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## Genotypes and Clinical Phenotypes in Children with Cytochrome c Oxidase Deficiency

### Abstract

Cytochrome *c* oxidase (COX) deficiency has been associated with a wide spectrum of clinical features and may be caused by mutations in different genes of both the mitochondrial and the nuclear DNA. In an attempt to correlate the clinical phenotype with the genotype in 16 childhood cases, mtDNA was analysed for deletion, depletion, and mutations in the three genes encoding COX subunits and the 22 tRNA genes. Furthermore, nuclear DNA was analysed for mutations in the *SURF1*, *SCO2*, *COX10*, and *COX17* genes and cases with mtDNA depletion were analysed for mutations in the *TK2* gene. *SURF1*-mutations were identified in three out of four cases with Leigh syndrome while a mutation in the mitochondrial tRNA<sup>trp</sup> gene was identified in the fourth. One case with mtDNA depletion had mutations in the *TK2* gene. In two cases with leukoencephalopathy, one case with encephalopathy, five cases with fatal infantile myopathy and cardiomyopathy, two cases with benign infantile myopathy, and one case with mtDNA depletion, no mutations were identified. We conclude that COX deficiency in childhood should be suspected in a wide range of clinical settings and although an increasing number of genetic defects have been identified, the underlying mutations remain unclear in the majority of the cases.

### Key words

■ please add key words

### Introduction

Mitochondrial encephalomyopathies caused by dysfunction of the mitochondrial respiratory chain, constitute a relatively common group of neurometabolic diseases in childhood. Their incidence has been estimated to be in the order of 1 in 11000 preschool children and cytochrome *c* oxidase (COX) deficiency is one of the most common defects [7].

The oxidative phosphorylation system, which consists of five multi-subunits enzyme complexes (complex I – V), is located in the mitochondrial inner membrane. COX (complex IV) is the terminal enzyme of the respiratory chain, catalysing the transfer of electrons from reduced cytochrome *c* to molecular oxygen and it couples this reaction to proton pumping across the inner mitochondrial membrane. The COX enzyme complex is composed of 13 structural subunits, three of which are encoded by genes of the mitochondrial DNA (mtDNA) and form the catalytic core of the enzyme. In addition, a number of proteins are involved in its assembly and maintenance [55].

COX deficiency should be kept in mind as a potential differential diagnosis in a broad range of clinical conditions in childhood with possible onset from the prenatal period to adolescence (Table ). A handful mutations have been identified in each of the three mtDNA-encoded COX subunits [5,16,24,34,46,67]. COX deficiency has also occasionally been associated with mutations in mitochondrial tRNA genes [23,51,56,59]. In contrast, no mutations have yet been identified in any of the nuclear DNA-encoded COX subunits [60]. More recently, mutations have been identified in an increasing number of nuclear genes involved in

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the assembly and maintenance of the holoenzyme complex; *SURF1* [62, 76], *SCO2* [42], *COX10* [69], *COX15* [3], and *SCO1* [68].

COX deficiency may also be associated with mtDNA depletion [75]. This group of autosomal recessive disorders shows a very variable phenotype, which may involve muscle, liver, brain, heart, and other organs [31, 75], including the syndromes of Leigh [1] and Alpers [38]. Mutations have recently been identified in the deoxyguanosine kinase gene (*dGK*) associated with a hepatocerebral form [33] and the thymidine kinase gene (*TK2*) described in a myopathic variant [49].

The purpose of this study was to investigate a series of childhood cases with COX deficiency for mutations in known disease-causing genes, attempting to correlate the clinical phenotype with the genotype as far as possible and to give a clinical overview based on these cases and an overview of the literature.

## Patients and Methods

### Patients

A total of 16 children with COX deficiency, diagnosed since 1984 at the Queen Silvia Children's Hospital in Göteborg, were included in the study. Siblings with a similar clinical course were considered to have the same diagnosis as their affected and investigated sibling. The Ethics Committee at the Faculty of Medicine at Göteborg University approved the study.

### Laboratory definitions

COX deficiency was defined as a generalized reduction in COX activity by enzyme histochemical analysis, affecting the majority of the muscle fibres, in addition to marked reduction of COX activity as determined by oxymetric and/or spectrophotometric analysis of muscle mitochondria with or without deficiency of other respiratory chain enzymes.

The demonstration of mtDNA depletion was based on quantification by Southern blot analysis and defined as a ratio of mtDNA/nuclear DNA less than 10% of the mean ratio in age-matched controls [27].

### Morphologic analysis of skeletal muscle

Muscle specimens obtained from the vastus lateralis of the quadriceps femoris muscle were frozen in a mixture of propane and propylene chilled by liquid nitrogen. Cryostat sections were stained with hematoxylin eosin and modified Gomori trichrome and incubated for succinate dehydrogenase [11] and COX [54] as well. Specimens were also fixed in 2.5% glutaraldehyde, post-fixed in OsO<sub>4</sub>, and embedded in Epon for electron microscopy. Ultrathin sections were contrasted with uranyl acetate and lead citrate.

### Biochemical analyses of skeletal muscle mitochondria

Hyperlactatemia was defined as blood lactate concentration > 2.5 mmol/L, measured on at least two occasions. CSF lactate was considered as increased when > 2.5 mmol/L. Isolation of mitochondria, oximetric measurements on fresh mitochondria, and spectrophotometric enzyme analyses were performed essentially as previously described [66].

Table 1 Clinical heterogeneity of COX deficiency in childhood

Onset	Clinical features	References
Prenatal	growth retardation and dysmorphic features	[25, 35]
	cerebellar hypoplasia ± optic nerve atrophy	[29, 39]
Neonatal	spinal muscular atrophy-like	[48]
	recurrent myoglobinuria	[53]
	asphyxia-like	[73]
	Respiratory distress-like	[58]
	ohtahara syndrome	[72]
Early childhood	fatal myopathy and LA	[41]
	– ± De Toni-Fanconi-Debré syndrome	[70]
	– renal tubular acidosis	[12]
	– cardiomyopathy	[47]
	– encephalopathy	[22]
	benign myopathy and LA with reversible COX deficiency	[9]
	hepatopathy	[4]
	cardioencephalopathy	[42]
	Leigh syndrome	[71]
	Alpers syndrome	[44]
Late childhood	MELAS	[34]
	MERRF	[30]
	leukoencephalopathy	[17]
	unspecific encephalomyopathy	[2]
	spinocerebellar syndrome	[56]
	sensorineural hearing impairment	[56]
	proximal myopathy	[46]
	exercise intolerance	[16]
	muscle cramps and myoglobinuria	[24]
	intestinal pseudoobstruction and neurogenic bladder	[15]
	renal Fanconi syndrome	[26]
bilateral cataracts	[5]	
endocrinopathy	[10]	

COX = cytochrome c oxidase, LA = lactic acidosis

### Molecular analysis

Total DNAs were isolated from frozen skeletal muscle, liver tissue, and blood samples by using a DNA extraction kit (Qiagen). Southern blotting of mtDNA was performed to detect large-scale rearrangements after cleavage with the restriction enzymes *Pvu* II or *Bam* HI. The genes of the nuclear-encoded mitochondrial proteins *SURF1*, *SCO2*, *COX10*, *COX17*, and *TK2* were analysed by PCR using intronic primers and sequencing of the PCR products as described [18, 32, 42, 62, 69]. Point mutations were searched for in 22 mitochondrial tRNA genes by PCR, followed by direct sequencing as previously described [19].

Long-expand PCR (LX-PCR) encompassing mitochondrial COX I to III was performed as previously described [36]. Mutations were searched for by direct sequencing of purified PCR products specific for mitochondrial COX genes.

Table 2 Clinical and genetic features in 16 children with COX deficiency

Case	Onset	Death */last assessment	Syndrome/clinical features	Genetic defect
1	birth	6 y*	LS/progressive motor deterioration with hypotonia and muscle weakness, ataxia, dystonia, choreoathetosis, and periodic breathing, hepatopathy, swallowing difficulties, growth retardation, hypertrichosis	SURF1
2	12 m	10 y	LS/progressive motor deterioration with hypotonia and muscle weakness, ophthalmoplegia, ataxia, choreoathetosis, nystagmus, swallowing difficulties, growth retardation	SURF1
3	5 m	2 y*	LS/progressive motor deterioration with hypotonia and muscle weakness, nystagmus, neuropathy, swallowing difficulties, growth retardation	SURF1
4	3 m	8 y	LS/progressive psychomotor retardation with SMR, spastic tetraparesis, optic atrophy, nystagmus, blindness, swallowing difficulties, epilepsy, hepatopathy	Mt-tRNA <sup>TP</sup>
5	2 y	9 y	leucodystrophy/spastic paraparesis, dystonia, ataxia, muscle weakness and swallowing difficulties	
6	4 y	5 y	leucodystrophy/spastic paraparesis, ataxia, muscle weakness, swallowing difficulties	
7	birth	6 y	progressive encephalopathy/with epilepsy, ataxia, dementia, autistic features, nystagmus, optic atrophy, papillary edema, neuropathy and myopathy, swallowing difficulties	
8	birth#	2 y*	congenital lactic acidosis, myopathy, hypertrophic cardiomyopathy and swallowing difficulties	
9	birth#	12 y*	congenital lactic acidosis, myopathy, hypertrophic cardiomyopathy, choreoathetosis and swallowing difficulties	
10	4 m#	5 m*	congenital lactic acidosis, myopathy, hypertrophic cardiomyopathy and swallowing difficulties	
11	birth	3 d*	congenital lactic acidosis, myopathy, hypertrophic cardiomyopathy	
12	birth	2 m*	congenital lactic acidosis, myopathy, hypertrophic cardiomyopathy	
13	< 1 m	9 y	congenital lactic acidosis and myopathy – benign variant	
14	< 1 m	14 y	congenital lactic acidosis and myopathy – benign variant	
15	7 m	1 y*	myopathy, hypertrophic cardiomyopathy, hepatopathy	MtDNA depletion
16	11 m	2 y*	myopathy	mtDNA depletion TK2

COX= cytochrome c oxidase, LS = Leigh syndrome, y = years, m = months, d = days, # = siblings

## Results

A total of 16 children with COX deficiency were diagnosed. A positive family history was identified in cases 8, 9, and 10 who were siblings, and in case 16 with an older sister who died at two years of age from a severe generalized myopathy. The male : female ratio was 1 : 1. Clinical onset was within the first 4 years of age. The course was progressive in 14 cases and nine of the children had died before thirteen years of age. A mild reversible phenotype was identified in two cases (cases 13 and 14). The clinical features are presented in Table 1.

The results from the genetic investigations are given in Table 2. We identified three cases with *SURF1* mutations, all with Leigh syndrome [37]. Patient 1 had a heterozygous mutation 688 C > T in exon 7 and a 15-bp duplication at nt 820/821 in exon 8. Patient 2 had a homozygous 10-bp deletion combined with AT insertion in exon 4 (312–321 del,insAT) and patient 3 had two heterozygous mutations, the 668 C > T substitution and a IVS7 + 1 G > T splice site mutation in exon 7. In addition, one case with Leigh syndrome had a 5537–5538 insT mutation in the mitochondrial tRNA<sup>TP</sup> gene [65]. Of the two cases with mtDNA depletion, case 16 with a pure myopathic form had two new compound hetero-

zygous mutations in the *TK2* gene (to be published). We could not identify any mutations in the other eleven cases.

Light microscopic investigations were normal in all of the cases with LS. Ragged-red fibres were identified in cases 11–13, abnormal lipid accumulation was found in cases 5–8, and pathological lipid and glycogen accumulation were detected in cases 11–16. Cases 9 and 10 were not investigated. All but one (case 1) of the investigated cases had abundant ultrastructurally abnormal mitochondria on electron microscopy (cases 2, 5–7, and 9 were not investigated).

The blood-lactate level was increased in all but one (case 2) of the cases (median 7.1; range: 2.4–32.0; control < 2.5). The spinal lactate level was increased in all but one (case 16) of the 10 investigated cases (median 3.6; range: 2.5–12.6; control < 1.8). Results from the polarographic and spectrophotometric investigations are presented in Table 3. There was a severe deficiency of COX activity in all the investigated cases, between 3 and 34% of the lower value of the reference interval, while the activities of complex I and complexes II–III were normal or less markedly decreased. The activity of complex I was normal in six and decreased to 16–69% of the lower reference interval in six, while

Table 3 Biochemical features in 16 children with COX-deficiency

Case	Oximetry $\text{nmol O} \times \text{min}^{-1} \times (\text{mg protein})^{-1}$			Spectrophotometry $\mu\text{mol} \times \text{min}^{-1} \times (\text{mg protein})^{-1}$		rate constant ( $k$ ) $\times$ $\text{min}^{-1} \times (\text{mg protein})^{-1}$ COX (6.1–15)
	Pyruvate + Malate (86–116)	Succinate + Rotenone (76–117)	Ascorbate + TMPD (207–302) (44–120) <sup>x</sup>	NADH ferricyanide reductase (4.5–8.6)	Succinate cytochrome- C reductase (0.16–0.40)	
1	56	43	57	5.2	0.12	0.88
2	67	78	157	6.2	0.26	1.4
3	55	59	101	5.4	0.19	1.0
4	22	60	81	2.2	0.15	0.62
5	78	79	173	7.3	0.32	1.4
6	84	69	149	6.2	0.28	1.0
7	56	60	137	5.2	0.20	1.6
8#	<10	<10	<10 <sup>x</sup>	0 <sup>*</sup>	0.01 <sup>*</sup>	0.2 <sup>*</sup>
9#	ND	ND	ND	ND	ND	ND
10#	ND	ND	ND	ND	ND	ND
11	<10	10	16	1.1	0.07	0.34
12	56	102	183	3.1	0.11	2.1
13	5	10	20	0.9	0.12	0.2
14	0	22	36 <sup>x</sup>	ND	ND	ND
15	9.5	26.5	74.9	0.7	0.14	0.34
16	5.6	7.0	20 <sup>x</sup>	ND	ND	ND

COX = cytochrome c oxidase, ND = not done. <sup>x</sup> The concentration of TMPD was 60  $\mu\text{mol/L}$  before 1987 and was thereafter raised to 500  $\mu\text{mol/L}$ . <sup>\*</sup> Analysis performed on autopsy tissue. # Siblings

the activity of complexes II – III was normal in five and decreased to 44 – 94% in seven of the cases.

## Discussion

In childhood, COX deficiency has been reported in a wide range of clinical conditions (Table 1). The finding of increased lactate levels in body fluids is a valuable support for the diagnosis and could guide further investigations. Indeed, blood or spinal lactate levels were increased in all of the cases in this study. A precise diagnosis, based on biochemical investigations, has become more and more important with the increasing number of known genetic defects underlying these conditions, thereby making genetic counselling possible.

Leigh syndrome was found in four of the cases. This distinctive neuropathological entity shows focal, symmetrical necrotic lesions, extending from the thalamus to the pons, the inferior olives, and the posterior columns of the spinal cord [28]. Characteristic features on neuroimaging are either symmetrical hypodensities in the basal ganglia on computed tomography (CT) or areas of increased signals in the basal ganglia on T<sub>2</sub>-weighted magnetic resonance imaging (MRI) [8]. Common clinical manifestations include psychomotor regression, brain stem dysfunction, ataxia, dystonia, and optic atrophy [45]. The *SURF1* gene, which is located on chromosome 9q34 and encodes a putative assembly or maintenance factor, is often involved in typical cases of LS with COX-deficiency [57,76,83] not in lit]. The 5537–

5538 insT mutation in the tRNA<sup>trp</sup> gene has previously been described in one case with LS [52]. In our study, the three identified cases with *SURF1* mutations showed similar clinical presentations without obvious cortical involvement. They differed clearly from the patient with LS due to a 5537–5538 insT mutation in the tRNA<sup>trp</sup> gene, who had signs of severe cortical involvement with severe mental retardation, spastic tetraparesis, epilepsy, and cortical blindness.

MtDNA depletion was diagnosed in two of our cases (cases 15 and 16). Both had onset in late infancy, a progressive fatal course, and myopathy with, in addition, involvement of heart and liver in one of the cases. Case 16 with myopathy had two new compound heterozygous mutations in the *TK2* gene (to be published). MtDNA depletion may be an under-diagnosed group of disorders in childhood [31]. Biochemistry often shows COX-deficiency, sometimes associated with decreased activities of other respiratory chain complexes [75]. A broad suspicion is warranted since these disorders may present prenatally with dysmorphic features [31] and with non-specific symptoms in infancy such as vomiting, failure to thrive, and developmental delay before multorgan involvement occurs [64].

*SCO1* and *SCO2* are believed to be involved in copper delivery for the copper centers of COX [14]. Mutations in the *SCO2* gene, located on chromosome 22q13, were initially identified in 5 unrelated infants with fatal cardioencephalopathy [21,42]. Recently however, three additional infants have been identified with a different phenotype of delayed infantile onset Leigh-like syndrome,

neurogenic muscular atrophy, and hypertrophic cardiomyopathy [20]. Compared to mutations in the *SURF1* gene, *SCO2* mutations seem to be a more rare cause of COX-deficiency, judged by the few cases reported in the literature, and we did not identify mutations in any of our cases. Mutations in the *SCO1* gene, localized on chromosome 17p13.1, have only been found in two siblings with neonatal-onset hepatic failure and encephalopathy [68], and were not analysed in our cohort of cases.

The *COX10* gene, located on chromosome 17p13.1-q11.1, encodes heme A:farnesyltransferase, which is required for the biogenesis of functional COX in yeast [40]. A mutation in this gene has previously been identified in three consanguineous siblings with mitochondrial encephalopathy and tubulopathy [69]. The *COX17* gene, located on chromosome 3q, encodes a cytoplasmic protein, which is involved in copper transport to the mitochondria [13] and has thus far not been associated with disease in human. Our negative screening for mutations in the *COX10* and *COX17* genes, together with the results from others [18,50], indicate that neither *COX10* nor *COX17* are common causes of COX-deficiency disorders.

Reported cases due to mutations in the mtDNA genes encoding COX subunits show a considerable phenotypic variation, ranging from fatal lactic acidosis in the neonatal period [74], preschool onset multisystem disease [6], Leigh-like [61], or MELAS-like [34] syndromes to proximal myopathy [46] or recurrent myoglobinuria in adolescence [24]. They seem to be uncommon in childhood, as suggested by our negative results and previous negative screening efforts [42,50].

We conclude that COX deficiency in childhood constitutes a broad group of disorders with very variable phenotype, which should be looked for in a wide range of clinical settings. When the diagnosis is established, even large-scale genetic screening efforts such as in this study, often fail to yield a molecular diagnosis. Accordingly, we were not able to identify the underlying gene defects in any of our cases characterized by infantile myopathy with COX-deficiency and congenital lactic acidosis or leukoencephalopathy. Thus, although an increasing number of genetic defects resulting in COX deficiency have been characterized, the molecular basis of a majority of the cases remains unidentified, a obvious incentive for searching other disease-causing genes.

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