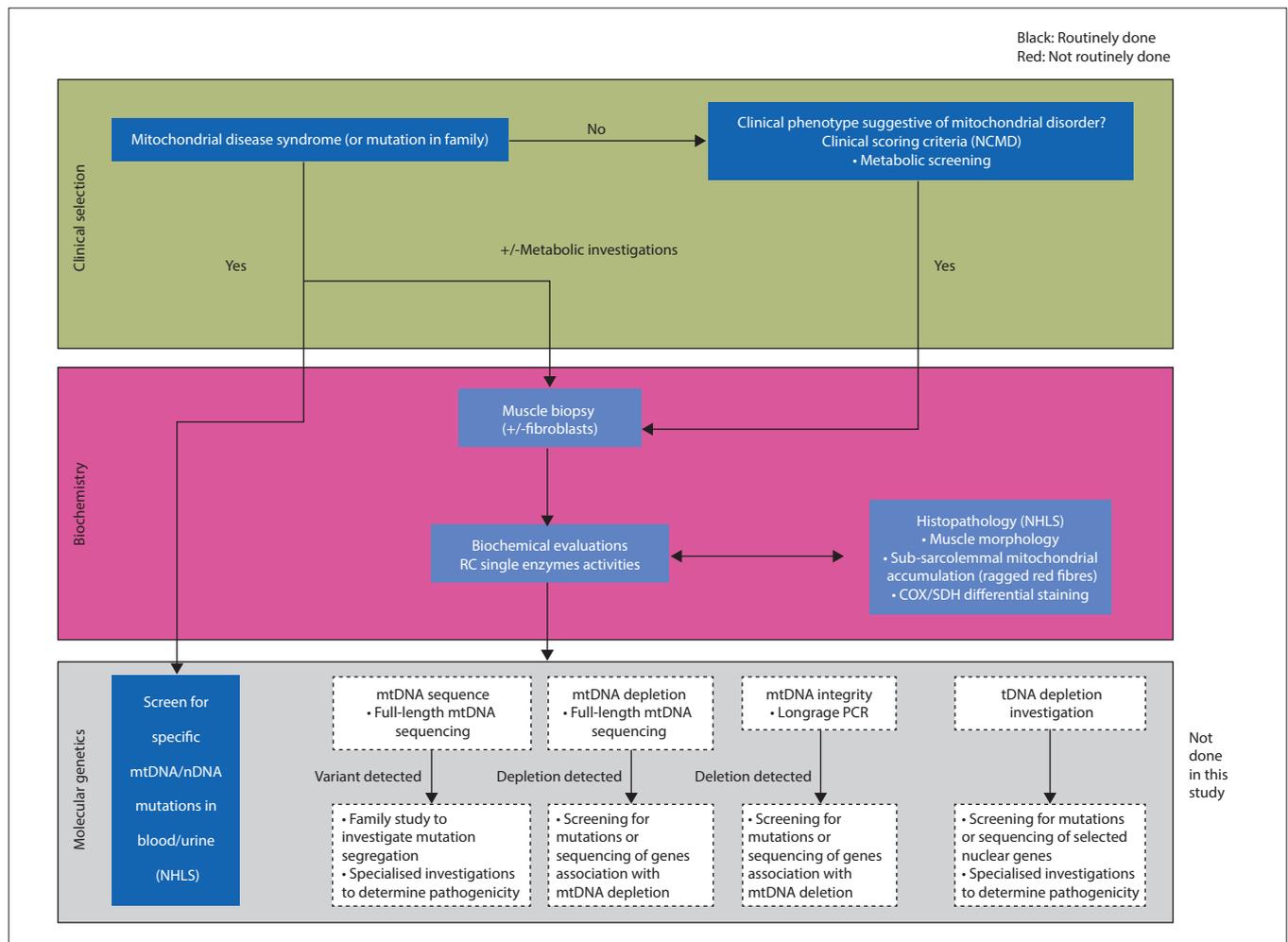

Fig. 1. Algorithm for diagnosis of mitochondrial disorders. (NCMD = Nijmegen Centre for Mitochondrial Disorders; RC = respiratory chain; NHLS = National Health Laboratory Service; COX = cytochrome C oxidase; SDH = succinate dehydrogenase.)

phosphate buffer pH 7.5 containing 0.25 M sucrose, 2 mmol ethylene diamine tetra acetic acid (EDTA) and 50 IU/mL heparin.[4]

Mitochondrial RCCs and CS enzymatic activities were determined by a spectrophotometric enzyme assay. Complex I (NADH: ubiquinone reductase; EC1.6.5.3) activity was measured by the rotenone-sensitive oxidation of NADH at 340 nm. Complex II/III (succinate: cytochrome *c* reductase; EC1.3.5.1 and EC1.10.2.2) activity was measured by antimycin A-sensitive succinate-dependent reduction of cytochrome *c* at 550 nm. Complex IV (cytochrome *c* oxidase; EC1.9.3.1) activity was measured by the potassium-cyanide-sensitive oxidation of reduced cytochrome *c* at 550 nm. CS (EC2.3.3.1) activity was determined by the formation of 5-to-2-nitrobenzoic acid following the incubation of tissue homogenate with acetyl-CoA, oxaloacetate, and 5,5'-dithio-*bis*-(2-nitrobenzoic acid) at 412 nm using UV/visible spectrophotometry (Ultra spec 2100 pro; Amersham, UK) supported by Swift-II software (version 2.06; Fisher Scientific, UK).[4]

Statistical analyses

Data were analysed using SPSS advanced statistics version 20 (IBM Corp., USA). Qualitative data were expressed using numbers and frequencies. Quantitative data were expressed using mean (standard deviation (SD)) or median (range) as appropriate and categorical variables were expressed as percentages. All mitochondrial enzymes were expressed as a ratio to the activity of CS and complex II as an internal mitochondrial marker enzyme to standardise the mitochondrial enrichment of the sample. Activities were expressed in nmol/mL/mg protein and the range, mean (SD) and Z-scores were calculated for each RCC enzyme activity. Statistical significance between patients' results and controls was expressed as the p-value (significance considered if $p<0.0001$).

Results

The present study included 38 paediatric patients (female, $n=18$ (47.4%); male, $n=20$ (52.6%)) and 38 controls (female, $n=24$ (63%); male, $n=16$ (37%)). The median (range) age of patients and controls was 6 (1 - 15) and 4.5 (1 - 16) years, respectively.

Positive consanguinity was remarkable in 84% patients ($n=32$) and negative consanguinity in 16% ($n=6$). The demographic data and the clinical presentations of the patients and controls are described in Table (1).

Atrophic brain changes were observed in the brain MRI of 10 cases (26.3%): 4 had normal RC enzyme activity ($n=4/10$; 40%), while the remaining 6 (60%) cases had variable degrees and types of enzyme deficiencies. Bilateral and symmetrical abnormal signal intensity in the basal ganglia was the main finding in 7 cases (18.4%) and all showed RCC enzyme deficiency. Bilateral white matter demyelination was seen in 5 cases (39.5%) of whom all had complex I deficiency, while bilateral cerebellar demyelination was seen in 4 patients (10.5%), with RCC enzyme deficiency in 3 patients (7.9%). Normal brain imaging was observed in 12 patients (32.6%). Radiological and neuroimaging findings of the patients are shown in Table 2.

Electron microscopic examination showed accumulation of sub-sarcolemmal aggregates of mitochondria in 14 patients (36.8 %), and biochemical RCC enzyme activity assays verified 8 out of 14 patients with complex I deficiency (57.2%): 2 patients had isolated complex II deficiency (14.3%); 2 had combined complex I, II+III, and IV deficiency (14.3%); 1 patient had isolated complex IV deficiency (7.1%); and 1 patient had normal enzyme activity (7.1%).

Histochemical investigations showed COX-negative ragged red fibers (RRFs) in 8 patients (21.1%), scattered COX-stain deficiency with RRF in 4 patients (10.5%), atrophied muscle changes with RRFs in 6 patients (15.8%) (Fig. 2), nonspecific muscle changes in 5 patients (13.2%) and a normal pattern in 15 patients (39.5%) (Table 3).

Biochemical measurement of RCC enzymes in muscle homogenates confirmed enzyme deficiency in 23 patients (60.5%) when compared with citrate synthase and complex II enzyme activities. Fifteen patients (65%) showed complex I deficiency (Fig. 3). All of the patients with complex I deficiency had lactic acidosis (mean (SD) plasma lactate 2.8 (1.18) mmol/L). Complex IV enzyme activity assays verified 2 cases (9%) (Fig. 3). Both patients presented with convulsions, epileptogenic focus on EEG, and Cox-stain deficient RRFs on histopathological examination. Two patients (9%) had combined complex I and complex II deficiencies, two patients (9%) had combined (complex I, II+III, complex IV) deficiencies, two patients (9%) (Fig. 3) had isolated complex II deficiency, and 15 patients had normal RCC enzyme activities (39.5%). Tables 3 and 4 show a summary of the results of the histochemical and biochemical analyses. The supplementary table (https://www.samedical.org/file/1956) summarises demographic, clinical, radiological, biochemical and histopathological data of the patients (N=38).

Discussion

Many Egyptian studies have discussed MDs from the clinical, radiological and pathological points without verifying the role of biochemical mitochondrial enzyme activity measurements as a step for determining isolated and combined complex deficiencies in individuals.

Table 1. Demographic data and the clinical presentation, *n* (%)*

Item	Patients, N=38	Controls, N=38
Age (years), median (range)	6 (1 - 15)	4.5 (1 - 16)
Gender		
Male	20 (52.6)	16 (37)
Female	18 (47.6)	24 (63)
Consanguinity		
Positive	32 (84)	20 (52.6)
Negative	6 (16)	18 (47.4)
Family history		
Positive	10 (26)	-
Negative	28 (74)	
Disease course		
Stationary	12 (31.6)	-
Progressive	26 (68.4)	
Clinical presentation		
Lactic acidosis	28 (73.7)	-
Convulsions	12 (31.6)	
Delayed motor development	10 (26)	
Peripheral neuropathy	6 (12.8)	
Acute encephalopathy	4 (10.5)	
Exercise intolerance	4 (10.5)	
Ataxia	4 (10.5)	
Hepatomegaly	3 (7.9)	
Cardiomegaly	3 (7.9)	
Opthalmoplegia	2 (5.3)	
Dystonia	2 (5.3)	
Bulbar palsy	2 (5.3)	
Loss of weight	1 (2.6)	

*Unless otherwise specified.

El-Bassyouni *et al.*[14] illustrated the clinical, pathological, and biochemical diagnosis of mitochondrial diseases in 19 Egyptian patients, in whom they only measured plasma lactate, lactate/pyruvate ratio, and serum cytochrome *c* using an enzyme-linked immunoassay (ELISA).[14] Selim *et al.* [8] performed their studies on 31 patients to confirm the role of brain MRI and MRS as non-invasive diagnostic procedures,[8] while El-Ettribi *et al.* [9] investigated the most common mtDNA point mutations based on clinical suspicion in only 8 Egyptian patients.[9] Selim *et al.*[10] discussed the role of clinical, pathological, biochemical and molecular diagnosis of mitochondrial neurogastrointestinal encephalopathy in 3 Egyptian patients.[10]

Diagnosing mitochondrial disorders remains a major challenge for the clinician owing to variable age at onset, clinical presentations and their genetic heterogeneity. The diagnosis often remains uncertain until muscle biopsy investigations are conducted and genetic tests confirm an abnormality. [15] Walker *et al.*[16] were the first to establish a set of major and minor criteria for the identification of MDs.[16] The Walker criteria were then modified by Bernier *et al.*,[17] and are now known as the modified Walker criteria.[17] Other attempts to define diagnostic criteria included the Nijmegen Centre for Mitochondrial Disorders scoring system and the Mitochondrial Disease Criteria.

In the present study, lactic acidosis was the most common finding (*n*=28 (73.7%)). Fatal infantile lactic acidosis (FILA) has been reported most frequently in children with complex I deficiency of the mitochondrial respiratory chain disorder.[18]

Convulsions were the second most frequent clinical presentation representing 31.6% (*n*=12) of patients. Lee *et al.*[21] performed a study of 125 patients clinically suspected of having Leigh syndrome. Among the patients, 25 were identified as having mitochondrial DNA associated with Leigh syndrome, while 14 out of the 25 (56%) presented with seizures.[21]

As Egypt is an African country, it was crucial to compare our findings with those of a study of African patients by Smuts *et al.*,[22] who reported that myopathy was the most frequent clinical presentation (62.5%), followed by encephalomyopathy (30%) and encephalopathy (7.5%), while encephalomyopathy was the predominant finding (60%) among the white patients, followed by an equal frequency of encephalopathy and myopathy (20%).[22]

In contrast, an audit of all mitochondrial disease genetic tests (*N*=1 614) performed in Cape Town, South Africa (SA), by Meldau *et al.*[23] found that muscle phenotypes are not commonly encountered in SA paediatric patients.[23]

This variation may be due to the difference in the examined sample size, with more conclusive results reported by Meldau *et al.*[23]

Histopathological findings matched biochemical assay results of mitochondrial enzymes, as 10 patients (66.7%) showed neurogenic muscle atrophy in muscle biopsies, with accumulation of sub-sarcolemmal aggregates of mitochondria visible in electron micrographs. Rollins *et al.*[24] evaluated 118
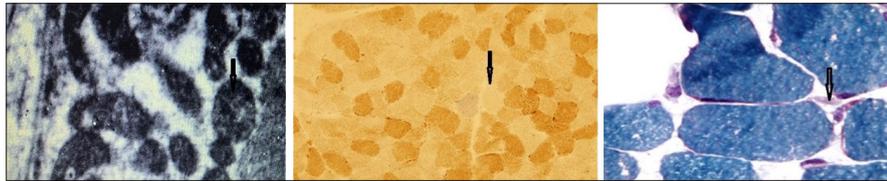

Fig. 2. Electron micrographs of sub-sarcolemmal mitochondrial aggregates, scattered COX-negative fibers in muscle biopsy from a patient with complex IV deficiency, and Gömöri trichrome stain (940×) showing moderate mitochondrial proliferation.

Table 2. Radiological and neuroimaging findings in patients (*N*=38)

Imaging modality	*n* (%)
Brain MRI	
Brain atrophy	10 (26.3)
Basal ganglia abnormal signal	7 (18.4)
Bilateral white matter demyelination	5 (13.2)
Bilateral cerebellar demyelination	4 (10.5)
Normal findings	12 (31.6)
Electromyelogram	
Myopathic changes in proximal muscles	17 (44.8)
Neurogenic muscle atrophy	6 (15.7)
Normal pattern	17 (39.5)
Electroencephalogram	
Epileptogenic dysfunction	16 (42.1)
Magnetic resonance spectroscopy	
High lactate peak	4 (10.5)
Echocardiography	
Hypertrophic cardiomyopathy	3 (7.9)
Cardiomegaly	2 (5.3)
Normal findings	33 (86.8)

MRI = magnetic resonance imaging.

Table 3. Bio- and histochemical results (*N*=38)

	n (%)*
Histochemical staining findings	
RRFs	8 (21.1)
COX stain deficiency with RRFs	4 (10.5)
Atrophic muscle changes with RRFs	6 (15.8)
Nonspecific muscle patterns	5 (13.2)
Normal pattern	15 (39.5)
Electron microscope	
Subsarcolemmal aggregated of mitochondria	14 (36.8)
Biochemical enzyme assay results, n/N (%)	
RCC enzyme deficiencies	23/38 (60.5)
Complex I deficiency	15/23 (65.2)
Complex IV deficiency	2/23 (8.7)
Combined complex I and complex II deficiency	2/23 (8.7)
Complex I, II, III and IV deficiency	2/23 (8.7)
Isolated complex II deficiency	2/23(8.7)
Normal RCC enzymes activities	15/38 (39.5)

RCC = respiratory chain complex; RRFs = ragged red fibers.
*Unless otherwise specified.

muscle biopsy specimens from patients of all ages with mitochondrial diseases and reported normal findings on light microscopy of 45% of the specimens, RRFs in 2.5% and cytochrome *c* oxidase-negative fibers were found in 7% (53).[24] A previous Egyptian study reported RRFs on modified Gömöri trichrome stain of muscle biopsies from all patients in the study as an associative finding with MCDs.[11] Selim *et al.* [10] also illustrated the importance of histochemistry in muscle biopsies obtained from suspected MD patients in increasing Morava scoring for clinical diagnosis.[10]

The present study verified complex I deficiency in 65% (*n*=15/23) of patients with RCC enzyme deficiency, a finding matching previously published studies in mitochondrial diseases.[10,23,24] In our study, all patients with complex I deficiency had lactic acidosis, but developmental delay was notable in only 6 patients (*n*=6/15; 40%), while RRFs were the main pathological finding in 9 patients (*n*=9/15; 60%). Owing to the clinical heterogeneity of complex I disorders, patients often do not present with the traditional hallmarks of

mitochondrial disease. This reinforces the importance of biochemical and metabolic screening as appropriate methods to diagnose complex I deficiency in paediatric patients.[24]

Mitochondrial complex II deficiency is an autosomal-recessive disorder with a highly variable phenotype. It is exclusively nuclear-encoded with some patients having multisystem involvement of the brain, heart, muscles, liver and kidneys. This may result in death in infancy, whereas some patients have only isolated cardiac or muscle involvement with onset in adulthood and normal cognition.[2] In the present study, 2 patients had isolated complex II deficiency (*n*=2/23, 8.7%), both with positive consanguinity: one presented with lactic acidosis and bulbar palsy with abnormal basal ganglia signals in the brain MRI, while the other presented with exercise intolerance, ptosis, and cardiomyopathy.

Isolated complex IV deficiency was notable in 2 patients (*n*=2/23; 8.7%). One patient with positive consanguinity presented with convulsions, peripheral neuropathy and hypertrophic cardiomyopathy, with white matter demyelination visible on brain MRI, as well as COX-stain deficiency and RRFs on histopathological investigation. The other patient presented with delayed motor development, lactic acidosis, convulsions, abnormal basal ganglia signals on brain MRI, and COX-stain deficiency with RRFs and sub-sarcolemmal aggregates on histopathology. Fatal infantile cardio-encephalomyopathy with COX-stain deficiency was the main finding in a previous study which determined that clinical phenotype caused by mutations in the human *SCO2* assembly gene causing complex IV deficiency presented mainly with Leigh syndrome.[25]

Although isolated complex deficiency was notable in this study, combined complex deficiency was a remarkable finding: 2 patients (*n*=2/23; 8.7%) had combined complex I and II deficiency; and 2 patients (*n*=2/23; 8.7%) had complex I, II, III and IV deficiency. On the other hand, in their study of 191 subjects, Smuts *et al.*[22]

Table 4. Individual RCC enzyme activities

	Patients, mean (SD)*	Controls, mean (SD)*
Protein concentration (mg/mL)	5.53 (1.05)	5.95 (1.23)
Complex I	20.98 (10.96)	35.00 (11.00)
Complex II	35.87 (7.60)	53.00 (16.00)
Complex III	49.21 (3.90)	54.00 (15.00)
Complex II+III	49.00 (7.10)	59.00 (20.00)
Complex IV	121.00 (19.00)	131.00 (32.00)
CS nmol/min/mg protein	172.95 (27.06)	182.00 (50.00)

RCC = respiratory chain complex; SD = standard deviation; CS = citrate synthase.
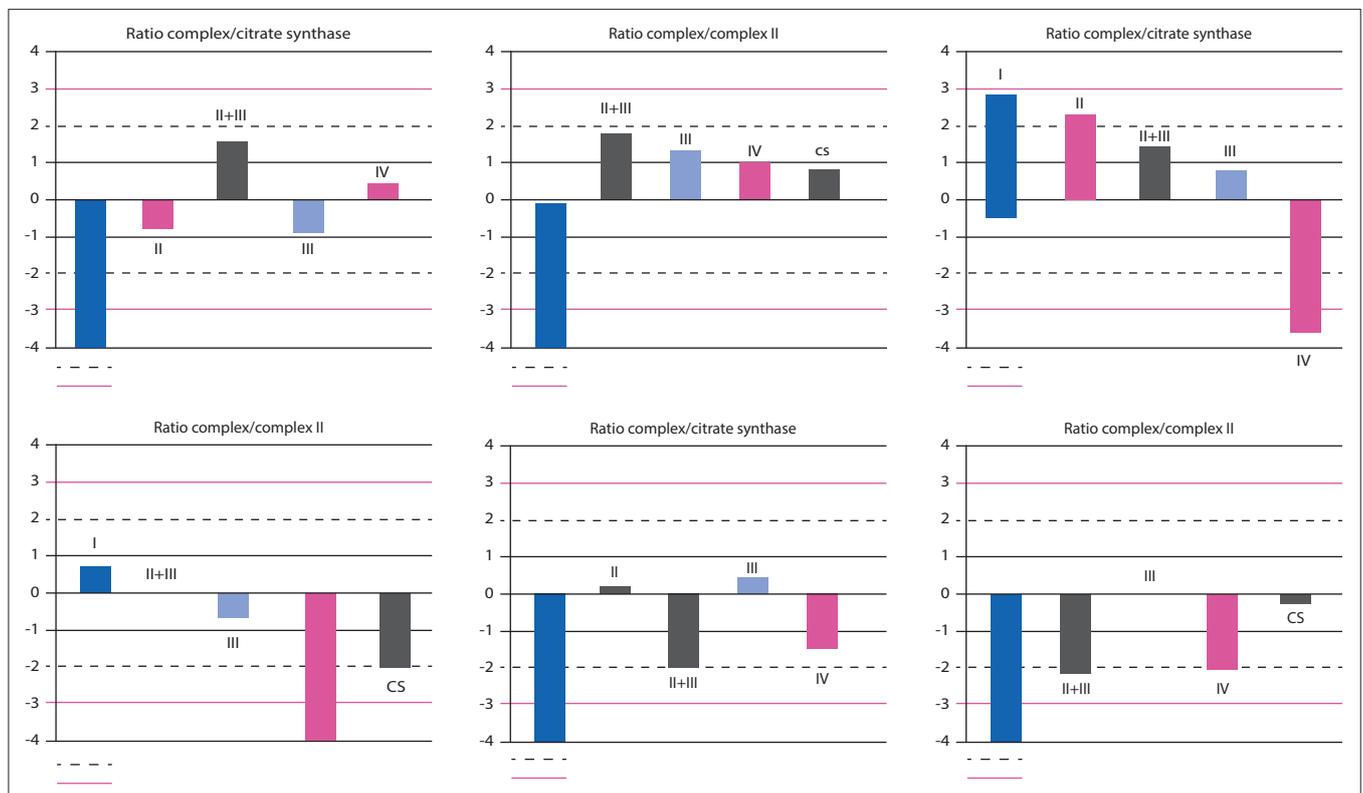*Measured in nmol/min/mg protein unless otherwise specified.

Fig. 3. Marked complex I enzyme deficiency (compared with citrate synthase and complex II), marked complex IV enzyme deficiency (compared with citrate synthase and complex II, and combined complex I, II+III, and IV deficiencies compared with citrate synthase and complex II.

(including both African and white patients) illustrated that single-enzyme deficiencies were more common than combined deficiencies in white patients (60.0%), but combined deficiencies were found in 67.5% of African patients.[22]

Numerous co-factors also play an essential role in mitochondrial energy metabolism and some of these cofactors are required for the function of RCC enzymes, such as coenzyme Q, iron-sulphur clusters, riboflavin and haem – deficiencies typically result in defects of more than one respiratory enzyme.[20]

The lack of genetic data limited the present study but important logistical and procedural challenges have been overcome, in addition to using control samples for refinement of analyses and reference values.

Conclusion

The results of the present study confirm the clinical heterogeneity of mitochondrial diseases and the importance of biochemical assays of RCC enzymes in muscle biopsies in suspected patients. Histopathological investigations of muscle biopsies will raise the confirmatory diagnosis (Morava score) and aids biochemical diagnosis.

Despite the limited sample size, the presence of 60.5% of patients with respiratory chain enzyme defects confirms that mitochondrial diseases are not rare among Egyptian paediatric patients and there is a need for proper selection of patients based on strict inclusion criteria. Histochemical, histopathological and biochemical respiratory chain enzymes assay in muscle biopsy are needed for better diagnosis and treatment of mitochondrial diseases. Molecular analysis of respiratory chain complex deficiencies is crucial for the elucidation of the underlying genetic basis of such disorders in Egyptian patients. National programmess are needed for early detection, intervention and management of such disorders in the Egyptian population.

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