doi:10.1093/brain/awm135 Brain (2007), 130, 2045–2054

# *ETFDH* mutations as a major cause of riboflavin-responsive multiple acyl-CoA dehydrogenation deficiency

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**Multiple acyl-CoA dehydrogenation deficiency (MADD) is a disorder of fatty acid, amino acid and choline metabolism that can result from defects in two flavoproteins, electron transfer flavoprotein (ETF) or ETF: ubiquinone oxidoreductase (ETF:QO). Some patients respond to pharmacological doses of riboflavin. It is unknown whether these patients have defects in the flavoproteins themselves or defects in the formation of the cofactor, FAD, from riboflavin. We report 15 patients from 11 pedigrees. All the index cases presented with encephalopathy or muscle weakness or a combination of these symptoms; several had previously suffered cyclical vomiting. Urine organic acid and plasma acyl-carnitine profiles indicated MADD. Clinical and biochemical parameters were either totally or partly corrected after riboflavin treatment. All patients had mutations in the gene for ETF:QO. In one patient, we show that the ETF:QO mutations are associated with a riboflavin-sensitive impairment of ETF:QO activity. This patient also had partial deficiencies of flavin-dependent acyl-CoA dehydrogenases and respiratory chain complexes, most of which were restored to control levels after riboflavin treatment. Low activities of mitochondrial flavoproteins or respiratory chain complexes have been reported previously in two of our patients with ETF:QO mutations. We postulate that riboflavin-responsive MADD may result from defects of ETF:QO combined with general mitochondrial dysfunction. This is the largest collection of riboflavin-responsive MADD patients ever reported, and the first demonstration of the molecular genetic basis for the disorder.**

**Keywords:** riboflavin-responsive multiple acyl-CoA dehydrogenation deficiency; electron transfer flavoprotein ubiquinone oxidoreductase; mutations; mitochondrial myopathy

**Abbreviations:** AMP = adenosine 5′-monophosphate; ETF = electron transfer flavoprotein; ETF:QO = ETF: ubiquinone oxidoreductase; FAD = flavin adenine dinucleotide; FMN = flavin mononucleotide; MADD = multiple acyl-CoA dehydrogenation deficiency; PTC = premature termination codon

*Received June 12, 2006. Revised May 14, 2007. Accepted May 18, 2007. Advance Access publication June 20, 2007*

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## Introduction

Multiple acyl-CoA dehydrogenation deficiency (MADD) is a disorder of oxidative metabolism with a wide range of clinical severity (Frerman and Goodman, 2001). The most severely affected patients have congenital anomalies (such as cystic renal dysplasia) and die in the newborn period. Other patients present with hypoglycaemia, encephalopathy, muscle weakness or cardiomyopathy in the newborn period or later in childhood. The most mildly affected patients present with muscle weakness as adults. Some of the less severely affected patients show a dramatic response to riboflavin.

In MADD, multiple dehydrogenation reactions are impaired because of defective transfer of electrons from a number of primary flavoprotein dehydrogenases to the mitochondrial respiratory chain. At least 12 flavoprotein dehydrogenase enzymes seem to be affected, all of which use flavin adenine dinucleotide (FAD) as the redox prosthetic group. Several of the dehydrogenases are involved in fatty acid oxidation and the clinical features described above resemble those seen in fatty acid oxidation disorders; other affected enzymes are involved in the oxidation of several amino acids, dimethyl glycine and sarcosine. From the FAD prosthetic group, electrons are passed to ubiquinone in the respiratory chain via two other flavoproteins—electron transfer flavoprotein (ETF) and ETF: ubiquinone oxidoreductase (ETF:QO).

ETF is localized in the mitochondrial matrix as a heterodimer of $\alpha$- and ß-subunits and contains one FAD prosthetic group and one adenosine 5′-monophosphate (AMP) (Roberts *et al.*, 1996); the two subunits are encoded by *ETFA* and *ETFB* genes (Olsen *et al.*, 2003). ETF:QO is a monomer located in the inner mitochondrial membrane, and contains a 4Fe4S cluster as well as FAD (Simkovic *et al.*, 2002); its gene is called *ETFDH* (Goodman *et al.*, 2002; Olsen *et al.*, 2003). Like the majority of mitochondrial enzymes, the ETF and ETF:QO proteins are imported from the cytosol. In the mitochondria the proteins fold into the native conformation (Ikeda *et al.*, 1986; Goodman *et al.*, 1994). For most mitochondrial flavoproteins, FAD binding occurs inside mitochondria (Nagao and Tanaka, 1992; Saijo and Tanaka, 1995; Brizio *et al.*, 2002), which agrees with recent lines of evidence suggesting that mitochondria can synthesize their own FAD (Barile *et al.*, 2000; Brizio *et al.*, 2006).

Many patients with MADD have been shown to have mutations in *ETFA*, *ETFB* or *ETFDH* (Frerman and Goodman, 2001; Goodman *et al.*, 2002; Olsen *et al.*, 2003; Schiff *et al.*, 2006). In patients with riboflavin-responsive forms of MADD, the molecular defect is still unknown. A disorder of mitochondrial flavin metabolism or transport has been proposed, for several reasons. Low intra-mitochondrial concentrations of FMN and FAD were found in fibroblasts from the first riboflavin-responsive patient described by Gregersen and co-workers in 1982 (Gregersen *et al.*, 1982; Rhead *et al.*, 1993), and have subsequently been found in skeletal muscles from three other unrelated patients with riboflavin-responsive MADD (Vergani *et al.*, 1999; Gianazza *et al.*, 2006). Moreover, studies in a number of patients have shown decreased activity and immunoreactive protein for several mitochondrial flavin-dependent enzymes, including acyl-CoA dehydrogenases (Turnbull *et al.*, 1988; DiDonato *et al.*, 1989; Antozzi *et al.*, 1994; Vergani *et al.*, 1999; Gianazza *et al.*, 2006). These abnormalities resemble those found in riboflavin-deficient rats (Veitch *et al.*, 1989), and they cannot easily be explained by primary defects of ETF or ETF:QO. We have studied patients from 11 pedigrees with riboflavin-responsive MADD, including the first patient described by Gregersen and co-workers. To our surprise, we have found mutations of ETF:QO in all cases.

## Methods

### Identification of patients

Between 1998 and 2006, mutation analysis has been undertaken in Aarhus on patients from 68 pedigrees with MADD. Patients from 13 pedigrees were reported to have a riboflavin-responsive phenotype. Eleven of the pedigrees are included in the present study. On reviewing the clinical and biochemical features, we were uncertain whether the other two patients had shown a genuine response. One excluded patient presented in infancy with weakness that improved with riboflavin, carnitine, glycine and dietary treatment but she deteriorated later despite continuing riboflavin (Olsen *et al.*, 2004). The other patient's organic acids improved on riboflavin treatment but there was no unequivocal clinical response or change in blood acylcarnitines. Both patients had *ETFDH* mutations on each allele.

### Fibroblast culture and measurement of fatty acid oxidation flux

Fibroblasts were cultured in Ham's F10 with 25 mM HEPES buffer plus glutamine, 12% foetal calf serum, penicillin and streptomycin. The riboflavin concentration of the Ham's F10 was 0.38 mg/l. Fatty acid oxidation flux was measured in cultured fibroblasts as previously described (Manning *et al.*, 1990; Olpin *et al.*, 1999). Flux results from riboflavin-responsive-MADD cell lines were expressed as a percentage of 3–5 simultaneous normal controls. Experience over many years has shown that precision and reproducibility are better using simultaneous controls rather than long-term control data.

### Preparation of mitochondrial fractions from skeletal muscles and enzyme activity assays

Mitochondria were isolated from fresh open muscle biopsy specimens from the vastus lateralis muscle. An aliquot of the mitochondrial preparation was directly used for polarographic analyses. The rest of the mitochondria were frozen in liquid nitrogen and stored at −80°C until used. Mitochondrial preparation, polarography and spectrophotometric analyses of the respiratory chain function were performed as described (Tulinius *et al.*, 1991).

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**Table 1** Clinical features for the patients reported

| Case | Sex | Age at diagnosis (years) | Episodes of encephalopathy (age in years) | Periods of severe weakness (age in years) | Other problems | Previously reported in |
|---|---|---|---|---|---|---|
| 1A | M | 3 | 2, 3 | None | | (Gregersen *et al*., 1982, 1986) |
| 1B | M | 13 | 2 | None | | (Gregersen *et al*., 1982, 1986) |
| 2 | M | 7 | None | 3–7 | Learning difficulties | (Ramos *et al*., 1995) |
| 3A | F | 14 | 14 | 14 | | (Henderson *et al*., 2002) |
| 3B | F | 19 | None | None | Vomiting | |
| 4A | F | 16 | 9, 16 | None | Cyclical vomiting | |
| 4B | F | 15 | 11 mild episodes between 8 and 14 | None | Cyclical vomiting | |
| 5 | F | 16 | None | 13–16 | | (Beresford *et al*., 2006) |
| 6A | F | 17 | 16, 17 (mild) | 16–17 | | |
| 6B | F | 14 | None | None | Cyclical vomiting | |
| 7 | F | 21 | 21 | 20–21 | Cyclical vomiting | (Moore *et al*., 1998) |
| 8 | F | 22 | None | 22 | | |
| 9 | F | 26 | 3 | 22–26 | Cyclical vomiting | |
| 10 | F | 31 | 31 | None | | (Stojanovic *et al*., 2000) |
| 11 | M | 42 | none | 33–42 | | |

*Note*: Preliminary molecular analysis on case 5 has been previously reported (Beresford *et al*., 2006).

For ETF:QO and acyl-CoA dehydrogenase activity assays, mitochondria were suspended in 0.6 ml 250 mM sucrose, 2 mM EDTA, 10 mM Tris–HCl, pH 7.4 and heparin 5000 IE/ml. The mitochondria were sonicated three times with the microtip on a Branson sonifier, Model 450, for 6 s at 30% duty using a setting of 3.0. The suspension was centrifuged for 30 min at $200\,000 \times g$ in a Beckman TLA 100.1 rotor. The supernatant was removed for assay of acyl-CoA dehydrogenase and citrate synthase activities. The membrane pellet was suspended in 0.2 ml of 20 mM Tris–HCl, pH 7.4, 10% glycerol, 50 mM NaCl, 0.1 mM dithiothreitol and frozen at −80°C until needed. The membrane fraction was thawed and extracted for 1 h with a 2.5 excess of dodecyl maltoside over protein (weight:weight), centrifuged at $200\,000 \times g$ as earlier and ETF:QO in the supernatant fraction was assayed as described later.

ETF:QO was assayed spectrophotometrically at 275 nm as described (Simkovic *et al*., 2002), following the reduction of CoQ by octanoyl-CoA in the presence of substrate levels of human ETF and medium chain acyl-CoA dehydrogenase. Citrate synthase was assayed spectrophotometrically as described by Srere (1969). Acyl-CoA dehydrogenase activities were assayed fluorimerically under anaerobic conditions (Frerman and Goodman, 1985) with porcine ETF as the electron acceptor. Because of the overlap in substrate specificities of the acyl-CoA dehydrogenases, activities are referred to as palmitoyl-CoA and octanoyl-CoA dehydrogenases. Protein concentrations were determined by the method of Bradford (1976).

## Molecular studies

cDNA- and genomic DNA-based PCR amplifications and sequence analysis of the human *ETFA*, *ETFB* and *ETFDH* genes were carried out as described previously (Olsen *et al*., 2003). All references to nucleotides or amino acids are based upon the cDNA sequence of *ETFDH* (NM_004453.1). The initiating ATG codon is numbered as bp 1–3, and the initiating methionine is numbered as amino acid 1.

# Results

## Clinical features

The patients' clinical features are summarized in Table 1. There are four sibling pairs (patients 1A and 1B, 3A and 3B, 4A and 4B, 6A and 6B); patients 4A and 4B are identical twins. Clinical features have been reported previously for patients 1–3B, 5, 7 and 10 (Table 1). The patients are all Caucasians; patients 1A and 1B are Danish, patient 2 is Spanish, patient 11 is Swedish and the others are from the UK. None of the patients have consanguineous parents but the parents of patient 2 and the grandparents of patients 4A and 4B come from small isolated villages.

Episodes of acute encephalopathy occurred in nine patients, generally precipitated by an infection. Only three patients had hypoglycaemia. Three patients required ventilation; imaging showed generalized cerebral oedema in one of these and pontine oedema in a second. All patients made a full recovery. Besides these episodes of frank encephalopathy, many patients suffered recurrent episodes of vomiting in mid-childhood.

Eight patients had severe muscle weakness, generally as young adults. Four other patients had mild weakness. Typically, strength deteriorated rapidly, but myoglobinuria was rare (only patient 3A). Some patients deteriorated after a minor infection but often there was no precipitant. The weakness affected proximal muscles and particularly the neck flexors and extensors; several patients could not lift their chins off their chests. A few patients had dysphagia or respiratory muscle weakness. Muscle histology sometimes showed a lipid storage myopathy but it was normal in one patient at the time of severe weakness (patient 6A).

Patient 2 differed from the other patients in having learning difficulties (IQ 50) as well as weakness. Magnetic resonance imaging showed white matter changes at 8 years

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of age, which resolved on treatment with riboflavin. A similar patient with riboflavin-responsive MADD has been reported previously (Uziel *et al*., 1995).

For each pedigree, the index case showed an unequivocal clinical response to treatment with riboflavin (100–400 mg/day). The patients with weakness all improved within days of starting riboflavin. Most patients now have normal muscle strength, including five patients who were bed-bound or wheelchair-bound prior to treatment. Patient 2 has mild residual weakness. Riboflavin treatment was started in four patients whilst they were encephalopathic. Three of these, who were comatose and deteriorating, responded dramatically, mechanical ventilation being withdrawn within 24 h. The other patient also recovered promptly, much faster than during a previous episode of encephalopathy managed without riboflavin (Gregersen *et al*., 1982). No patient has become encephalopathic whilst taking riboflavin. Carnitine and glycine were started at the same time as riboflavin in two of the encephalopathic patients; carnitine and glycine have subsequently been withdrawn without problems. No drugs were started at the same time as riboflavin in any of the other patients; in some, carnitine had previously been given without benefit.

In pedigrees where the index case had an affected sibling, the latter had milder symptoms and the response to riboflavin was, therefore, less striking. Patients 3B, 4A, 4B and 6B had recurrent vomiting, which resolved with riboflavin treatment. Patient 1B was asymptomatic after a single episode of encephalopathy (Gregersen *et al*., 1982).

## Urine organic acid and blood acylcarnitine analysis

Urine organic acids were abnormal in all patients at the time of presentation, typically with C5-10 dicarboxylic aciduria and hexanoylglycine as well as other short-chain glycine conjugates. If the patient was encephalopathic or vomiting, ketone bodies were present, often in massive amounts. Patient 11 excreted only ethylmalonic and 2-hydroxyglutaric acids. On treatment with riboflavin, the abnormalities resolved completely in nine patients. The other patients usually excreted ethylmalonic acid and occasionally small amounts of other dicarboxylic acids. The improvements could be due to riboflavin but resolution of catabolism will have been another factor in some cases. In some patients, organic acid analysis was undertaken *when the patient was stable* before and after the introduction of riboflavin. In six cases, this established that the improvements were due to riboflavin (e.g. patients 1A and 1B (Gregersen *et al*., 1982, 1986)). In four patients, however, there were no abnormalities when the patient was stable, even before treatment with riboflavin.

Blood acylcarnitine results are not available for patients 1A, 1B, 4A, 4B or 11. Other patients had raised concentrations of acylcarnitines, particularly chain lengths 6–12, before riboflavin was started, whether the patient was stable or acutely unwell. Several patients also had raised levels of C14:1, C16:1 and C18:1 acylcarnitines. The mildest abnormalities were in patients 3A and 3B, who only had increased C8 and C10 (±C4) acylcarnitines. After treatment with riboflavin, the acylcarnitine profile showed residual abnormalities in all patients but the abnormalities were often very mild and, in some patients (cases 3A and 3B) they were only intermittently present. At presentation, serum carnitine concentrations were low in seven of the eight patients in whom it was measured. Patient 11 had normal serum carnitine, but pronounced muscle carnitine deficiency.

**Table 2** ß-Oxidation flux in cultured fibroblasts, expressed as a percentage of simultaneous means to controls

| Patient | Myristate | Palmitate | Number of duplicate assays |
|---|---|---|---|
| 1A | 32 ± 9 | 32 ± 2 | 4 |
| 2 | 78 ± 16 | 77 ± 9 | 4 |
| 3A | 100 | 103 | 2 |
| 4A | 129 ± 8 | 125 ± 10 | 3 |
| 4B | 38 | 48 | 2 |
| 5 | 34 | 44 | 1 |
| 6A | 62 ± 12 | 62 ± 13 | 4 |
| 6B | 96 | 91 | 2 |
| 7 | 148 ± 10 | 127 | 3 (myristate), 1 (palmitate) |
| 9 | 58 | 71 | 1 |
| 10 | 55 ± 6 | 59 ± 7 | 5 |

*Note*: Results are expressed as a percentage of 3–5 simultaneous controls. Means ± standard deviations are given for the number of duplicate assays shown.

## Fatty acid oxidation flux

The results of fatty acid oxidation flux studies are summarized in Table 2. Several cell lines showed normal flux activity and in none was it severely reduced. In contrast, in 30 patients with riboflavin-*un*responsive-MADD, we found severely reduced flux activity for myristate (6.8 ± 5.4% controls) and palmitate (8.9 ± 9.1% controls). All cell lines were grown in medium with a high riboflavin concentration (0.38 mg/l). Growing fibroblasts in severely riboflavin depleted medium resulted in very low flux activity in both riboflavin-responsive-MADD patients and controls (data not shown). We were unable to demonstrate a threshold riboflavin concentration that limited flux in riboflavin-responsive-MADD patients but not in controls. Fatty acid oxidation flux studies were not performed on fibroblasts from patient 11. However, palmitoyl-carnitine oxidation in isolated muscle mitochondria measured by polarography was only 10% of that of controls before riboflavin treatment and increased to 20% of that of controls after treatment with riboflavin (Table 3).

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**Table 3** ETF:QO, acyl-CoA dehydrogenase and respiratory chain activities in skeletal muscle mitochondria from patient 11

| | Patient (% of control mean) | | Controls[a] | |
|---|---|---|---|---|
| | Before treatment | After treatment | Mean | Range |
| ETF:QO (μmol/min/mg) | 0.0036 (1) | 0.0476 (12) | 0.3922 | 0.349–0.461 |
| Acyl-CoA dehydrogenases (nmol/min/mg) | | | | |
| Octanoyl-CoA | 31.3 (38) | 28.0 (34) | 82.4 | 33.8–129.6 |
| Palmitoyl-CoA | 24.5 (39) | 48.7 (77) | 63.22 | 36.6–99.4 |
| Citrate synthase (μmol/min/mg)¤ | 1.27 (95) | 1.30 (97) | 1.34 | 0.61–1.81 |
| Polarography (nmol/min/mg) | | | | |
| Pyruvate + malate | 15 (11) | 120 (89) | 135 | 111–147 |
| Glutamate + malate | 17 (11) | 144 (96) | 150 | 127–186 |
| Palmitoyl-carnitine + malate | 8 (10) | 16 (20) | 80 | 51–102 |
| Succinate + rotenone | 55 (35) | 150 (96) | 157 | 130–187 |
| Ascorbate + TMPD | 160 (48) | 320 (96) | 335 | 281–365 |
| Respiratory chain enzymes | | | | |
| NADH-ferricyanide reductase (nmol/min/mg) | 1600 (21) | 7000 (92) | 7580 | 6100–8880 |
| Succinate-cytochrome c reductase (nmol/min/mg) | 130 (32) | 370 (92) | 401 | 288–481 |
| Cytochrome oxidase (k/mg) | 4 (29) | 12 (88) | 14 | 11–16 |
| Citrate synthase (μmol/min/mg)# | 2.1 (87) | 2.3 (95) | 2.4 | 1.7–2.9 |

[a] Age and storage matched control samples ($n = 5$).
Citrate synthase was measured simultaneously either with ETF:QO and acyl-CoA dehydrogenase activities (¤), or with respiratory chain activities (#).

## ETF:QO, acyl-CoA dehydrogenase and respiratory chain activities in skeletal muscle mitochondria

The results of ETF:QO, fatty acid oxidation and respiratory chain function measurements in skeletal muscle mitochondria before and after 1.5 years of riboflavin treatment in patient 11 are summarized in Table 3. Before treatment there was a pronounced deficiency of ETF:QO (1% of control mean) and moderate decrease in octanoyl-CoA and palmitoyl-CoA dehydrogenase activities. The respiratory chain function was generally low but the decrease was most pronounced for complex I and ETF/ETF:QO-dependent activities. After treatment, the ETF:QO activity increased greater than 10-fold to 12% of normal mean, resulting in a palmitoyl-carnitine oxidation of 20% of normal mean. Octanoyl-CoA dehydrogenase activity did not change significantly but the respiratory chain function was normalized.

The increase of ETF:QO activity to 12% normal mean would be compatible with the clinical improvement, given that mild, late-onset patients sometimes have this level of activity and asymptomatic obligate heterozygotes have 34–59% activity (Frerman and Goodman, 1985; Loehr *et al.*, 1990).

## Molecular studies

All 15 patients were demonstrated to have mutations in the *ETFDH* gene (Table 4). Mutations were identified in both alleles in all but two patients. For patient 7, only one heterozygous mutation was identified. No abnormalities in the quantity or processing of *ETFDH*-specific mRNA could be detected by sequence analysis of cDNA from this patient (data not shown). Nevertheless, the second allele may have a mutation in regulatory regions or in intronic splicing regulatory elements undetected by our assay. Sequence analysis of genomic DNA from patient 11 identified a single heterozygous c.1285G>C mutation at the last position of exon 10. Sequence analysis of *ETFDH*-specific cDNA from patient 11 revealed two *ETFDH* transcripts; one of full length with a C at position c.1285 and a truncated transcript with a G at position c.1285 lacking exons 2 and 3 (data not shown). This illustrates that a mutation causing abnormal exons 2 and 3 splicing of *ETFDH* is missed by our assay. No mutations were identified in the *ETFA* and *ETFB* genes from any of the 15 patients.

Fourteen different mutations were identified. Eleven are missense mutations. None of the mutations were present in a screen of 106 Caucasian European control chromosomes, thus reducing the likelihood that these mutations represent neutral common variants.

Two mutations introduce a premature termination codon (PTC) (c.51_52insT and c.1060G>T). We have previously demonstrated that the c.51_52insT mutation, which introduces a PTC in exon 2, leads to nearly undetectable levels of *ETFDH* mRNA, probably as a result of degradation by the nonsense-mediated decay pathway (Olsen *et al.*, 2003). The c.1060G>T mutation introduces a PTC in exon 9: RT-PCR studies of *ETFDH*-specific mRNA from patient 10 revealed that no normally processed *ETFDH* transcript is produced from the allele with this mutation; the c.1060G>T mutation seems mainly to cause nonsense-mediated mRNA decay of the corresponding transcripts (Garneau *et al.*, 2007) and to a minor degree to cause skipping of exon 9 (data not shown).

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**Table 4** Mutations identified in *ETFDH*

| Patient | Allele 1 | | Allele 2 | |
|---|---|---|---|---|
| | cDNA | Protein | cDNA | Protein |
| 1A & B | c.806A>T | p.Gln269Leu | **c.1448C>T** | **p.Pro483Leu** |
| 2[a] | **c.244T>C** | **p.Ser82Pro** | **c.244T>C** | **p.Ser82Pro** |
| 3A & B[a] | **c.1367C>T** | **p.Pro456Leu** | **c.1367C>T** | **p.Pro456Leu** |
| 4A & B | **c.1351G>C** | **p.Val451Leu** | **c.1351G>C** | **p.Val451Leu** |
| 5 | c.334C>T | p.His112Tyr | c.1366C>A | p.Pro456Thr |
| 6A & B | c.1001T>C | p.Leu334Pro | **c.1367C>T** | **p.Pro456Leu** |
| 7 | c.1445A>T | p.Glu482Val | – | – |
| 8[a] | c.51_52insT | p.Ala18fs | **c.508G>T** | **p.Gly170Cys** |
| 9 | c.51_52insT | p.Ala18fs | **c.1367C>T** | **p.Pro456Leu** |
| 10[a] | c.1060G>T | p.Glu354X | **c.1351G>C** | **p.Val451Leu** |
| 11 | – | p.Ala12fs | **c.1285G>C** | **p.Gly429Arg** |

*Note*: Mutations that are expected to confer a mild MADD phenotype and potentially be responsible for the observed riboflavin-responsiveness are shown **bold**.
[a]Indicates that sequence analyses of parental samples have demonstrated that both of the identified alleles are located in separate alleles. Samples were not available from the remaining parents.

Abnormal splicing caused by an unknown mutation was also observed in mRNA from patient 11. The mutation causes skipping of exons 2 and 3 resulting in a truncated transcript with a PTC in exon 4, which is not efficiently recognized and degraded by the nonsense-mediated decay pathway as evidenced by RT-PCR studies of *ETFDH*-specific mRNA from patient 11 (data not shown). The truncated transcript will, however, be translated into a protein lacking a substantial portion of the protein to cause no or very low residual ETF:QO activity. The c.1285G>C mutation is located at the last position of exon 10 and may therefore cause abnormal splicing. According to the NNSPLICE program (http://www.fruitfly.org/seq_tools/splice.html), the c.1285G>C mutation only has a minor effect on the function of the donor splice site of intron 10, changing the splice score from 1.0 to 0.94. In agreement with this, RT-PCR studies of mRNA from patient 11 did not reveal any evidence of skipping of exon 10 and/or flanking exons or activation of nearby cryptic splice sites. Even though such abnormal transcripts could be left undetected because they may be recognized and degraded by the nonsense-mediated mRNA decay pathway, the large amounts of full length *ETFDH* transcripts with the c.1285G>C mutation in mRNA from patient 11 suggests that the c.1285G>C mutation mainly results in substitution of glycine-429 by arginine.

## Discussion

The aetiology of riboflavin-responsive MADD remains uncertain, 25 years after it was first described (Gregersen *et al.*, 1982). An underlying disorder of mitochondrial FAD transport or metabolism has been proposed. In the current study, we show that riboflavin-responsive MADD is associated with defects in the *ETFDH* gene. Moreover, in one of the patients, we demonstrated decreased ETF:QO activity, which is improved greater than 10-fold after riboflavin therapy.

*ETFDH* mutations have been identified in every patient referred to us with riboflavin-responsive MADD. None of the mutations have been found in control subjects, and all affect amino acids conserved in most higher eukaryotes. Our results suggest, therefore, that riboflavin-responsive MADD is caused by *ETFDH* mutations, at least in a large proportion of cases.

In a recessive disorder, the phenotype tends to be determined by the milder mutation if there are different mutations in the two alleles. Each of our patients should, therefore, have at least one mild 'riboflavin-responsive' *ETF:QO* mutation. Table 5 shows which mutations are likely to account for the response to riboflavin, along with the supporting evidence. Three mutations are found in homozygous form, implying that they are responsible for the riboflavin-responsive phenotype. Patient 5 is compound heterozygous for two novel missense mutations. There is no evidence to indicate which of the two mutations confers riboflavin responsiveness, but it is interesting that one (p.Pro456Thr) involves the same amino acid as a 'riboflavin-responsive' mutation (p.Pro456Leu) identified in patients 3A, 3B, 6A, 6B and 9.

Why should the mutations we have found give rise to a riboflavin-responsive phenotype? Flavin binding is important for the catalytic activity of flavoproteins and also for their folding, assembly and/or stability (Nagao and Tanaka, 1992; Saijo and Tanaka, 1995; Sato *et al.*, 1997; Muralidhara *et al.*, 2006). Riboflavin supplements likely increase the intra-mitochondrial FAD concentration and thereby promote FAD binding. This could ameliorate the effect of mutations that reduce the affinity of ETF:QO for FAD. According to the 3D structure of porcine ETF:QO (Zhang *et al.*, 2006), none of the mutations, associated with a riboflavin-responsive MADD phenotype, change amino acids directly involved in FAD binding, making it difficult

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**Table 5** Analysis of which mutations may account for the patients' response to riboflavin

| Mutation | Evidence |
|---|---|
| *Probably responsible for riboflavin-responsiveness* | |
| p.Pro483Leu | Compound heterozygous with non-responsive mutation in patients 1A and 1B |
| p.Ser82Pro | Homozygous in patient 2 |
| | Previously reported in an infantile-onset patient (clinical course and response to riboflavin not mentioned) (Goodman et al., 2002) |
| | A p.Ser82Phe mutation has been identified in a 19-year-old woman with progressive muscle weakness from which she died. A biochemical response to treatment with riboflavin and carnitine was demonstrated in urine (Goodman et al., 2002) |
| | We have identified a p.Ser82Phe mutation in 3-year-old male twins. The patients are asymptomatic on treatment with carnitine and riboflavin (unpublished data) |
| p.Pro456Leu | Homozygous in patients 3A and 3B |
| | Compound heterozygous with severe mutations in patients 6A, 6B and 9 |
| | Previously reported in a mildly affected patient with a PTC mutation on the other allele (Goodman et al., 2002) |
| p.Val451Leu | Homozygous in patients 4A and 4B |
| | Compound heterozygous with a severe mutation in patient 10 |
| p.Gly170Cys | Compound heterozygous with severe mutation in patient 8 |
| *Probably **not** responsible for riboflavin-responsiveness* | |
| p.Gln269Leu | Previously reported in a patient with a nonsense mutation on the other allele who died at 10 months despite treatment with riboflavin (Olsen et al., 2003, 2005) |
| p.Leu334Pro | Previously reported homozygous in a patient who died aged 19 days with cystic renal dysplasia (Goodman et al., 2002) |
| p.Ala18fs | Introduces PTC, leading to nonsense mediated decay of transcript (Olsen et al., 2003) |
| | Previously reported in three neonatal-onset patients (Goodman et al., 2002) |
| p.Glu354X | Introduces PTC, leading mainly to nonsense mediated decay of transcript |
| p.Ala12fs | Introduces PTC, leading to nonsense mediated decay of transcript |
| *Uncertain* | |
| p.His112Tyr | – |
| p.Pro456Thr | p.Pro456Leu is associated with riboflavin-responsiveness (see above) |
| p.Glu482Val | Likely to be mild as valine is found at this position in the yeast protein |

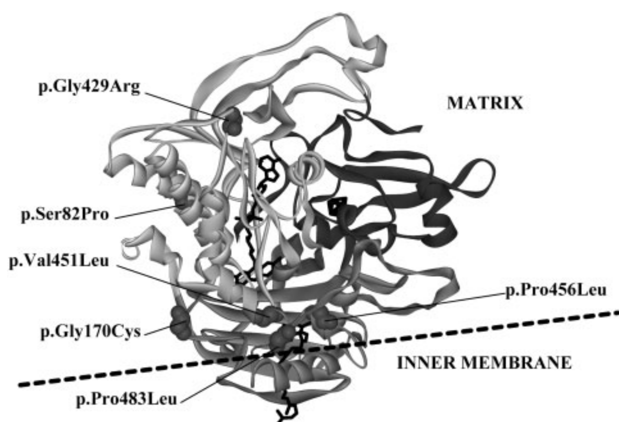


**Fig. 1** *'Riboflavin sensitive' missense mutations in the ETF:QO structure.* Ribbon representation of the ETF:QO protein based on the crystal structure of porcine ETF:QO (Zhang et al., 2006). The 4Fe4S cluster domain is in dark grey, the ubiquinone-binding domain in medium grey and the FAD domain in light grey. The three redox centres are shown in stick models. The human residues mutated in riboflavin-responsive MADD are shown in space filling. Figure prepared with the ViewerLite 5.0 program.

to assign them a probable mechanism of action. Two of the mutations (p.Ser82Pro and p.Gly429Arg) are located in the FAD-binding domain of ETF:QO, but the affected amino acids do not make direct interactions with FAD. The remaining mutations are all clustered in a small region in the ubiquinone-binding domain (Fig. 1). The mutations may cause long-distance conformational changes that make the FAD-binding site less accessible to FAD, or they may affect catalysis by impairing flavin-ubiquinone electron transfer. It has been shown recently that flavins may bind to the unfolded state of certain flavoproteins and chaperone their folding (Higgins *et al*., 2005; Muralidhara *et al*., 2006). In this respect raising the intra-mitochondrial FAD concentration may compensate for a decreased folding capacity of the mutant proteins.

*ETFDH* mutations alone cannot easily explain all the findings in our patients. In particular, multiple mitochondrial enzyme deficiencies have been found in all our patients for whom such studies have been possible (patients 1A, 5 and 11). Studies of patient 11's muscle prior to riboflavin treatment showed partial deficiencies of multiple mitochondrial flavoproteins (acyl-CoA dehydrogenases and complexes I and II of the respiratory chain) and of complex IV (which does not use a flavin cofactor); all the activities except octanoyl-CoA dehydrogenase returned to normal after treatment with riboflavin. Partial deficiency of octanoyl-CoA dehydrogenase, unresponsive to riboflavin, has also been demonstrated in fibroblast mitochondria from patient 1A (Rhead *et al*., 1993), and complex I, II and IV deficiencies have been reported in pre-treatment muscle from patient 5 (Beresford *et al*., 2006). Riboflavin-sensitive defects of multiple acyl-CoA dehydrogenases have been

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reported previously in a number of riboflavin-responsive MADD patients (summarized in Antozzi *et al*., 1994), sometimes accompanied by impairment of complex I and II (Antozzi *et al*., 1994; Zerbetto *et al*., 1997; Vergani *et al*., 1999, Russell *et al*., 2003; Gianazza *et al*., 2006) and complex IV (Mongini *et al*., 1992; Antozzi *et al*., 1994).

It is possible that the primary deficiency of ETF:QO in these patients may cause secondary impairment of other mitochondrial enzymes, due to the accumulation of toxic metabolites and/or increased oxidative stress. Secondary mitochondrial dysfunction is thought to occur in a number of inborn errors of metabolism, including fatty acid oxidation disorders (Ventura *et al*., 1995; Gregersen *et al*., 2005; Schwab *et al*., 2006). Defects of the respiratory chain have, for example, been demonstrated in patients with long-chain 3-hydroxyacyl-CoA dehydrogenase deficiency (Tyni *et al*., 1996).

Alternatively, patients with riboflavin-responsive MADD may have *ETFDH* mutations combined with a disturbance of mitochondrial flavin and flavoprotein homeostasis. Low mitochondrial concentrations of flavins have been found in four unrelated patients with riboflavin-responsive MADD including patient 1A of this study (Rhead *et al*., 1993; Vergani *et al*., 1999; Gianazza *et al*., 2006). Moreover, the observed mitochondrial dysfunction is similar to that seen in riboflavin-deficient rats (Veitch *et al*., 1989). The impairment of non-flavin-dependent complex IV in patients 5 and 11 of this study and in two previously reported riboflavin-responsive-MADD patients (Mongini *et al*., 1992; Antozzi *et al*., 1994; Beresford *et al*., 2006) might be explained by an impairment of FAD-containing protoporphyrinogen oxidase. Protoporphyrinogen oxidase is the penultimate mitochondrial enzyme of the heme biosynthetic pathway (Dailey and Dailey, 1998), and heme deficiency has been shown to decrease the assembly and activity of complex IV (Atamna *et al*., 2001).

Our patients are unlikely to have an autosomal recessive disorder of flavin metabolism in addition to ETF:QO deficiency, particularly as they come from non-consanguineous families. They might, however, carry a common genetic susceptibility factor that predisposes to disturbances of mitochondrial flavin homeostasis. The main candidate genes would be those encoding enzymes of mitochondrial flavin metabolism and transport (Barile *et al*., 2000; Spaan *et al*., 2005; Brizio *et al*., 2006), such as mitochondrial FAD pyrophosphatase. Increased activity of this enzyme was found in muscle from three unrelated riboflavin-responsive MADD patients (Vergani *et al*., 1999; Russell *et al*., 2003; Gianazza *et al*., 2006).

Defective mitochondrial flavin and flavoprotein homeostasis could also be related to diet. Diet histories and biochemical tests suggest that subclinical riboflavin deficiency is common in adults in the UK and particularly in adolescent girls (Gregory and Lowe, 2000; Moat *et al*., 2003). This could explain the markedly skewed sex ratio of our patients (12 of the 15 patients are females), as well as their age at presentation. Most of the patients described here first developed severe symptoms as adolescents or young adults, and it is possible that symptoms may have been precipitated by a deterioration in their riboflavin status.

The data presented in this study suggest that *ETFDH* mutations and general disturbances of mitochondrial protein integrity are important factors in riboflavin-responsive MADD. Our data, however, do not allow us to conclude whether the observed mitochondrial dysfunction is secondary to *ETFDH* mutations or if it is caused by additional genetic and/or diet-related disturbances of flavin metabolism. Since muscle biopsy specimens, especially in children, are always very limited, we have not been able to investigate flavin and flavoprotein integrity of all our patients, and previously reported patients showing deficiencies of multiple mitochondrial flavoproteins have not been investigated for *ETFDH* mutations. Thus, it is possible that the aetiology of riboflavin-responsive MADD may be heterogeneous: some may have *ETFDH* mutations in addition to abnormalities of mitochondrial flavin/flavoprotein homeostasis and others may have isolated genetic defects of ETF:QO, or of proteins responsible for mitochondrial flavin homeostasis.

We suspect that riboflavin-responsive MADD may be under-diagnosed for two reasons. First, some patients may not have a trial of riboflavin. Our patients showed a wide range of clinical severity, suggesting that all MADD patients should have a trial of riboflavin (except, perhaps, those with congenital anomalies). Second, patients with riboflavin-responsive MADD may be missed because a fatty acid oxidation disorder is not suspected. This is particularly likely for adults presenting with muscle weakness but even in patients presenting with encephalopathy the diagnosis may be missed. In fatty acid oxidation disorders, encephalopathy is usually accompanied by hypoketotic hypoglycaemia but most of our patients had normal blood glucose concentrations and they all excreted large quantities of ketone bodies. Moreover, several patients excreted no abnormal organic acid between acute episodes. Wider use of blood acylcarnitine analysis should reduce the number of missed diagnoses. A valuable clinical clue to the diagnosis may also be the characteristic distribution of weakness with prominent neck involvement.

In summary, we have identified *ETFDH* mutations in all members of this large series of patients with riboflavin-responsive MADD. Partial deficiencies of other mitochondrial enzymes were found in three patients for whom suitable samples were available. It is not clear whether these deficiencies can be explained by the *ETFDH* mutations or whether the patients have a second abnormality affecting mitochondrial flavin metabolism. We suspect that this treatable condition may be missed in a number of patients, especially those presenting with weakness.

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## Acknowledgements

We thank the patients and their families for their contribution. We are grateful to all the clinicians and biochemists, who referred patients to us, particularly Drs M. Cleary, J. Davidson, W. Gatling, N. Haites, M. Henderson, J. Kirk, P. Lee, M. Martinez-Pardo and V. Walker. We are indebted to Shirely Clark for tissue culture and fatty acid oxidation studies and to Hans Eiberg for contributing DNA from normal controls. This work was supported by grants from the Danish Medical Research Council, the March of Dimes (1-FY2003-688) and Fundación Ramón Areces.

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