

The myopathic form of coenzyme Q10 deficiency is caused by mutations in the electron-transferring-flavoprotein dehydrogenase (*ETFDH*) gene

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Coenzyme Q10 (CoQ10) deficiency is an autosomal recessive disorder with heterogenous phenotypic manifestations and genetic background. We describe seven patients from five independent families with an isolated myopathic phenotype of CoQ10 deficiency. The clinical, histological and biochemical presentation of our patients was very homogenous. All patients presented with exercise intolerance, fatigue, proximal myopathy and high serum CK. Muscle histology showed lipid accumulation and subtle signs of mitochondrial myopathy. Biochemical measurement of muscle homogenates showed severely decreased activities of respiratory chain complexes I and II + III, while complex IV (COX) was moderately decreased. CoQ10 was significantly decreased in the skeletal muscle of all patients. Tandem mass spectrometry detected multiple acyl-CoA deficiency, leading to the analysis of the electron-transferring-flavoprotein dehydrogenase (*ETFDH*) gene, previously shown to result in another metabolic disorder, glutaric aciduria type II (*GAI*). All of our patients carried autosomal recessive mutations in *ETFDH*, suggesting that *ETFDH* deficiency leads to a secondary CoQ10 deficiency. Our results indicate that the late-onset form of *GAI* and the myopathic form of CoQ10 deficiency are allelic diseases. Since this condition is treatable, correct diagnosis is of the utmost importance and should be considered both in children and in adults. We suggest to give patients both CoQ10 and riboflavin supplementation, especially for long-term treatment.

Keywords: coenzyme Q10 myopathy; *ETFDH* mutations; riboflavin and CoQ10 supplementation; late-onset glutaric aciduria type II

Abbreviations: TMS = tandem mass spectrometry

Received November 21, 2006. Revised February 16, 2007. Accepted February 26, 2007. Advance Access publication April 5, 2007

Introduction

Ubiquinone (coenzyme Q10 or CoQ10) is a lipid-soluble component of the cell membranes, where it functions as a mobile electron and proton carrier. Primary coenzyme Q10 (CoQ10) deficiency is thought to be an autosomal recessive disorder with five major phenotypes described: (1)

an encephalomyopathic form with exercise intolerance, mitochondrial myopathy, myoglobinuria, seizures and ataxia (Ogasahara *et al.*, 1989; Sobreira *et al.*, 1997); (2) a multisystem infantile variant with encephalopathy, cardiomyopathy, ataxia, optic nerve atrophy, deafness and

nephrotic syndrome resulting in renal failure (Rotig *et al.*, 2000); (3) a predominantly cerebellar form with ataxia and cerebellar atrophy (Lamperti *et al.*, 2003); (4) Leigh syndrome with growth retardation, ataxia and deafness (Maldergem *et al.*, 2002) and (5) an isolated myopathic phenotype (Lalani *et al.*, 2005; Horvath *et al.*, 2006).

The concept that this clinical heterogeneity may reflect blocks at different levels in the complex biosynthetic pathway of CoQ10 is supported by the increasing number of molecular defects that are being identified in different clinical variants. Thus, two siblings of consanguineous parents with the multisystem infantile form of CoQ10 deficiency had autosomal recessive mutations in the *COQ2* gene (Quinzii *et al.*, 2006), which encodes para-hydroxybenzoate-polyprenyl transferase, whereas an infant with Leigh syndrome and nephrosis had autosomal recessive mutations in the *PDSS2* gene, which encodes a subunit of *COQ1*, the first enzyme of CoQ10 biosynthesis (López *et al.*, 2006). These defects were detected in single families, and the frequencies of mutations in *COQ1* and *COQ2* are still unknown. On the other hand, severe CoQ10 deficiency could be secondary to other genetic defects, as shown by a family with ataxia and CoQ10 deficiency harbouring mutations in the aprataxin gene (*APTX*) previously associated with ataxia oculomotor apraxia syndrome (AOA) [MIM606350] (Quinzii *et al.*, 2005).

We have reported three patients with pure myopathy and CoQ10 deficiency in muscle manifesting as proximal myopathy with exercise intolerance and increased serum CK and lactate levels (Horvath *et al.*, 2006). In two of the three patients, tandem mass spectrometry (TMS) showed increased levels of short-, medium- and long-chain acyl-carnitines, as described in multiple acyl-coenzyme-A deficiency (glutaric aciduria type II, GA II). These findings prompted us to search for mutations in the nuclear genes that cause multiple acyl-CoA deficiency.

Here, we describe pathogenic mutations in the electron-transferring-flavoprotein dehydrogenase (*ETFHDH*) gene in seven patients from five independent families fulfilling the clinical, histological and biochemical criteria of the myopathic form of coenzyme Q10 deficiency. Our results indicate that the late-onset form of GAI1 and the myopathic form of CoQ10 deficiency may be allelic diseases, which has important implications for therapy.

Patients and methods

Patients

Patients 1 and 2 were reported previously (Horvath *et al.*, 2006). Briefly, patient 1 is a 34-year-old German woman with no family history of neuromuscular disease, in whom insulin-dependent diabetes mellitus was diagnosed at 14 years of age. Proximal muscle weakness started at age 32 during her first pregnancy and progressed rapidly. Serum CK was 2.000 U/l (normal <180) and increased further (up to 5000 U/l) after high doses of

oral carnitine (3 g/day), lactate was 2.7 mmol/l (normal <2.0). Oral supplementation with high dose (500 mg/day) CoQ10 resulted in subjective improvement within a few days. After 1 month of supplementation, CK levels returned to normal and the patient reported a dramatic improvement of muscle strength, which was confirmed by manual and quantitative testing. After 6 months of supplementation, she was able to take care of her baby and all household chores. However, after 8 months of CoQ10 intake, the muscle symptoms reappeared, serum CK (2446 U/l) and lactate were again increased, and there was also an unexplained increase of the liver enzymes (GOT 259 U/l, normal <32; GPT 226 U/l, normal <35; LDH 2175 U/l, normal <230). Serum TMS examinations showed multiple acyl-CoA derivatives and low carnitine. A repeated muscle biopsy was performed. Carnitine supplementation and a low-protein low-fat diet were ineffective, and associated with further worsening of symptoms. After sequencing the three genes responsible for GAI1, we combined CoQ10 supplementation with riboflavin (100 mg/day), with marked and rapid improvement of her symptoms for the next 3 months. The elevation of LDH, CK and liver enzymes decreased to normal values and she was able to work and to take care of her 2-year-old daughter. CoQ10 supplementation was stopped and she was maintained on riboflavin therapy, which resulted in another deterioration of her symptoms within 3 weeks and prompted us to continue combined supplementation with CoQ10 and riboflavin (Table 1). Currently (12 months after the initiation of the combined CoQ10 and riboflavin treatment), she only has slight residual neck flexor weakness, and CK-levels are normal.

Patient 2 is a 29-year-old man of Turkish origin. His parents are consanguineous but there is no family history of similar disorders. Six months prior to examination, he developed premature fatigue, difficulty walking and weakness of shoulder, hip and neck muscles. Neurological examination showed bilateral scapular winging, waddling gait and proximal muscle weakness with decreased tendon reflexes. EMG showed myopathic alterations in proximal muscles. Nerve conduction velocities and brain MRI were normal. Serum CK was elevated up to 1000 U/l (normal <180), liver enzymes were normal and serum lactate was normal at rest but increased excessively after exercise (more than 10-fold). Oral supplementation with CoQ10 (150 mg/day) resulted in subjective improvement within 2 months. After documenting muscle CoQ10 deficiency, the dose of CoQ10 was increased to 500 mg/day. Within 3 months, CK levels normalized and the patient resumed work. After 1 year on CoQ10 alone, he is symptom-free, only his CK is mildly increased (320 U/l, normal <155). However, TMS examination showed accumulation of multiple acyl-CoA derivatives.

Patient 3, a 13-year-old boy, is the third of five children in a consanguineous Kurdish family. His parents and three of four siblings (age 19, 11 and 9 years) are healthy, while a 17-year-old sister is similarly affected (patient 4). Onset in the sister was at 14 years, with exercise intolerance, premature fatigue and proximal weakness. Serum CK was elevated at 1484 U/l (normal <180). There was no history of myoglobinuria. Her proximal muscle strength was 3+/5 according to the MRC scale. There was no muscle hypertrophy. Our working diagnosis was either a limb-girdle dystrophy or a metabolic muscle disease. After reviewing the muscle biopsy from her affected brother (see later), supplementation with CoQ10 was initiated. This resulted in full recovery within 3 weeks. At the 4-month follow-up visit,

Table 1 Follow-up TMS spectra of serum acylcarnitines in patient 1

Follow-up	Before diagnosis	CoQ10 1000 mg/day	CoQ10 + low-protein, low-fat diet	CoQ10 + riboflavin without l-carnitine
Clinical symptoms	Subacute muscle Weakness, fatigue	Marked improvement	Weakness, ↑ liver Enzymes, re-biopsy	Complete recovery
Serum CK U/l normal <180	Up to 2000	Normal	2446	Normal
C0 x-fold lower limit	Normal	Normal	0.5	0.2
C4 x-fold upper limit	3.8	Normal	1.9	Normal
C5 x-fold upper limit	16.5	1.4	3.6	Normal
C8 x-fold upper limit	5.0	6.5	2.8	Normal
C10 x-fold upper limit	5.8	5.8	3.6	Normal
C14 : I x-fold upper limit	8.9	1.8	6.4	Normal

Note: The combined elevation of short (C4, C5), medium (C8, C10) and long-chain (C14 : I) acylcarnitines is characteristic of GA-II. Free carnitine (C0) was repeatedly diminished reflecting excretion of acylcarnitines in the urine. Reference ranges for free carnitine (C0) and acylcarnitines ($\mu\text{mol/l}$): C0 30–63; C4 < 0.6; C5 < 0.2; C8 < 0.3; C10 < 0.5; C14:I < 0.3).

her CK was 323 U/l. However, 9 months later her symptoms reappeared in the form of muscle cramps and weakness. At the time, her CK was significantly elevated (4824 U/l). No hepatopathy was observed. Interestingly, addition of riboflavin (100 mg/day) resulted in full recovery within a few days, with serum CK dropping to 141 U/l. Since then, several months have elapsed and she is totally symptom free. Her 13-year-old brother (patient 3) had just emerging symptoms. He recently began to experience muscle cramps and premature fatigue. No muscle weakness was detected. His CK was 2745 U/l. A muscle biopsy was performed from the vastus lateralis muscle. He was put on CoQ10, 200 mg/day, and his muscle complaints have ceased. However, 6 months after normalization of CK, a repeat test showed a CK of 373 U/l. In view of his sister's response, he was also initiated on riboflavin 100 mg/day.

Patient 5 is a 12-year-old girl, the first child of a consanguineous Turkish family. Just 1 month prior to submission, she had subacute onset of muscle weakness and pain resembling polymyositis. She was weak and had a Gowers sign. Serum CK was 672 U/l at onset, and rose to 4188 U/l (normal <180), while liver enzymes were normal. EMG was normal. A metabolic myopathy was suspected and a muscle biopsy was performed. TMS examination showed accumulation of multiple acyl-CoA derivatives. Riboflavin, 100 mg/day, was introduced. This resulted in complete cessation of the muscle symptoms. One younger sister was found to be similarly affected (patient 6).

Patient 7 is a 13-year-old girl of a consanguineous Turkish family. She presented at age 12 with subacute onset of muscle weakness, myalgia and loss of ambulation within 20 days. Her serum CK was 538 U/l (normal <180), and liver enzymes were normal. A muscle biopsy was performed. TMS examination showed accumulation of multiple acyl-CoA derivatives. Riboflavin (100 mg/day) led to improvement over a few days and she started to walk again. At a 6-month follow-up visit she was doing fine. Several weeks later, her symptoms re-appeared when she incidentally stopped riboflavin. With re-initiation of riboflavin, she was back to normal. Two of her siblings had expired at ages 6 and 9 months with recurrent vomiting of unknown origin.

Muscle histology, biochemistry, CoQ10 measurement and tandem mass spectrometry (TMS)

Six micrometre thick serial cross-sections of muscle biopsy specimens were obtained for histochemical stains according to

standard procedures. A frozen portion of the biopsy was used for biochemistry. Respiratory chain (RC) complexes I–IV activities were determined in skeletal muscle homogenate spectrophotometrically (Cary 50, VARIAN) as described (Fischer *et al.*, 1986). In brief, frozen muscle specimen was first homogenized in SETH buffer (250 mM saccharose, 2 mM EDTA, 10 mM Tris-HCl pH 7.4, 50 U/ml heparin). For measuring NADH oxidation (complex I), we added 4 μg antimycin A to 480 μl test mix [28 mM KPO_4 , 2.825 mg/ml BSA, 0.56 mM MgCl_2 , 0.0225 mM NADH (Roche)] and after 10 min preincubation at 30°C, 15 μl decylubiquinone (1 mg/ml, Sigma) was added. Six minutes after, 20 μl muscle homogenate was added and the measurement was run for 2–5 min (till linearity). After adding 4 μg rotenone (Sigma), the estimated activity was corrected for rotenone-insensitive NADH activity. Succinate cytochrome *c* oxidoreductase (complexes II + III) activity was estimated spectrophotometrically after activation of the succinate dehydrogenase complex by preincubation for 10 min at 30°C of 460 μl of a test mix [20 mM KPO_4 (pH 7.5), 2 mM EDTA, 2 mM NaN_3 , 0.7 $\mu\text{g}/\mu\text{l}$ succinate, 0.004 $\mu\text{g}/\mu\text{l}$ rotenon] and 8.4 mg cytochrome *c* (Sigma). The activity was estimated with and without 5 min preincubation with 125 μM CoQ1 on ice at 550 nm (cytochrome *c*).

In muscle homogenates, CoQ10 was measured by an HPLC method with UV detection (275 nm), using the Coenzyme Q10 Kit (Chromsystems). The control range was determined on muscle biopsies of different age groups, without histological and biochemical evidence of a respiratory chain disease ($n=25$). As disease controls, we measured CoQ10 in muscle of patients with primary respiratory chain disease and with fatty acid oxidation deficiency ($n=16$). TMS was performed as described (Gempel *et al.*, 2002).

Molecular genetic analysis

Genomic sequence of the *ETFDH* genes (Goodman *et al.*, 2002) was performed in all five index patients, *ETFA* and *ETFB* genes were sequenced in two additional patients as described elsewhere (Goodman *et al.*, 2002). RFLP analysis for the codon 1130 T>C (L377P) mutation in families of patients 2, 3, 4, 5 and 6 was performed by amplifying exon 10 of *ETFDH* with a forward mismatch primer, 5'-TGT-TTC-CTC-AGT-CTA-TAC-CAA-GA-3' and with the intronic exon 10 reverse primer (Goodman *et al.*, 2002). The PCR product was digested with the *MlyI* restriction

enzyme, which cuts the 227 bp wild-type product into two bands (204 and 23 bp) and mutant remains uncut.

Results

Muscle histology, biochemistry, CoQ10 measurement and TMS

Muscle histology in all five index cases revealed similar findings: moderate to severe myopathy with small vacuoles in most type 1 fibres (Fig. 1A, E, H and K). However, the vacuolar change was most prominent in patient 5 (Fig. 1K). Sudan black/oil-red-O stains showed excessive lipid droplets, predominantly in type 1 fibres (Fig. 1D, G, J and L). A few COX negative fibres were detected in all cases (Fig. 1C and F), but ragged red fibres were seen only in patients 1 and 2 (Fig. 1I). SDH stain was faint in two cases (patients 5 and 7, data not shown).

Biochemically, the activities of RC complexes I and II+III and COX were decreased in all patients, especially when referred to the elevated mitochondrial marker enzyme citrate synthase (CS). Muscle CoQ10 was decreased in all five cases. The biochemical results are summarized in Table 2. Because of clinical progression on long-term CoQ10 monotherapy, patient 1 underwent a second muscle biopsy. Muscle histology was similar to the first biopsy; however, biochemical measurements of respiratory chain enzymes and CoQ10 were within normal range.

To document that CoQ10 deficiency was reasonably specific in our patients and not a general finding in mitochondrial myopathies, we measured CoQ10 in 16 patients with different primary respiratory chain or fatty acid oxidation defects (single mtDNA deletions, $n=3$; multiple mtDNA deletions, $n=2$; A3243G MELAS mutation, $n=2$; novel mtDNA tRNA^{Glu} mutation, $n=1$; complex III deficiency caused by CYTB mutation, $n=1$; complex IV deficiency caused by SURF1 mutation, $n=1$; autosomal dominant POLG1 mutation, $n=1$; isolated complex III deficiency, $n=1$; CPTII deficiency, $n=1$; lipid storage myopathy of unknown origin, $n=1$; myoglobinuria of unknown origin, $n=1$; ataxia of unknown cause, $n=1$). Notably, most of these patients had severe isolated or combined respiratory chain defects. No significant decrease in CoQ10 levels was observed in any of them, CoQ10 was slightly below the normal range (70% of mean control) only in the patient with CPTII deficiency. TMS was performed in four of our five index patients and showed a combined elevation of short (C4, C5), medium (C8, C10) and long-chain (C14:1) acylcarnitines (Table 1). This TMS result is characteristic of GAIL. Free carnitine (C0) was repeatedly diminished reflecting excretion of acylcarnitines in the urine (Table 1).

Molecular genetic analysis

Sequence analysis of *ETFDH* in patient 1 revealed two heterozygous missense mutations, c.1367 C>T (P456L)

and c.1768 A>G (K590E), in compound heterozygosity. The mutation c.1367 C>T (P456L) had been previously described in late-onset GAIL patients (Goodman *et al.*, 2002; Beresford *et al.*, 2006). The mutation c.1768 A>G (K590E) affects a highly conserved amino acid residue (Fig. 2) in the C-terminal part of the protein. Each parent was heterozygous for one of the mutations. Patients 2, 3, 4, 5 and 6 harboured the same homozygous missense mutation, c.1130 T>C (L377P). As all these patients belonged to three families of Turkish or Kurdish origin, this implies that L377P is more frequent in this region. This mutation was not detected in 50 Turkish normal control samples and it co-segregated with the disease phenotype within the families (Fig. 3). One healthy sibling of patient 3 also carried the mutation in homozygous form, but this child was below the age of onset in his siblings. This mutation had not been described previously, but it affects a conserved amino acid (Fig. 2) in the carboxyl-terminal domain of the protein. Patient 7 harboured a novel homozygous missense mutation, c.1448 C>T (P483L) in the carboxyl-terminal domain, which had not been detected, previously. This mutation affects a conserved amino acid (Fig. 2) and is in close proximity to a previously described mutation (P456L). Mutations in this part of the protein affect most likely the catalytic activity and the stability of the tetramer (Westover *et al.*, 2003).

Discussion

We present seven patients from five independent families with isolated myopathy, severe CoQ10 deficiency, and mutations in the *ETFDH* gene, previously associated with GAIL. Our data indicate that *ETFDH* is the fourth gene associated with CoQ10 deficiency. Mutations in two genes involved in CoQ10 biosynthesis cause primary CoQ10 deficiency with infantile mitochondrial encephalomyopathy. Mutations in the aprataxin gene cause secondary CoQ10 deficiency associated with ataxia oculomotor apraxia (AOA1) and mutations in the *ETFDH* gene cause secondary CoQ10 deficiency associated with isolated myopathy.

Multiple acyl-CoA dehydrogenase deficiency or glutaric aciduria type II (GAIL) is an autosomal recessive disorder of fatty acid and amino acid metabolism, due to a defect of electron transport from FAD-containing CoA dehydrogenases to CoQ in the mitochondrial electron transport chain (Przyrembel *et al.*, 1976). The heterogeneous clinical features of patients with GAIL fall into three main variants (Olsen *et al.*, 2003). A neonatal-onset form (GAIL type I) is characterized by congenital anomalies, severe non-ketotic hypoglycaemia, metabolic acidosis and excretion of large amounts of fatty acid- and amino acid-derived metabolites. A second neonatal-onset form (GAIL type II) does not have congenital anomalies. A late-onset form (GAIL type III) is characterized by hepatomegaly, lipid storage myopathy and recurrent episodes of lethargy, vomiting, hypoglycaemia and metabolic acidosis often triggered by metabolic stress.

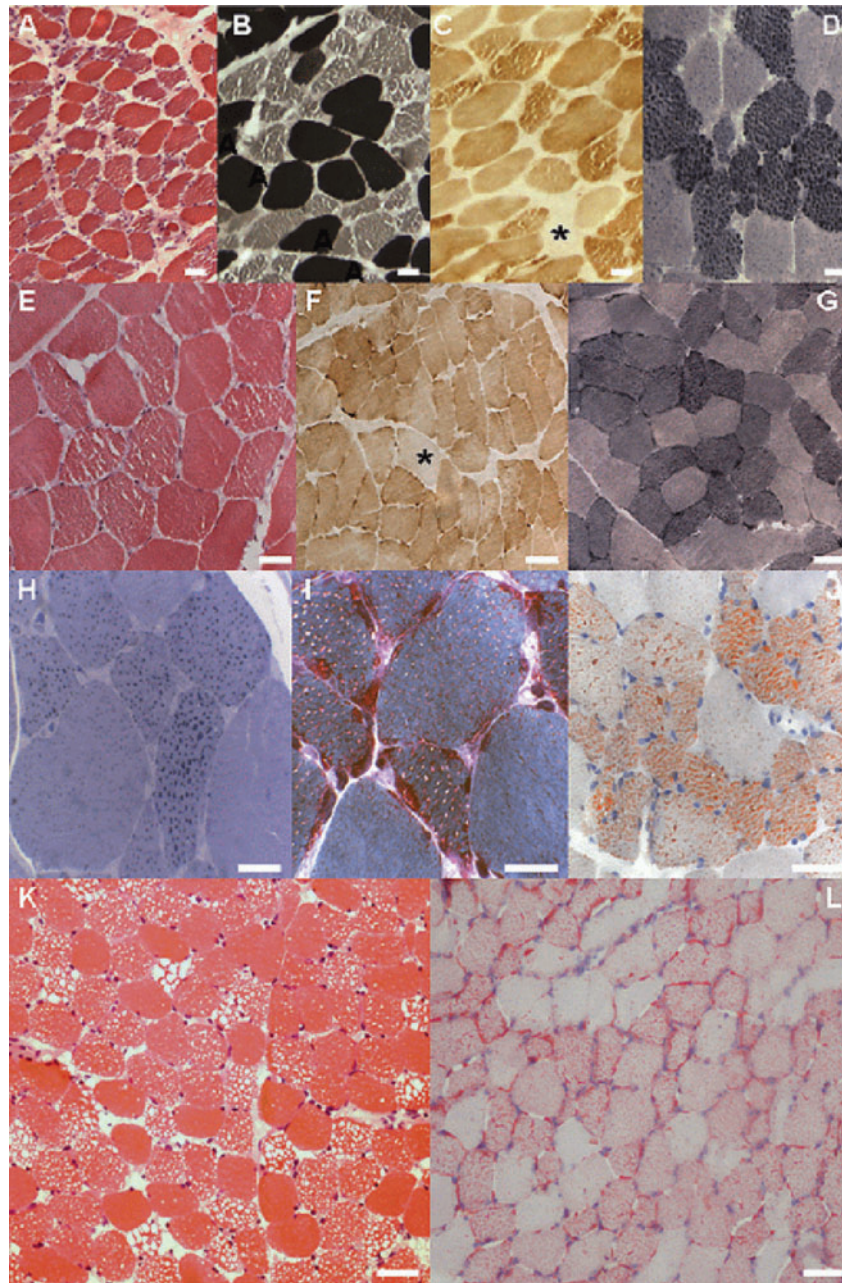


Fig. 1 Histological findings of patient 1 (A–G), patient 2 (H–J), patient 3 (L) and patient 5 (K). Muscle biopsies of all patients show moderate to severe vacuolar myopathy (H&E stain: A, E, K; semithin section, toluidin blue: H); the vacuolar change was most prominent in type I fibers (ATPase pH 9.4: B). There are few COX negative fibres (asterix in C, F). Sudan black (D, G) or Oil-Red-O (J, L) stain shows lipid accumulation in type I fibres of all patients. Ragged red fibres were seen rarely (Gomori trichrome stain: I). There is no significant difference between the first and second biopsy specimens of patient 1 (A–D; E–G) Bars in A–L adjusted to 30 μ m.

Patients complain of muscle pain, exercise intolerance and proximal weakness, but glutaric aciduria is usually intermittent and coincides with worsening of the symptoms. All three clinical variants are caused by mutations in one of the three genes, two of which encode the alpha- and beta-subunits of the electron transfer flavoprotein (*ETFA*, OMIM : 608053; *ETFB* OMIM : 130410), while the third encodes ETF ubiquinone oxidoreductase (*ETFDH* OMIM 231675). All our patients harboured missense mutations in *ETFDH* and affected the C-terminal of the protein.

Mutations in this protein domain most likely impair the catalytic activity and the folding/stability of the protein (Olsen *et al.*, 2003; Westover *et al.*, 2003). Nonsense mutations and missense mutations in the N-terminal part of the protein usually lead to the more severe neonatal GAIL type I or type II. However, some cases of GAIL are due to as yet unidentified disturbances of riboflavin metabolism. Riboflavin (vitamin B2) is the co-factor shared by *ETF*, *ETFDH* and all acyl-CoA dehydrogenases. Therapy with riboflavin, carnitine and low-fat, low-protein diet is

Table 2 Summary of the clinical, histological, biochemical and genetic data of our five patients carrying mutations in *ETFDH*

Patient	Family history	Disease onset(years)	Clinical signs	Muscle histology	RC I (normal 0.17–0.56)	RC II+III (normal 0.08–0.45)	RC IV (normal 1.1–5.0)	CS (normal 45–100)	CoQ10 nmol/UCS (normal 2.7–70)	<i>ETFDH</i> mutations
1 F	Negative	32	Proximal myopathy, exercise intolerance, hepatopathy, CK up to 2000 U/L	Lipid-accumulation RRF	0.05	0.03	0.7	173	0.6	het P456L het K590E
2 M	Consanguinous parents	29	Proximal muscle weakness, scapular winging, myalgia, CK up to 1000 U/L	Lipid-accumulation RRF	0.09	0.04	0.9	172	0.8	hom L377P
3 M	Consanguinous parents, one affected sister	12	Exercise intolerance, fatigue, CK 2745 U/L	Lipid-accumulation COX-ve fibers	0.00	0.01	0.1	149	0.8	hom L377P
5 M	Consanguinous parents, one affected sister	12	Subacute muscle weakness, exercise intolerance, myalgia, CK up to 4188 U/L	Lipid-accumulation	0.02	0.02	0.7	173	1.28	hom L377P
7 F	Consanguinous parents	12	Proximal muscle weakness, myalgia, CK 538 U/L	Lipid-accumulation	0.00	0.01	0.4	193	0.87	hom P483L

Note: RC complex activities are normalized to citrate synthase (CS); for the RC complexes and for CS: 1 unit means 1 $\mu\text{mol}/\text{min}$; for the coenzyme Q10 from control measurements ($n = 25$) median value was 4.7, CK normal <180 U/L.

beneficial, although the long-term treatment of patients with late-onset GAI is still challenging (Olsen *et al.*, 2003).

The discovery of pathogenic *ETFDH* mutations in our patients with myopathic CoQ10 deficiency shows that late-onset GAI, usually caused by less severe mutations in the *ETFDH* gene, probably are one and the same disease, although the severe defect of CoQ10 in muscle was initially considered a primary aetiological event. This concept provides important clues to pathophysiology and useful tools for therapy.

How can *ETFDH* deficiency result in CoQ10 deficiency? As CoQ10 is the direct acceptor of electrons from ETF, it stands to reason that lack (or dysfunction) of the reducing enzyme could—via some feedback mechanism—downregulate the synthesis of CoQ10. Alternatively, faulty binding of the enzyme to CoQ10 could result in excessive degradation of the acceptor molecule (Olsen *et al.*, 2003). CoQ10 had not been measured in previous patients with GAI, although it was repeatedly reported that respiratory chain complexes were decreased (Beresford *et al.*, 2006). Respiratory chain dysfunction and clinical presentation suggest that the physiopathology of GAI may well be related to CoQ10 deficiency. Regarding the crucial point of ‘primary’ versus ‘secondary’ CoQ10 deficiency, our position is that the term ‘primary CoQ10 deficiency’ should be restricted to defects of CoQ10 biosynthesis, such as those due to *COQ1* or *COQ2* mutations, whereas defects of CoQ10 due to mutations in aprataxin or *ETFDH* are examples of secondary CoQ10 deficiency.

Not surprisingly, the clinical presentation of late-onset GAI patients (de Visser *et al.*, 1986; Di Donato *et al.*, 1986; Bell *et al.*, 1990; Olsen *et al.*, 2004) is very similar to that of our cases, with subacute (3–6 months) exercise intolerance and proximal weakness affecting predominantly hip and shoulder girdle muscles. Muscle weakness fluctuates and often worsens during intermittent infections, fasting, catabolic stress or pregnancy. Neck flexor weakness is relatively typical and was prominent in all patients described here. Episodes of hepatopathy, vomiting and somnolence or stupor (Reyès syndrome-like crises) were common in previously described patients with GAI (de Visser *et al.*, 1986; Di Donato *et al.*, 1986; Bell *et al.*, 1990; Olsen *et al.*, 2004), but muscle weakness and exercise intolerance usually preceded these events. Some patients also had respiratory failure requiring assisted ventilation. Of our patients, only one (patient 1) had an episode of weakness accompanied by LDH and liver transaminase elevation, which resolved over several weeks, whereas the other six had isolated myopathy, with no evidence of hepatopathy or encephalopathy, and none of our patients showed involvement of the respiratory muscles. The lack of extramuscular symptoms explains why initially we did not suspect GAI. Conversely, our cases show that GAI may present as a pure myopathy without clinical signs of a systemic metabolic disease.

	L377P	P456L	P483L	K590E
homo sapiens	SIP KLTFPGG	RNIRPSCHGV	RGMEPWTLKH	CVHCKTCDIK
bos taurus	CIP KLTFPGG	RNIRPSCHSI	RGMEPWTLKH	CVHCKTCDIK
canis familiaris	SIP KLTFPGG	RNIRPSCHGI	RGMEPWTLKH	CVHCKTCDIK
mus musculus	SIP KLTFPGG	RNIRPSCHGI	RGMEPWTLKH	CVHCKTCDIK
xenopus	SLP KLTFPGG	RNIRPSCHGP	RGKEPWTLKH	CVHCKTCDIK
drosophila	SLPQKLSFPGG	RNVHPSFHNP	GGREPWTLLKH	CI HCKTCDIK
saccharomyces c.	SVP KLNFPGG	RNIRPSFSGK	KGKVPWTLKF	CI HCKTCDIK

Fig. 2 Conservation of the *ETFDH* mutations L377P, P456L, P483L and K590E.

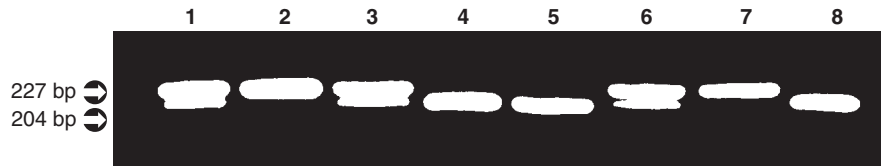


Fig. 3 RFLP analysis for the mutation L377P in the family of patient 2. Presence of a homozygous state in muscle DNA (lane 2) and blood DNA (lane 7) of the patient. The mutation is heterozygous in both parents (mother—lane 1; father—lane 3) and in one healthy sibling (lane 6), and absent in another healthy sibling (lane 4) and in the patient's wife (lane 5) as well as in a normal control (lane 8).

In the late-onset form of GAI and in previous cases with the myopathic variant of CoQ10 deficiency, onset of symptoms was before age 15 years. Thus, it is noteworthy that two of our patients were 32 and 29 years old at presentation, implying that this diagnosis should be considered even in adult-onset cases.

Muscle histology in the five patients who were biopsied showed lipid storage myopathy with subtle signs of mitochondrial dysfunction, and biochemical measurement of respiratory chain enzymes showed reduced activities of complexes I and II + III and increased activity of citrate synthase. Thus, lipid storage myopathy and respiratory chain dysfunction are hallmarks of the disease. TMS suggested multiple acyl-CoA dehydrogenase deficiency in the four patients in whom it was performed.

In patient 1, the TMS profile and the low level of free carnitine in serum suggested a block in mitochondrial fatty acid oxidation and led to the genetic diagnosis of GAI.

The therapy and follow-up of our patients led us to important conclusions. After 3–6 months of CoQ10 supplementation, all patients showed dramatic clinical improvement and normalization of serum CK and lactate levels. This was also true in all other reported cases with the myopathic phenotype (Lalani *et al.*, 2005; Horvath *et al.*, 2006). As our initial diagnosis was primary myopathic CoQ10 deficiency, in four of our patients we initiated high-dose CoQ10, which resulted in prominent clinical and biochemical improvement. After diagnosing GAI, we added to the therapy of patient 1 a low-protein low-fat diet: far from improving, after 3 weeks on the diet, she became weaker, was unable to walk more than 100 m, had frequent vomiting and liver enzymes increased. After stopping the diet and introducing riboflavin supplementation (100 mg/day) she recovered within a few days. After 3 months of combined CoQ10 and riboflavin therapy, she was completely normal. Because of the good condition

and cooperation of the patient, we stopped CoQ10 supplementation and continued with riboflavin monotherapy, but after 3 weeks the reappearance of proximal muscle weakness prompted us to continue with combined riboflavin and CoQ10 supplementation. It seems that patients with *ETFDH* deficiency in long-term need both CoQ10 and riboflavin to maintain a good muscle function. Because of the additional carnitine deficiency, carnitine supplementation was repeatedly tried, but never resulted in improvement, rather worsening of symptoms.

For cases 5 and 7, riboflavin was given alone as a single agent just based on the pattern of TMS screening which originally denoted a GAI pattern. This scheme really worked well, and they currently are not in need of CoQ10. Of course, longer follow-up is required.

We sequenced *ETFDH* in 10 other patients with CoQ10 deficiency. Eight of these patients presented with ataxia and epilepsy and only two showed myopathy. One of them was a 7-year-old boy with normal TMS result (patient 3 in Horvath *et al.*, 2006), and the other was a 35-year-old woman with delayed motor milestones, partial complex seizures, exercise intolerance and recurrent myoglobinuria (Sobreira *et al.*, 1997). Mutations of the *ETFDH* gene were not detected in any of these cases, suggesting further genetic heterogeneity.

Since CoQ10 deficiency/late-onset GAI is treatable, correct diagnosis is of utmost importance and should be considered both in children and in adults. The association of high-serum CK, proximal myopathy (with or without hepatopathy or encephalopathy), multiple acyl-CoA deficiency on TMS, lipid storage myopathy and decreased activity of respiratory chain complexes I and II + III (and IV) attributable to lack of CoQ10 in muscle homogenate are key tell-tale signs of the disease. We would suggest that patients should be kept on both CoQ10 and riboflavin supplementation, especially on the protracted course.

Acknowledgements

The authors thank Ira Kaus, Manja Thorwirt, Andrea Zöllner and Eva Schmidtmeyer for technical assistance. KG is supported by a grant from the Stiftung Pathobiochemie der Deutschen Gesellschaft für Klinische Chemie und Labormedizin (DGKL). BGS, PS and HL are members of the German network on muscular dystrophies (MD-NET, 01GM0302) funded by the German ministry of education and research (BMBF, Bonn, Germany). MD-NET is a partner of TREAT-NMD (EC, 6th FP, proposal # 036825; www.treat-nmd.eu). SDM is supported by a grant from the Muscular Dystrophy Association. HP is supported by the German National Genome Network (BMBF O1GR0411). RH is supported by a grant from the Deutsche Forschungsgemeinschaft (HO 2505/2-1).

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