



Case Report

Myocerebrohepatopathy spectrum disorder due to *POLG* mutations: A clinicopathological report

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Abstract

We report on the clinical, neuropathological, and genetic findings of a Japanese case with myocerebrohepatopathy spectrum (MCHS) disorder due to polymerase gamma (*POLG*) mutations. A girl manifested poor sucking and failure to thrive since 4 months of age and had frequent vomiting and developmental regression at 5 months of age. She showed significant hypotonia and hepatomegaly. Laboratory tests showed hepatocellular dysfunction and elevated protein and lactate levels in the cerebrospinal fluid. Her liver function and neurologic condition exacerbated, and she died at 8 months of age. At autopsy, fatty degeneration and fibrosis were observed in the liver. Neuropathological examination revealed white matter-predominant spongy changes with Alzheimer type II glia and loss of myelin. Enzyme activities of the respiratory chain complex I, III, and IV relative to citrate synthase in the muscle were normal in the biopsied muscle tissue, but they were reduced in the liver to 0%, 10%, and 14% of normal values, respectively. In the liver, the copy number of mitochondrial DNA compared to nuclear DNA was reduced to 3.3% of normal values as evaluated by quantitative polymerase chain reaction. Genetic analysis revealed compound heterozygous mutations for *POLG* (I1185T/A957V). This case represents the differential involvement of multiple organs and phenotype-specific distribution of brain lesions in mitochondrial DNA depletion disorders.

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Keywords: Alpers syndrome; Mitochondrial DNA depletion; Myocerebrohepatopathy spectrum disorder; *POLG*

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1. Introduction

Mitochondrial DNA (mtDNA) depletion syndrome (MDDS), first described in 1991, is defined as a reduction in the mtDNA copy number in different tissues, leading to insufficient synthesis of respiratory chain complexes (RCC) [1]. Clinical manifestations of MDDS involve many organ systems including the central and peripheral nervous system, liver, muscle, and gastrointestinal tract [2]. Human polymerase gamma (*POLG*) is the common causative gene involved in MDDS, whose mutations result in a diverse group of phenotypes, such as Alpers syndrome and myocerebrohepatopathy spectrum (MCHS) disorders, which typically show disease onset during early childhood. Further, several *POLG*-related phenotypes manifesting during adolescence and adulthood are recognized, including progressive external ophthalmoplegia, ataxia-neuropathy spectrum disorders, myoclonus epilepsy myopathy sensory ataxia, and sensory ataxic neuropathy with dysarthria/dysphagia and ophthalmoplegia. Some overlaps in the symptoms between these adult phenotypes exist, and can be additionally accompanied by tremor, parkinsonism, hearing loss, stroke-like episodes, and gastrointestinal symptoms, which are reminiscent of symptoms of mitochondrial diseases with pathomechanisms other than MDDS [3,4].

MCHS, the most severe phenotype of *POLG* disorders, was recently identified and is defined by the clinical triad of (1) myopathy or hypotonia, (2) developmental delay or dementia, and (3) liver dysfunction [3,5]. Severe, intractable epilepsy is included in the diagnostic hallmarks of Alpers syndrome, but is not characteristic of MCHS. As the number of patients with MCHS disorders is small and detailed clinicopathological findings are unavailable, we herein report the case of a girl with MCHS disorders due to *POLG* mutations. As far as we know, this is the first Japanese case of MCHS disorders with *POLG* mutation.

2. Case report

A girl was born at 40 weeks of gestation to healthy non-consanguineous parents without any abnormalities. The birth weight, height, and head circumference were normative. Early development and growth were unremarkable. At 4 months of age, she developed poor weight gain, emesis, hypotonia, developmental delay, and lethargy. She was admitted to our hospital because of recurrent vomiting at 6 months of age.

On admission, body length was 60.9 cm [−2.2 standard deviation (SD)], body weight was 5600 g (−2.3 SD), and head circumference was 42 cm (+0.2 SD). Hepatomegaly of a hard consistency was observed approximately 3 cm under the costal margin with no associated splenomegaly. She was alert and could

establish good eye contact and smile. She showed severe hypotonia and proximal dominant muscular weakness. She could hold neither her head nor limbs up. All deep tendon reflexes were weak.

Although complete blood count and urinalysis were unremarkable, hepatocellular dysfunction was obvious at the time of hospitalization, with the following laboratory test values: aspartate aminotransferase, 390 U/L; alanine aminotransferase, 218 U/L; total bilirubin, 1.6 mg/dL; total bile acids, 172 μmol/L; γ-glutamyl transpeptidase, 179 IU/L; leucine aminopeptidase, 268 IU/L; and cholinesterase, 73 IU/L. Levels of serum creatine kinase and blood glucose were normal. Cerebrospinal fluid (CSF) examination showed elevated protein levels of 304 mg/dL and normal cell count and glucose levels. Lactic acid was elevated in both plasma and CSF, at 15.9 mg/dL and 30.3 mg/dL, respectively. Pyruvic acid was normal in both plasma and CSF. Metabolic screening tests, including urine organic acids, plasma, and urine amino acids, were unremarkable. Initial brain computed tomography (CT) and magnetic resonance imaging performed at 6 months of age were unremarkable. The electroencephalogram showed generalized slow wave activity. Only wave I was identifiable on auditory evoked potentials. Motor nerve and sensory conduction were mildly delayed.

Muscle biopsy findings at 6 months of age showed a variation in fiber type; ragged-red fiber was not observed. Lipid and glycogen storage were not observed. Cytochrome c oxidase staining showed normal findings. Analysis of the RCC enzyme activity revealed no abnormality. No mtDNA mutations were identified.

Soon after admission, difficulty in feeding and vomiting aggravated, and tube feeding along with parenteral nutrition was required. She experienced bouts of diarrhea. Consciousness level decreased progressively, and myoclonic jerks of the right and left arms were infrequently observed. Follow-up CT revealed mild cerebral atrophy at 7 months of age. Hepatocellular dysfunction exacerbated progressively, and she died of multiple organ failure caused by hepatic failure at 8 months of age, despite supplementation of multiple vitamins and coenzyme Q 10, and was autopsied. Two years later, another girl was born to the parents. She had the same clinical course and laboratory findings observed in the present patient and died at 7 months of age. Valproic acid was not used in either patient.

2.1. Postmortem examinations

Body weight was 6.0 kg (mean ± SD, 8.0 ± 0.88 kg). The weight of the atrophic liver was 200 g, and the surface was yellowish, irregular, and hard. The lungs were congested and adrenal glands were atrophic. The other visceral organs were unremarkable on macroscopic

examination. The brain weighed 760 g and showed massive edema and caudal necrosis. Microscopically, hepatocytes and adrenal cortical cells were swollen, and renal tubular cells contained phospholipids and diffuse foam cells. Similar foam cells were also seen in the lungs and cardiac muscle fibers. In the liver, hepatic fibrosis, microvesicular steatosis, and fatty degeneration were observed (Fig. 1). In the central nervous system, a spongy change was noted predominantly in the cerebral white matter, and neuronal loss in the cerebral and cerebellar cortex was mild. Alzheimer type II glia was observed in massive numbers in the cerebral and cerebellar white matter, with a smaller amount in the cerebral cortex and deep gray matter. Neuronal loss, capillary proliferation, and sponginess were prominent in the substantia nigra (Fig. 2). Recent linear necrosis was present in the bilateral caudate nucleus.

2.2. Assay of respiratory chain complex enzyme activity in the liver

The liver samples were immediately frozen at autopsy and stored at -70°C . Activities of RCC I, II, III and IV were assayed as described previously [6,7]. The percentages of RCC I, II, III and IV activities relative to that of citrate synthase (CS) as a mitochondrial enzyme marker

were calculated. Relative enzyme activities of RCC I, III, and IV to CS in the liver were reduced to 0%, 10%, and 14% of normal values, respectively, while that of RCC II was reduced to 29%.

2.3. Analysis of quantitative polymerase chain reaction of mtDNA and DNA sequence of POLG gene

Written informed consent was obtained from the patient's parents in order to perform gene analysis. The quantitative estimation of mtDNA was performed by real-time amplification of fragments of *ND1* in the mtDNA genome, as previously described [7,8]. To determine the overall abundance of mtDNA, we compared the real-time amplification of *ND1* with a single-copy nuclear reference gene (exon 24 of the *CFTR* gene) [7,9]. The ratio of *ND1* to *CFTR* in the liver was reduced to 3.3% (SD, 1.2%) as compared to the control.

Mutation analysis was performed on the genomic DNA using primers designed to amplify the coding exons and the exon-intron boundaries of *POLG* (NM_002693.2). Fragments were analyzed by direct sequencing using ABI 3130XL (Applied Biosystems, Tokyo, Japan). The genetic analysis revealed compound heterozygous mutations in *POLG* (c.2870C>T, p.A957V and c.3554T>C, p.I1185T). The two DNA mutations

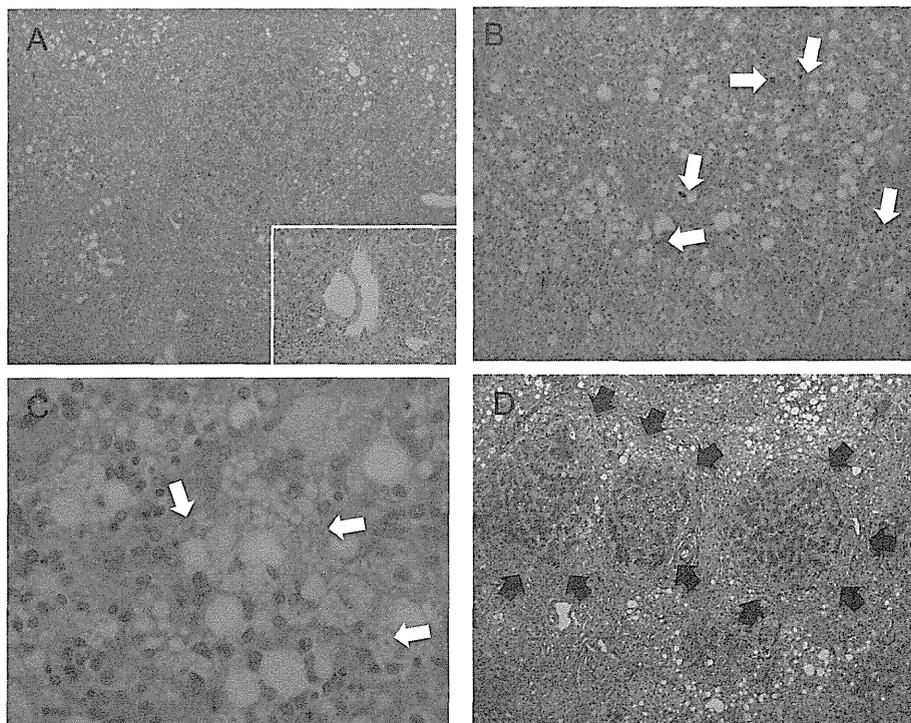


Fig. 1. Pathological findings of the postmortem liver (A–C: hematoxylin & eosin staining, D: Masson trichrome staining). (A) Moderate inflammatory cell infiltration (inset) with destroyed limiting plates and a rather progressive fibrosis with bridging formation in the portal tracts were observed (original magnification, $\times 40$). (B) Swollen hepatocytes containing lipid droplets of various sizes were found. Bile plugs (white arrows in B and C) were noted in the cytoplasm of hepatocytes and dilated canaliculi ($\times 100$). (C) Swollen hepatocytes containing lipid droplets of various sizes were found. Bile plugs were noted in the cytoplasm of hepatocytes ($\times 400$). (D) A rather progressive fibrosis with bridging formation (arrows) in the portal tracts was found ($\times 40$).

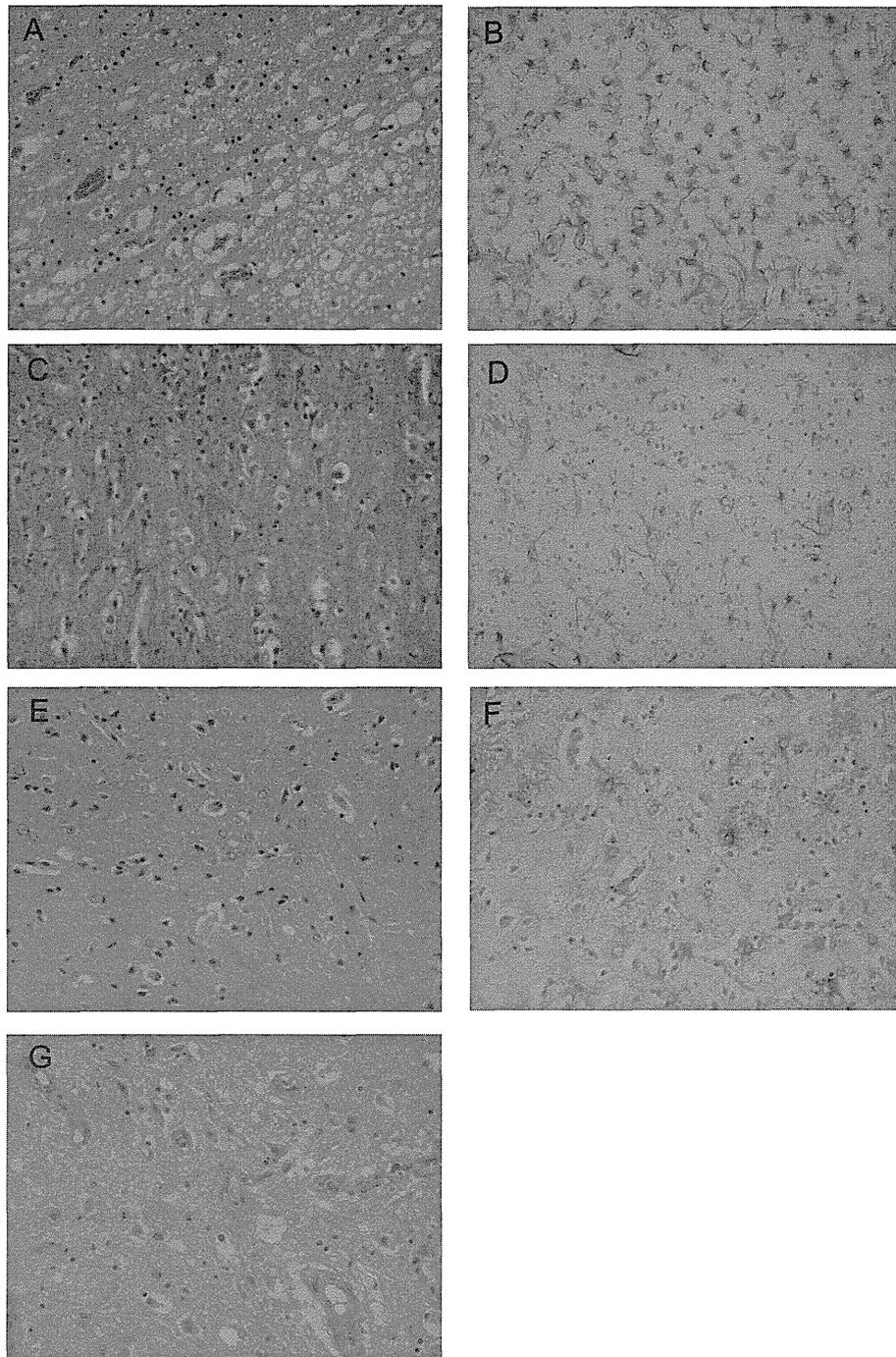


Fig. 2. Pathological findings of the postmortem brain (A, C, E, and G: hematoxylin & eosin staining; B, D, and F: immunohistochemical staining against glial fibrillary acidic protein; original magnification, $\times 400$). Marked spongy changes (A) with Alzheimer type II astrocytosis (B) was observed in the cerebral white matter, and less prominently in the cerebral cortex (C and D) and striatum (E and F). Neuronal loss, sponginess, and capillary proliferation, which were reminiscent of the findings of Leigh syndrome, were noted in the substantia nigra (G).

were not registered in neither of the 1000 Genomes Project Database (<http://www.1000genomes.org/>), ESP6500 database (<http://evs.gs.washington.edu/EVS/>) or HGVD (<http://www.genome.med.kyoto-u.ac.jp/SnpDB/index.html>). The amino acid sequences of these two sites (p.A957V and p.I1185T) are well conserved across species, suggesting their importance (Fig. 3). *In*

silico analyses were performed using the prediction algorithms SIFT (<http://sift.jcvi.org/>) and PolyPhen2 (<http://genetics.bwh.harvard.edu/pph2/>). These mutations are predicted to be deleterious by SIFT (0 and 0, respectively) and PolyPhen2 (0.985 and 0.991, respectively) programs. The results of mutation analysis have been reported previously (patient 6 in Ref. [9]).

		V	
NM_002693:c.2870C>T, p.A957V			
Human	924	RKSRGTDLHSKTATTVGISREHAKIENYGRIGAGQPPFAE	963
Chimpanzee	921	RKSRGTDLHSKTATTVGISREHAKIENYGRIGAGQPPFAE	960
Cow	912	RKSRGTDLHSKTAAATVGISREHAKIENYGRIGAGQPPFAE	951
Dog	926	RKSRGTDLHSKTAAATVGISREHAKIENYGRIGAGQPPFAE	965
Mouse	902	RKSRGTDLHSKTAAATVGISREHAKIENYGRIGAGQSPFAE	941
Rat	901	RKSRGTDLHSKTAAATVGISREHAKVENYGRIGAGQSPFAE	940
Chicken	618	KKSDGTDLHSKTAAATVGISREHAKVENYGRIGAGQPPFAE	657
Zebrafish	885	KKSQGTDLHSRTADAVGISREHAKVENYGRIGAGQPPFAE	924
Drosophila	842	SKSNGSDMHSITAKAVGISRDHAKVINYARIYAGQLFAE	881
S. cerevisiae	726	TKNEGTDLHTKTAQILGCSRNEAKIENYGRIGAGAKFAS	765

		T	
NM_002693:c.3554T>C, p.I1185T			
Human	1158	LLTRCMFAYKLGGLNDLPQSVAFFSAVDIDRCLRKEVTMDC	1197
Chimpanzee	1155	LLTRCMFAYKLGGLNDLPQSVAFFSAVDIDRCLRKEVTMDC	1194
Cow	1146	LLTRCMFAHKLGLNDLPQSVAFFSTIDIDQCLRKEVTMDC	1185
Dog	1160	LLTRCMFAYKLGGLNDLPQSVAFFSTVDIDQCLRKEVTMDC	1199
Mouse	1136	LLTRCMFAYKLGGLNDLPQSVAFFSAVDIDQCLRKEVTMDC	1175
Rat	1135	LLTRCMFAYKLGGLNDLPQSVAFFSAVDIDQCLRKEVTMDC	1174
Chicken	853	LLTRCMFAYKLGGLQDLQSVAFFSAVDIDRCLRKEVTMNC	892
Zebrafish	1122	LLTRCMFAFKLGMDLPQSVAFFSAVDIDKCLRKEVTMDC	1161
Drosophila	1062	LMTRSFVSRIGLQDLQSVAFFSSVEVDIVLRKEVTMDC	1101
S. cerevisiae	915	IWTRAMEFCQQMGINELPQNCAFFSQVDIDSVIRKEVNDC	954

Fig. 3. Conservation analysis of mutation sites in *POLG*. The sites of compound heterozygous amino acid mutations (p.957A and p.1185I) are well conserved across species.

3. Discussion

The hetero compound mutations in *POLG* were not found in either of the 1000 Genomes Project Database, ESP6500 database nor HGVD, suggesting that these are pathogenic mutations. The amino acid sequences of these two sites (p.A957V and p.I1185T) are well conserved across species including *Saccharomyces cerevisiae*, indicating their importance (Fig. 3). *In silico* analyses also predicted that these two amino acid mutations are deleterious. Furthermore A957V has been reported by Tang et al. [10]. They reported A957V allele was shared in three unrelated patients and concluded this mutation is pathogenic. The pathogenic mutations in the flanking region of p.1185I; p.1184D [11,12] and p.1186D [13] have been reported, suggesting this region is also important. Thus, we conclude the compound heterozygous mutations of this patient cause the disease.

Alpers syndrome is defined as the clinical triad of (1) refractory, mixed-type seizures that often include a focal component, (2) psychomotor regression, often triggered by intercurrent infection, and (3) hepatopathy with or without acute liver failure. There is an overlap between the phenotypes of MCHS and Alpers syndrome; however, the former usually shows an earlier onset age and more rapid disease progression, while the latter is characterized by intractable epilepsy. Using the “myo-” prefix in MCHS may be confusing since the pathological findings of muscles in this disorder often shows no evidence of mitochondrial myopathy; instead, the hypotonia observed in the triad can be regarded as a symptom of brain dysfunction. Thus, the clinical features of the patient discussed herein were typical of MCHS.

Although Wong et al. [3] “...excluded classical Alpers hepatopathy by liver biopsy” in MHCS, exact pathological findings were not provided by the authors. Differences in the hepatopathy observed in these two phenotypes have not been established; pathological characteristics of the liver in Alpers syndrome include fibrosis, regenerative nodules, hepatocyte dropout, bile duct proliferation, fatty changes, and bile stasis [14]. The findings of the present patient were compatible with those of Alpers syndrome, similar to the case of *POLG*-related MDDS previously observed [15]. As for the neuropathological findings, Alpers syndrome usually shows a preferential involvement of gray matter, characterized by gliosis, nerve cell loss, spongy degeneration, and accumulation of neural lipids in the cerebral cortex [16]. Alzheimer type II glia, representing hepatic encephalopathy, was also distributed predominantly in the gray matter [17].

A patient exhibiting a clinical evolution from MCHS to Alpers phenotype showed gray matter involvement and microscopic findings similar to those in Leigh syndrome [5], and brain biopsy in another Alpers patient with prominent white matter signal change revealed pathological characteristics typical of Alpers disease with intractable seizures [18]. On the other hand, marked gliosis and sponginess of the white matter without pathological changes in the cerebral cortex was observed in a patient with probable MCHS [17]. Apart from these, we could not find any MCHS cases with a neuropathological description in the literature. The white matter-predominant spongy degeneration with Alzheimer type II astrocytosis in the present patient may therefore be characteristic of MCHS.

POLG disorders often show elevated levels of lactate both in the serum and CSF as well as elevated levels of hepatic enzymes. However, these findings are not specific for POLG disorders; rather, they are hallmarks of mitochondrial disorders. Analysis of the RCC enzyme activity is the most valuable test for diagnosis of MDDS. However, RCC enzyme activity varies among muscle, liver, kidney, and brain tissues in the same patient [1,19], presumably due to the differential degree of DNA depletion among individual organs. The constituents of complex II are coded by genes in the nuclear, not mitochondrial DNA. In the present patient, the decreased complex II enzyme activity in the biopsied liver may either result from augmented activity of control CS enzyme due to an increase of mitochondria in number, or may be secondary to the damage of hepatocytes with necrotic and fibrotic changes [19]. It is very important to keep in mind that morphological findings and RCC enzyme activities in the muscle are sometimes unremarkable in MCHS patients, even though they show hypotonia or muscular weakness, as in the present case [5,15,20]. Therefore, analysis of RCC enzyme activities in the liver should be considered when Alpers syndrome or MCHS disorders are suspected, even when the morphological findings of muscle or enzyme assay results are unremarkable.

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COQ4 Mutations Cause a Broad Spectrum of Mitochondrial Disorders Associated with CoQ₁₀ Deficiency

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Primary coenzyme Q10 (CoQ₁₀) deficiencies are rare, clinically heterogeneous disorders caused by mutations in several genes encoding proteins involved in CoQ₁₀ biosynthesis. CoQ₁₀ is an essential component of the electron transport chain (ETC), where it shuttles electrons from complex I or II to complex III. By whole-exome sequencing, we identified five individuals carrying biallelic mutations in *COQ4*. The precise function of human *COQ4* is not known, but it seems to play a structural role in stabilizing a multiheteromeric complex that contains most of the CoQ₁₀ biosynthetic enzymes. The clinical phenotypes of the five subjects varied widely, but four had a prenatal or perinatal onset with early fatal outcome. Two unrelated individuals presented with severe hypotonia, bradycardia, respiratory insufficiency, and heart failure; two sisters showed antenatal cerebellar hypoplasia, neonatal respiratory-distress syndrome, and epileptic encephalopathy. The fifth subject had an early-onset but slowly progressive clinical course dominated by neurological deterioration with hardly any involvement of other organs. All available specimens from affected subjects showed reduced amounts of CoQ₁₀ and often displayed a decrease in CoQ₁₀-dependent ETC complex activities. The pathogenic role of all identified mutations was experimentally validated in a recombinant yeast model; oxidative growth, strongly impaired in strains lacking *COQ4*, was corrected by expression of human wild-type *COQ4* cDNA but failed to be corrected by expression of *COQ4* cDNAs with any of the mutations identified in affected subjects. *COQ4* mutations are responsible for early-onset mitochondrial diseases with heterogeneous clinical presentations and associated with CoQ10 deficiency.

Coenzyme Q (CoQ), or ubiquinone, is a lipophilic component of the electron transport chain (ETC), where it shuttles electrons derived from NADH and FADH₂ to ETC complex III (cIII) or ubiquinone-cytochrome c reductase. The main electron donors to CoQ are ETC complexes I (cI) and II (cII) but also include other mitochondrial flavoproteins, for instance, electron transfer flavoprotein-ubiquinone oxidoreductase, mitochondrial (ETF-dehydrogenase [ETF_{MDH}]), which is the terminal component of fatty acid β -oxidation and branched-chain amino acid oxida-

tion pathways. CoQ can also act as an antioxidant and a membrane stabilizer, is a cofactor of additional mitochondrial enzymes (e.g., uncoupling protein UCP1),^{1,2} and plays an indispensable role in the de novo pyrimidine biosynthesis as the electron acceptor from dihydroorotate dehydrogenase.^{3–5}

CoQ is a 1,4-benzoquinone with a tail of 10 isoprenyl units in humans (CoQ₁₀) but of variable length in other species (e.g., CoQ₆ in yeast). The synthesis of the isoprenoid moieties proceeds via either mevalonate or

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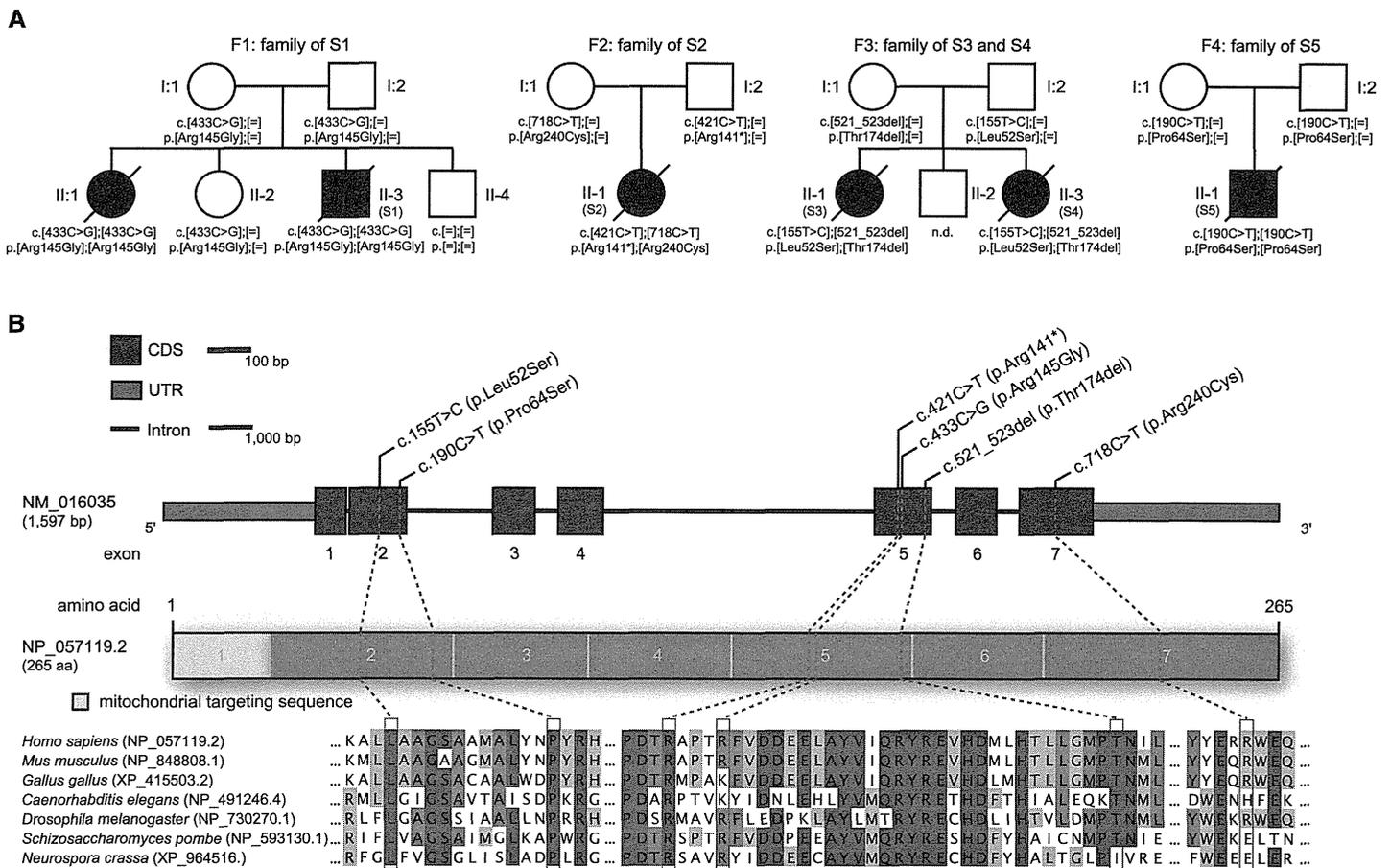


Figure 1. Pedigrees of Investigated Families and COQ4 Structure and Conservation of Identified Mutations
 (A) Pedigrees of four families affected by mutations in COQ4. The mutation status of affected and unaffected family members is indicated by closed and open symbols, respectively.
 (B) COQ4 structure showing the identified mutations. The structure of the gene product, COQ4, is also shown with known domains and localization and conservation of amino acid residues affected by the mutations. Intronic regions are not drawn to scale.

2-C-methyl-D-erythritol 4-phosphate pathways, whereas the aromatic precursor of the CoQ benzoquinone ring is p-hydroxybenzoate, derived from tyrosine.⁶ After the isoprenoid “tail” is bound to the aromatic “head,” the ring undergoes sequential modification. At least ten enzymes participate in CoQ biosynthesis; in yeast, and possibly mammals as well, these enzymes are all localized in mitochondria.

Primary CoQ₁₀ deficiency is the biochemical signature of a group of rare, clinically heterogeneous autosomal-recessive disorders caused by mutations in several genes encoding proteins involved in CoQ₁₀ biosynthesis.⁷ Mutations in COQ2 (MIM 609825), COQ6 (MIM 614647), ADCK3 (COQ8 [MIM 606980]), ADCK4 (MIM 615573), COQ9 (MIM 612837), PDSS1 (MIM 607429), and PDSS2 (MIM 610564) have been reported in subjects with severe infantile mitochondrial syndromes associated with severe tissue CoQ₁₀ deficiency, whereas the genetic bases underpinning adult-onset CoQ₁₀ deficiency remain mostly undefined.^{8,9} COQ4 (MIM 612898) codes for a ubiquitously expressed 265-amino-acid protein that is peripherally associated with the mitochondrial inner membrane on the matrix side;¹⁰ the precise function of human COQ4 is not known, but the yeast ortholog seems to play a structural

role crucial in the stabilization of a multiheteromeric complex including several, if not all, of the CoQ biosynthetic enzymes.¹¹

We report here the identification of pathogenic biallelic COQ4 mutations in a total of five individuals from four families; these subjects were part of a cohort of severe mitochondrial cases where the CoQ₁₀ defect was not anticipated. The family pedigrees are shown in Figure 1A.

Subject 1 (S1; II-3, family 1), a boy, was the third of four siblings and was born to healthy, non-consanguineous Italian parents after an uncomplicated pregnancy and elective cesarean delivery. His oldest sister (II-1), who presented with bradycardia and hypotonia, died at birth, and his 16-year-old second sister and his 5-year-old brother are alive and well. At birth, S1 had a weight of 3,410 g, a length of 49.5 cm, and a head circumference of 34.5 cm. Apgar scores were 7 and 10 at 1 and 5 min after birth, respectively. At birth, his condition appeared critical, given that he showed severe hypotonia, areflexia, acrocyanosis, bradycardia, and respiratory insufficiency. Ultrasound examination revealed markedly decreased motility of the left ventricle with an ejection fraction of 20%–25%. No evidence of hepatic or renal impairment was observed. Dobutamine infusion via an umbilical venous catheter was

Table 1. Mitochondrial ETC Activities in Muscle

	Subject	cI/CS ^a	cI+cIII/CS ^a	cII/CS ^a	cII+cIII/CS ^a	cIII/CS ^a	cIV/CS ^a	CS ^b
Muscle biopsy	S1 ^c	36	24	N	34	N	N	64
	S2 ^c	6	ND	42	43	10	30	57
	S3	N	N	N	55	N	50	54
	S4	145	N	N	N	222	189	109
	S5	<5	ND	N	30	50	N	65

Abbreviations are as follows: N, value in the control range; ND, not done; cI, complex I; cII, complex II; cIII, complex III; cIV, complex IV; cI+cIII, coupled activity of complexes I and III; and cII+cIII, coupled activity of complexes II and III. The analyses were performed in different laboratories, and the reference values are diverse (they usually range between 60% and 140% of the mean control value). The values of ETC complex activities out of the control range (specific to each enzymatic activity and to each laboratory) are reported.

^aMean control value (%) of CS-normalized ETC complex activities.

^bPercentage of mean control value.

^cSample from autopsy.

ineffective, and the baby died 4 hr after birth. His blood glucose level was normal, as were renal and hepatic parameters; plasma creatine kinase was moderately elevated (861 U/l; normal value [n.v.] < 400), and blood lactate was extremely high (20.1 mM; n.v. < 2). Analysis of urinary organic acids showed elevated levels of 2-OH glutaric acid, whereas plasma and urinary amino acids were within normal ranges. The autopsy examination revealed left ventricular hypoplasia with septum hypertrophy and a patent ductus arteriosus. No brain examination was performed.

The activities of the ETC complexes in autoptic skeletal-muscle homogenate showed severe defects of both coupled cI+cIII and cII+cIII reactions, normalized to citrate synthase (CS), and a decrease in CS-normalized cI (Table 1). In both liver and cultured fibroblasts, the CS-normalized activities of each of the individual ETC complexes were in the control range. Although the coupled cI+cIII activity cannot be reliably assayed in cultured cells,¹² the coupled cII+cIII activity was clearly decreased in S1 fibroblasts (65% of the control mean).

S2 (II-1, family 2) was born at the 34th week of gestation and was the female first child of non-consanguineous Japanese parents. Her birth weight was 1,120 g (−2.2 SDs). Apgar scores were 7 and 8 at 1 and 5 minutes after birth, respectively. There was no family history of neurological or cardiac disease. The pregnancy was complicated by severe intrauterine growth delay and ultrasound-documented hypertrophic cardiomyopathy. On S2's first day of life, she became apnoeic and was intubated as a result of respiratory failure. She initially displayed moderate lactic acidosis, but soon after her admission to Neonatal Medical Center, her lactic acidosis rapidly worsened (blood lactate = 11.2–18.8 mM; n.v. < 2); her hypertrophic cardiomyopathy evolved into severe heart failure, leading to death at the age of 1 day.

The metabolic profile (urinary and plasmatic amino acids, organic acids, and acylcarnitines) showed no significant findings. A liver autoptic specimen showed a severe deficiency of cI (cI/CS ratio = 2.9%); autoptic skeletal-muscle homogenate also showed a cI deficiency together with less pronounced reductions of other ETC complexes (Table 1).

Sisters S3 (II-1, family 3) and S4 (II-3, family 3) are the first and third, respectively, of three siblings and were born to healthy, non-consanguineous Austrian parents. Their brother (II-2) is a healthy, unaffected boy. S3 and S4 were born prematurely at gestational ages of 32 weeks (birth weight = 1,550 g) and 34 weeks (birth weight = 2,170 g), respectively.

Performed at the 20th week of gestation, prenatal organ screening of S3 revealed a suspected malformation of the cerebellum. A postnatal cranial ultrasound showed cerebellar hypoplasia. After birth, she showed distal arthrogyria, but no other dysmorphic features. At birth, she suffered from respiratory-distress syndrome, and a few hours later, a severe myoclonic epileptic encephalopathy ensued; blood lactic acid at 36 hr of age was 6.4 mM and rose to 14 mM prior to her death by multiorgan failure on the third day of life. Echocardiography showed a normal heart. Metabolic investigations (amino acids in plasma, acylcarnitine profile, and standard newborn screening) were essentially normal. Analysis of organic acids in urine showed excretion of glycerol and 2-OH-glutarate. In frozen postmortem muscle (obtained within 30 min after death), ETC enzyme activities were slightly decreased (Table 1). An autopsy of the brain revealed severe olivopontocerebellar and thalamic hypoplasia and scattered cavitations in the white matter; the visceral organs appeared normal for the gestational age.

Six years later, prenatal organ screening of the sister, S4, showed cerebellar hypoplasia, suggesting the same disease as in S3. Similar to her sister, S4 suffered from neonatal respiratory distress. No dysmorphic features were present. Echocardiography was normal. A cranial ultrasound confirmed cerebellar hypoplasia. Six hours after birth, epileptic encephalopathy ensued; blood lactic acid was 3.5 mM at 2 hr of age and rose to 9 mM at death on the second day of life. Metabolic investigations showed normal newborn-screening results and a normal acylcarnitine profile. Amino acids in plasma were grossly elevated but showed no specific pattern. Analysis of urinary organic acids showed excretion of a "mitochondrial dysfunctional pattern" with malate, fumarate, and 2-OH-glutarate, as

well as vitamin B6 metabolites and N-acetyl-tyrosine. Analysis of frozen postmortem muscle showed elevated levels of ETC activities (Table 1). In both girls, blood glucose concentration and renal and hepatic parameters were in the normal range.

S5 (II-1, family 4) is an 18-year-old young man and is the only offspring of healthy Italian parents who deny consanguinity and originate from a medium-size town in southern Italy. Pregnancy was normal, and delivery was via cesarean section because of a podalic presentation. He was born at term, and his weight at birth was 4,100 g. Weight and motor development were reportedly normal in his first year of life, but he started to show slowly progressive motor deterioration after the age of 10 months, when he manifested unsteadiness in maintaining acquired sitting position. He achieved the ability to walk with a spastic ataxic gait at 3 years of age but lost ambulation by 6 years of age and has been wheelchair bound since then. At 12 years of age, he started manifesting epileptic seizures in the form of prolonged right-side hemiclonic seizures. MRI showed bilateral increased signal intensity in fluid-attenuated-inversion-recovery and T2-weighted sequences in both occipital-cortical and juxtacortical areas (Figures S1A–S1D). Around the same period, he started to have swallowing difficulties. He was admitted for extensive investigation. Thorough blood tests excluded liver and kidney involvement and did not show lactic acidosis. A specific pattern of organic aciduria was excluded. Electrophysiological examination showed a sensory motor polyneuropathy with slowed conduction velocities. During a 5-year follow-up, he showed a slowly progressive downhill course with recurrent treatment-resistant seizures, worsened swallowing impairment, progressive scoliosis, and cognitive deterioration. A muscle biopsy was performed when he was 12 years old. Spectrophotometric assays of the ETC complexes in muscle homogenate showed virtually undetectable cI/CS ratios and reduced cII+cIII/CS and cIII/CS ratios. The other ETC complex activities were within control limits (Table 1). Since the age of 15 years, he has used a percutaneous-endoscopic-gastroscopy tube and has developed severe scoliosis with a Cobb angle of 75°. Control MRI performed when he was 17 years old showed cerebellar atrophy, widening of ventricular brain spaces, and scars from cortical necrotic lesions in both occipital areas (Figures S1E–S1H).

In agreement with the Declaration of Helsinki, informed consent for genetic and biochemical studies was signed by the parents of all subjects, and the ethics committee of the Technische Universität München approved the study.

We performed whole-exome sequencing (WES) to investigate the molecular bases of the mitochondrial disease presentations of S1, S4, and S5, as described previously.¹³ Coding DNA sequences were enriched with a SureSelect Human All Exon 50 Mb V4 or V5 Kit (Agilent) and subsequently sequenced on a HiSeq2500 system (Illumina). Read alignment to the human reference assembly (UCSC Genome Browser hg19) was done with the Burrows-

Wheeler Aligner (version 0.7.5), and single-nucleotide variants and small insertions and deletions were identified with SAMtools (version 0.1.19). On the basis of the rare disease phenotype and a pattern concordant with autosomal-recessive inheritance, we sought genes carrying rare (minor allele frequency [MAF] < 0.1% in 4,500 control exomes) variants predicted to be compound heterozygous or homozygous. We then prioritized variants in genes coding for proteins with known or predicted mitochondrial localization.¹⁴ This filtering strategy led to the identification of recessive variants in *COQ4*, coding for a mitochondrial protein involved in CoQ₁₀ biosynthesis,¹⁰ in all three subjects. In S2, we used the SeqCap EZ Library (version 1.0; Roche NimbleGen). Details on the bioinformatics pipeline and variant filtering have been reported recently.¹⁵ Sequencing statistics are provided in Table S1.

We identified *COQ4* mutations (RefSeq accession number NM_016035.3) in four individuals (Figure 1). In S1, we identified a homozygous missense variant, c.433C>G (p.Arg145Gly). Both parents and a healthy sister are heterozygous carriers, and a healthy brother has two reference alleles. No material was available from the deceased sister. S2 was found to be compound heterozygous for a nonsense variant on the paternal allele and a missense variant on the maternal allele: c.[421C>T];[718C>T], p.[Arg141*]; [Arg240Cys]. S4 was found to be compound heterozygous for a missense mutation and an exon 5 in-frame deletion: c.[155T>C];[521_523delCCA], p.[Leu52Ser];[Thr174del]. Both variants were also confirmed in the DNA of S3, whereas the parents are heterozygous for only one variant each (the father carries the missense mutation, and the mother carries the deletion). In S5, we identified a homozygous mutation, c.190C>T (p.Pro64Ser). Both parents are heterozygous for this mutation.

None of the identified variants are present in our exome database, which contains 4,500 samples, or in public SNP databases, including dbSNP, the NHLBI Exome Sequencing Project Exome Variant Server, and the Exome Aggregation Consortium (ExAC) Browser. The only exception is the c.718C>T variant (rs143441644), which is reported to have an extremely low frequency (MAF = 0.00023; 28/12,0330 alleles) in the ExAC Browser. Moreover, all missense changes are predicted to be deleterious by several bioinformatics tools (Table S2).

Because of the identified genetic defects, we tested CoQ₁₀ levels in available specimens from the subjects. In a muscle biopsy from S1, we detected a clear reduction of CoQ₁₀ (32.9 nmol CoQ₁₀/g protein; n.v. = 101–183; 1.16 nmol CoQ₁₀/CS; n.v. = 1.75–3.46). In fibroblasts from S1, the levels of CoQ₁₀ were also lower than CoQ₁₀ levels in neonatal control fibroblasts (54% of control mean). In frozen muscle from S3, CoQ₁₀ was reduced (13.5 nmol CoQ₁₀/g protein; n.v. = 160–1,200; 0.3 nmol CoQ₁₀/CS; n.v. = 2.7–7); in muscle from S4, CoQ₁₀ was profoundly reduced (25.7 nmol CoQ₁₀/g protein; n.v. = 160–1,200; 0.1 nmol CoQ₁₀/CS; n.v. = 2.7–7), whereas in S5 muscle, the amount of CoQ₁₀ was slightly decreased

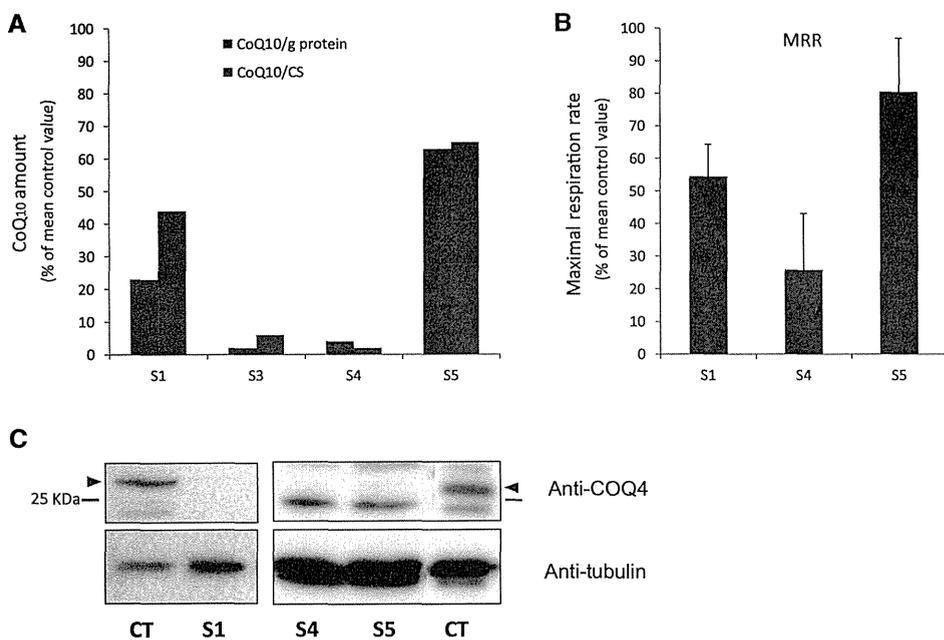


Figure 2. Biochemical Studies in COQ4 Mutant Muscle and Fibroblasts

(A) CoQ₁₀ in muscle from affected subjects S1 and S3–S5 is reported as a percentage of the mean of control values (the analyses were performed in different laboratories, and the reference values are diverse; see text). Data are reported after normalization to protein content or CS activity.

(B) Maximal respiration rate (MRR) measured in fibroblasts from subjects S1, S4, and S5; MRR values are expressed as percentages of MRR values obtained in control fibroblasts. The graphs represent the mean values from two independent experiments, each with six to eight replicates. Error bars represent the SD.

(C) Immunoblot analysis of COQ4 in fibroblasts from subjects S1, S4, and S5 and control individuals (Ct). Arrowheads indicate the band corresponding to COQ4. An antibody against tubulin was used as a loading control.

(88.9 μ g CoQ₁₀/g protein; n.v. = 101–183; 1.70 μ g nmol CoQ₁₀/CS; n.v. = 1.75–3.46) (Figure 2A). No residual sample from the muscle biopsy of S2 was available. Together, these findings are consistent with a deleterious role of the mutations identified in COQ4.

By Seahorse micro-oxygraphy,¹⁶ we detected that maximal respiratory rates were lower in S1, S4, and S5 fibroblasts than in control cells (Figure 2B). Moreover, a drastic decrease in the amount of COQ4 was detected by immunoblot analysis in S1, S4, and S5 fibroblasts (Figure 2C), confirming that the identified COQ4 nucleotide variants are deleterious.

The *Saccharomyces cerevisiae* ortholog of human COQ4 is *yCOQ4*; *yCOQ4*-null strains have been reported to be effectively complemented by human COQ4.¹⁰ In order to functionally test the effect of all the mutations found in our cohort, we transformed a COQ4-null strain (Δ coq4) by inserting the following *hCOQ4* variants into the multicopy pYES2.1 vector: pYES:*hCOQ4*^{WT} (human wild-type [WT]), pYES2.1 (empty vector), pYES:*yCOQ4*^{WT} (positive control), pYES:*hcoq4*^{p.Arg145Gly} (mutation c.433C>G), pYES:*hcoq4*^{p.Arg141*} (c.421C>T), pYES:*hcoq4*^{p.Arg240Cys} (c.718C>T), pYES:*hcoq4*^{p.Leu52Ser} (c.155T>C), pYES:*hcoq4*^{p.Thr174del} (c.521_523delCCA), and pYES:*hcoq4*^{p.Pro64Ser} (c.190C>T). In addition, to replicate the compound-heterozygous condition found in probands of families 2 and 3, we transformed the Δ coq4 strain via a pYES construct harboring both the c.155T>C and the c.521_523delCCA mutations (pYES:*hcoq4*^{p.Leu52Ser/p.Thr174del}) and a pYES construct expressing the c.421C>T and c.718C>T mutations (pYES:*hcoq4*^{p.Arg141*/p.Arg240Cys}). A WT strain transformed with the pYES2.1 empty vector was also included as an additional control. In order to reveal a possible respiratory defect, we compared the growth of our transformant strains cultured in either glucose (a fermentable carbon source) or glycerol (a non-fermentable carbon source) after

inducing gene expression with galactose for 4 hr. Notably, whereas the growth of the pYES2.1:*hCOQ4*^{WT} transformant strain was comparable to that of the pYES2.1:*yCOQ4*^{WT} transformant strain, the strains transformed with the *hCOQ4* mutant vectors grew as slowly as that transformed with pYES2.1 (Figure 3A). This result clearly indicates that each mutation reported in our probands leads to a virtually complete loss of function of the corresponding protein, COQ4. Next, we found that the CoQ₆ content in one Δ coq4 mutant strain, *hcoq4*^{p.Arg145Gly}, was markedly decreased, whereas Δ coq4 strains transformed with either pYES2.1:*yCOQ4* or pYES2.1:*hCOQ4* had CoQ₆ levels similar to those in the WT strain (Figure 3B). This result indicates that mutant *hcoq4*^{p.Arg145Gly} impairs CoQ biosynthesis.

Primary CoQ₁₀ deficiency, caused by genetic defects in CoQ₁₀ biosynthesis, is a clinically heterogeneous condition associated with a spectrum of different phenotypes, including encephalomyopathic forms with seizures and/or ataxia,^{17–19} multisystem infantile forms with encephalomyopathy and renal failure,²⁰ nephrotic syndrome with sensorineural deafness,^{21,22} adult Leigh syndrome,²³ and isolated myopathic forms.²⁴ Mutations in seven genes encoding proteins involved in CoQ₁₀ biosynthesis have been reported in single families or in a few singleton cases;²⁵ the genetic defect has not been determined in most of the cases of CoQ₁₀ deficiency, and only a few data are available regarding specific genotype-phenotype correlations. Secondary CoQ₁₀ deficiency has been reported in association with glutaric aciduria type IIC (MIM 231680), caused by mutations in *ETFDH* (MIM 231675; encoding electron-transfer dehydrogenase); ataxia-oculomotor apraxia syndrome (MIM 208920), caused by mutations in *APTX* (MIM 606350; encoding aprataxin); a cardiofacio-cutaneous syndrome caused by a mutation in *BRAF* (MIM 115150; encoding serine/threonine-protein kinase B-Raf)²⁶; and glucose transporter GLUT1 deficiency.²⁷

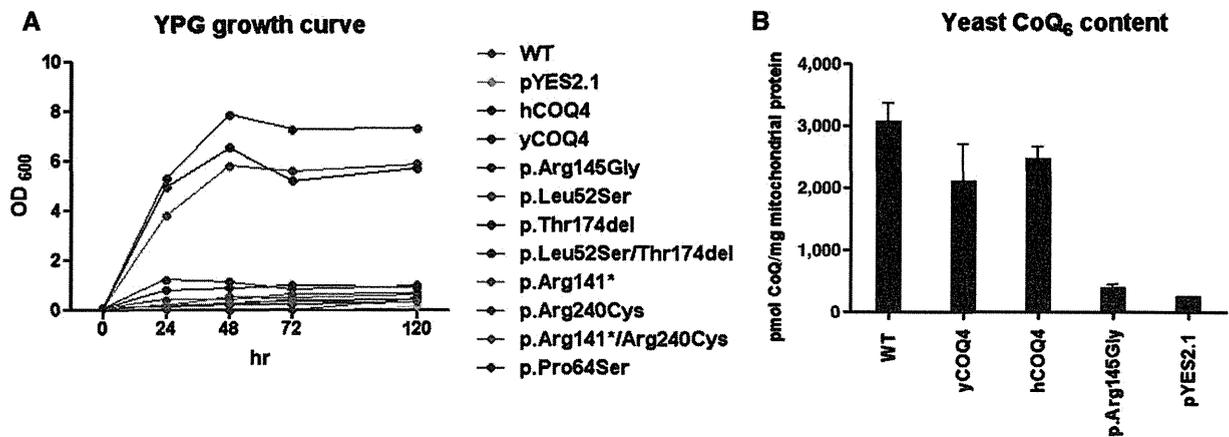


Figure 3. Yeast Studies

(A) Glycerol (YPG) growth of transformed $\Delta COQ4$ yeast with the different mutated versions of human *COQ4* (pYES2.1, empty vector; hCOQ4, pYES:hCOQ4^{WT}; yCOQ4, pYES:yCOQ4^{WT}; c.433C>G, pYES:hcoq4^{p.Arg145Gly}, c.421C>T, pYES:hcoq4^{p.Arg141*}, c.718C>T, pYES:hcoq4^{p.Arg240Cys}; c.155T>C, pYES:hcoq4^{p.Leu52Ser}; c.521_523delCCA, pYES:hcoq4^{p.Thr174del}, c.190C>T, pYES:hcoq4^{p.Pro64Ser}; c.155T>C and c.521_523delCCA, pYES:hcoq4^{p.Leu52Ser/p.Thr174del}; and c.421C>T and c.718C>T, pYES:hcoq4^{p.Arg141*/p.Arg240Cys}). WT indicates the wild-type yeast transformed with the YES2.1 empty vector. Cells were grown in selective medium for 16 hr, induced in galactose for 4 hr, and inoculated in YPG at 0.1 U of optical density (OD) at 600 nm. Growth at 30°C was monitored over 5 days by measurement of OD cultures at 600 nm.

(B) Yeast mitochondrial CoQ₆ levels. Purified mitochondria lipid extraction and high-performance-liquid-chromatography quantification of CoQ₆ was performed in the $\Delta COQ4$ strain transformed with the empty vector (pYES2.1), WT yeast (yCOQ4), or human (hCOQ4) or hcoq4^{p.Arg145Gly} (c.433C>G) *COQ4* genes. A WT strain transformed with the empty vector was included as a positive control. Error bars represent the SD.

Interestingly, although the mechanisms linking these heterogeneous genetic conditions to a decrease in CoQ₁₀ remain obscure, most of these individuals benefitted from CoQ₁₀ supplementation.^{28,29}

We found six *COQ4* mutations in five affected subjects from four unrelated families. All these individuals carried homozygous or compound-heterozygous mutations, clearly indicating that the resulting disease is an autosomal-recessive trait. Two alleles carried nonsense mutations, which are both transmitted by descent in combination with missense *COQ4* mutations to different individuals (S2 and sisters S3 and S4) and are predicted to lead to a truncated and aberrant COQ4. Given that the heterozygous parents carrying the nonsense mutations are alive and well, it is unlikely that COQ4 haploinsufficiency is pathogenic, even though a previous study reported on a boy carrying a de novo heterozygous deletion, including *COQ4*, in chromosomal region 9q34.³⁰ Because the biosynthetic pathway of CoQ is conserved throughout evolution from human to *Saccharomyces cerevisiae*, we modeled in yeast the mutations found in our subjects. Using this system, we demonstrated that each mutation, or the allelic combinations found in S2 and siblings S3 and S4, was associated with a severe defect of oxidative growth. In parallel, we also showed that COQ4 was strongly reduced in mutant fibroblast cell lines from S1, S4, and S5. In the skeletal muscle of S1 and S3–S5, the CoQ₁₀ content was reduced as well. Taken together, these results demonstrate the pathogenic role of the *COQ4* mutations found in our cohort.

In keeping with the essential role of COQ4, four of our five subjects had a prenatal or perinatal onset with a fatal outcome in the first days of life. S1 and S2 presented

with severe hypotonia, bradycardia, and respiratory insufficiency at birth; in S2, hypertrophic cardiomyopathy had been evident since fetal development. A markedly different, albeit equally severe, clinical presentation dominated by premature delivery, antenatal cerebellar hypoplasia, neonatal respiratory-distress syndrome, and epileptic encephalopathy characterized sisters S3 and S4. Rapidly progressive, severe lactic acidosis was a common feature in all four affected newborn subjects and is likely to have determined their fatal outcome. Involvement of the heart has been very rarely documented in CoQ₁₀-deficient subjects, often as part of multisystem phenotypes, where cardiomyopathy develops later than brain, muscle, or kidney impairment.²⁰ For instance, a homozygous nonsense mutation in *COQ9* was described in a baby who presented with neonatal lactic acidosis and later developed hypertrophic cardiomyopathy as part of a multisystem disease including intractable seizures, global developmental delay, and renal tubular dysfunction.⁹ In spite of his early onset, the clinical course of S5 was slowly progressive and dominated by neurological deterioration with hardly any involvement of other organs, including the heart and kidneys.

Although the link between specific genetic defects and phenotypes is often unclear in mitochondrial disorders, organs with the highest energy requirements, such as the heart, kidneys, and brain, have the highest CoQ₁₀ concentrations³¹ and are the most frequently affected by CoQ₁₀ deficiency. The level of expression of COQ genes in different cells seems to correlate poorly with the primarily affected tissue or organ; for instance, *COQ2*, mutations of which typically cause renal impairment, has expression

levels that are relatively higher in skeletal muscle and the heart than in other organs,³² whereas *COQ4*, mutated in our subjects with cardiac or brain failure, is ubiquitously expressed and has relatively higher levels in the liver, lungs, and pancreas.

Because cardiomyocytes have a remarkably high energy requirement, and cardiomyopathy is quite common in individuals with various inherited mitochondrial disorders, the cardiac involvement in subjects with mutations in *COQ* genes can be overlooked. Indeed, the crucial role of CoQ₁₀ in cardiomyocyte function has been recognized for a very long time; for instance, myocardial biopsies from individuals with congestive heart failure³³ or cardiomyopathy^{34,35} show low CoQ₁₀ levels, which correlate with the severity of heart damage.³⁶ Moreover, statins, cholesterol-lowering drugs that inhibit HMG-CoA reductase (the key enzyme common to the biosynthesis of both cholesterol and CoQ₁₀) can cause CoQ₁₀ deficiency, ultimately leading to cardiomyopathy;³⁷ interestingly, this harmful side effect can be overcome by oral CoQ₁₀ supplementation.³⁸ Moreover, long-term CoQ₁₀ treatment of individuals with chronic heart failure is safe, improves symptoms, and reduces major adverse cardiovascular events.³⁹ These observations all converge on a strict association between CoQ₁₀ deficiency and cardiomyopathy.

Notably, S3–S5 showed no sign of heart involvement, whereas the clinical phenotype was dominated by encephalopathy with seizures and a more progressive, but mainly neurological, syndrome is the clinical hallmark of S5, indicating the heterogeneity of the clinical presentations associated with *COQ4* defects. The variable specificity of organ failure (e.g., heart versus brain) in the neonatal cases of our cohort could be due to the fulminant course of the disease, which prevented the deployment of multisystem involvement. In support of this view, although cardiomyopathy dominated the clinical picture, the presence of severe hypotonia and hyporeflexia suggests concomitant involvement of the nervous system in S1 and S2 as well. Clinical heterogeneity was accompanied by an equally striking variability of the biochemical findings, which ranged from multiple (S1 and S5) to isolated (S2 and S3) ETC defects in muscle and fibroblasts to hardly any detectable defect at all (S4). This biochemical diversity could be due to differences in individual adaptive responses to reduced CoQ₁₀ availability or could reflect the striking tissue specificity observed in the clinical presentations, but at the moment, a mechanistic explanation for these observations is lacking. Poor correlation with the clinical and biochemical phenotypes has also been reported for other genes related to CoQ₁₀ biosynthesis. For instance, mutations in *COQ2*, the first mutated gene identified in affected individuals with primary CoQ₁₀ deficiency, have been associated with a wide range of clinical presentations, often including nephrotic syndrome but also including fatal neonatal multisystemic disorder, Leigh syndrome, myoclonic epilepsy, hypertrophic cardiomyopathy, deafness, and adult-onset

multisystem atrophy.^{25,40} In any case, the identification of *COQ4* mutations in subjects with such a wide spectrum of clinical and biochemical abnormalities is a further indication of the advantage of unbiased screening such as WES for the identification of genes newly associated with mitochondrial disorders.

Unfortunately, the fulminant fatal outcome in S1–S4 was so rapid that it prevented both the diagnosis of CoQ₁₀ deficiency and the start of CoQ₁₀ supplementation. Prompt diagnosis is a main challenge for syndromes of primary CoQ₁₀ deficiency but is very important given that co-factor deficiencies are virtually the only group of mitochondrial disorders for which beneficial pharmacological treatment is currently available. Treatment of the long-surviving subject, S5, has now started and will hopefully provide some useful indication of its efficacy in the near future.

Supplemental Data

Supplemental Data include one figure and two tables and can be found with this article online at <http://dx.doi.org/10.1016/j.ajhg.2014.12.023>.

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Web Resources

The URLs for data presented herein are as follows:

Exome Aggregation Consortium (ExAC) Browser, <http://exac.broadinstitute.org/>
NHLBI Exome Sequencing Project (ESP) Exome Variant Server, <http://evs.gs.washington.edu/EVS/>
OMIM, <http://www.omim.org>
UCSC Genome Browser, <http://genome.ucsc.edu>

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**COQ4 Mutations Cause a Broad Spectrum
of Mitochondrial Disorders Associated
with CoQ₁₀ Deficiency**

Gloria Brea-Calvo, Tobias B. Haack, Daniela Karall, Akira Ohtake, Federica Invernizzi, Rosalba Carrozzo, Laura Kremer, Sabrina Dusi, Christine Fauth, Sabine Scholl-Bürgi, Elisabeth Graf, Uwe Ahting, Nicoletta Resta, Nicola Laforgia, Daniela Verrigni, Yasushi Okazaki, Masakazu Kohda, Diego Martinelli, Peter Freisinger, Tim M. Strom, Thomas Meitinger, Costanza Lamperti, Atilano Lacson, Placido Navas, Johannes A. Mayr, Enrico Bertini Kei Murayama, Massimo Zeviani, Holger Prokisch, and Daniele Ghezzi

SUPPLEMENTAL DATA

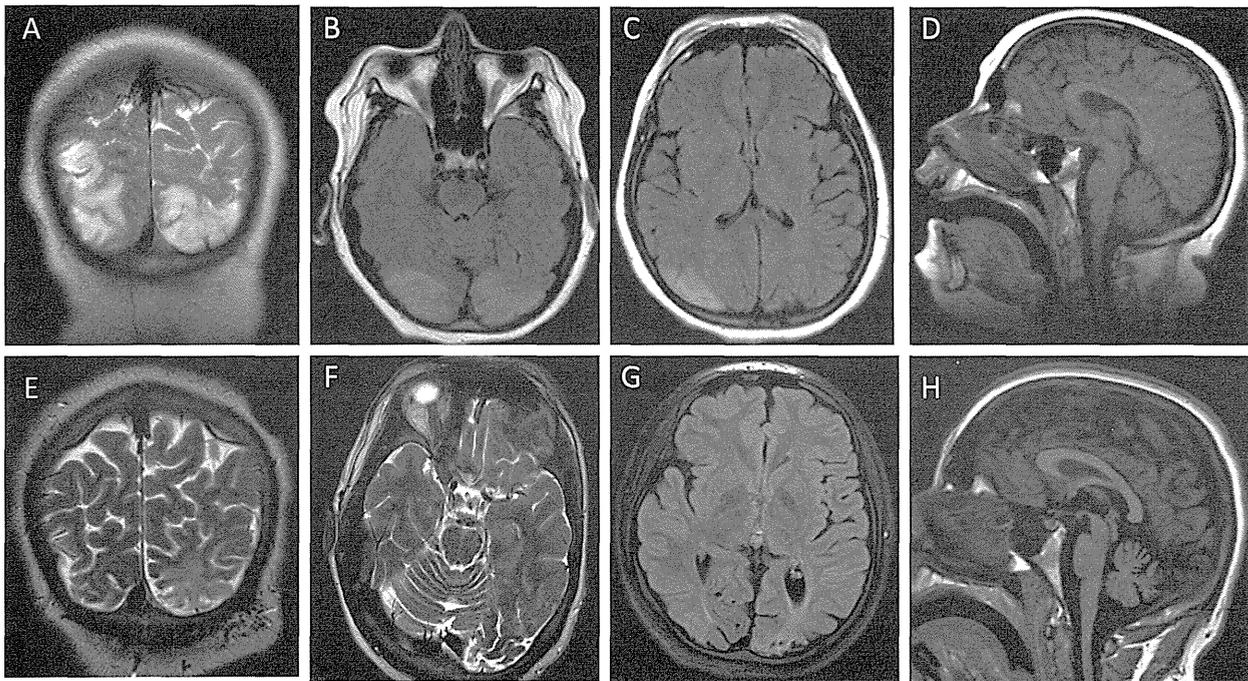


Figure S1. Brain MRI of subject 5.

Two serial brain MRI images of subject S5 obtained at age 12 years (A-D) and at age 17 years (E-H). A and E are coronal T2 weighted sections to show abnormal hyperintense cortical areas corresponding to infarct-like lesions of the occipital lobes at two different stages of progression to cortical atrophy with scars (E-G); The same occipital abnormal hyperintense cortical areas are shown in axial sections B and C (FLAIR weighted), F (T2 weighted) and G (FLAIR weighted). Notice that no abnormality is present in basal ganglia and brainstem. Finally, figures D and H are sagittal T1 sections showing progressive cerebellar atrophy of the vermis.

Id	Type	Reads	Mapped	Percent	Seq (Gb)	on bait	Avg cov	Cov 1x	Cov 4x	Cov 8x	Cov 20x
S1	SureSelect50Mbv5	106627996	105933934	99.35	10.77	78.48	132.44	99.92	99.72	99.35	97.38
S2	SeqCapEZ V1	130889538	125611825	95.97	13.81	62.71	155.83	98.36	97.32	96.41	93.80
S4	SureSelect50Mbv5	90821087	90214603	99.33	9.17	78.99	113.62	99.80	99.52	99.05	96.55
S5	SureSelect50Mbv4	125822167	124979728	99.33	12.71	73.98	146.50	99.95	99.80	99.55	98.22

Table S1: Exome Sequencing Statistics

Avg: average; Cov: coverage

Nucleotide Change	Amino acid Change	Subject	Status	Polyphen2	SIFT	PMUT	Mutation taster
c.[433C>G]	p.[Arg145Gly]	S1	Homozygous	Probably damaging	Deleterious	Pathological	Disease causing
c.[718C>T]	p.[Arg240Cys]	S2	CE with a nonsense mutation p.[Arg141*]	Probably damaging	Deleterious	Pathological	Disease causing
c.[155T>C]	p.[Leu52Ser]	S3, S4	CE with a deletion p.[Thr174del]	Probably damaging	Deleterious	Neutral	Disease causing
c.[190C>T]	p.[Pro64Ser]	S5	Homozygous	Probably damaging	Deleterious	Neutral	Disease causing

Table S2: In silico prediction of pathogenicity for *COQ4* mutations

CE: compound heterozygous.

RESEARCH PAPER

New *MT-ND6* and *NDUFA1* mutations in mitochondrial respiratory chain disorders

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Abstract

Objective: Mitochondrial respiratory chain disorder (MRCD) is an intractable disease of infants with variable clinical symptoms. Our goal was to identify the causative mutations in MRCD patients. **Methods:** The subjects were 90 children diagnosed with MRCD by enzyme assay. We analyzed whole mitochondrial DNA (mtDNA) sequences. A cybrid study was performed in two patients. Whole exome sequencing was performed for one of these two patients whose mtDNA variant was confirmed as non-pathogenic. **Results:** Whole mtDNA sequences identified 29 mtDNA variants in 29 patients (13 were previously reported, the other 13 variants and three deletions were novel). The remaining 61 patients had no pathogenic mutations in their mtDNA. Of the 13 patients harboring unreported mtDNA variants, we excluded seven variants by manual curation. Of the remaining six variants, we selected two Leigh syndrome patients whose mitochondrial enzyme activity was decreased in their fibroblasts and performed a cybrid study. We confirmed that m.14439G>A (*MT-ND6*) was pathogenic, while m.1356A>G (*mitochondrial 12S rRNA*) was shown to be a non-pathogenic polymorphism. Exome sequencing and a complementation study of the latter patient identified a novel c.55C>T hemizygous missense mutation in the nuclear-encoded gene *NDUFA1*. **Interpretation:** Our results demonstrate that it is important to perform whole mtDNA sequencing rather than only typing reported mutations. Cybrid assays are also useful to diagnose the pathogenicity of mtDNA variants, and whole exome sequencing is a powerful tool to diagnose nuclear gene mutations as molecular diagnosis can provide a lead to appropriate genetic counseling.

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Introduction

The mitochondrial respiratory chain (RC) is a pathway for vital energy generation in which ATP is generated as a form of energy by the substrates generated from glycolysis and β -oxidation. The pathway is composed of five multi-enzyme complexes (complexes I–V), two electron carriers, a quinone (coenzyme Q), and a small hem-containing protein (cytochrome c) that are located in the inner mitochondrial membrane. These RC complexes are formed from subunits encoded by both mitochondrial DNA (mtDNA) and nuclear DNA (nDNA), with the exception of complex II, which is entirely encoded by nDNA.

mtDNA is a circular double-stranded DNA molecule ~16 kb in length that encodes 37 genes comprising 13 proteins, 22 mitochondrial tRNAs, and 2 rRNAs.^{1,2} Defects in mitochondrial function are associated with numerous neurodegenerative diseases, such as Parkinson's disease, Alzheimer's disease, and Huntington's disease, and, in particular with mitochondrial respiratory chain disorder (MRCD). MRCD is genetically, clinically, and biochemically heterogeneous, and it can give rise to any symptoms, in any organs or tissues, at any age and with any mode of inheritance.³ One in 5000 births is a conservative realistic estimate for the minimum birth prevalence of MRCD.⁴ Especially in children, MRCD is an intractable disease and can be regarded as the most common group of inborn errors of metabolism.^{5,6}

Some MRCD patients have typical clinical findings that are caused by specific point mutations or large deletions of mtDNA. Typical clinical features include mitochondrial myopathy, encephalopathy, lactic acidosis, and stroke-like episodes (MELAS), myoclonus epilepsy associated with

ragged-red fibers (MERRF), Leber's hereditary optic neuropathy (LHON), and chronic progressive external ophthalmoplegia (CPEO).² Although mtDNA mutations or deletions are usually found in adults showing typical clinical findings, they account for only a minority of children with MRCD. Therefore, the diagnosis of MRCD in children by screening known mtDNA mutations is rather difficult.⁷ Hence, a combination of general biochemical study, histological study, and genetic analysis is essential for the diagnosis of MRCD, especially in children.⁶

In this study, we performed whole mtDNA sequencing for 90 children diagnosed with MRCD by RC enzyme assay with the aim of identifying causative mtDNA mutations.

Subjects, Materials, and Methods

Patients

Ninety Japanese pediatric patients diagnosed with MRCD and without characteristic clinical syndromes were studied. The primary diagnosis for these patients was definite or probable MRCD based on the criteria of Bernier et al.,⁸ and a mitochondrial RC residual enzyme activity of <20% in a tissue, <30% in a fibroblast cell line, or <30% in two or more tissues (Data S1). Informed consent was obtained from the patients and their families before participation in the study.

Patient summaries are shown in Tables 1, 2. The details of the two patients studied in the cybrid assay are as follows: Patient (Pt) 377 is a 1-year-old girl born after a normal pregnancy to non-consanguineous parents. She has a normal brother and sister. She was hospitalized with gait difficulties at the age of 1 year. Blood lactate levels were high. Brain magnetic resonance imaging (MRI)

Table 1. Distribution of mtDNA variants and clinical features.

Characteristics		Non-pathogenic mutations	Low probability variants	New pathogenic deletions	Known variants	Total
Number of subjects		61 (100%)	13 (100%)	3 (100%)	13 (100%)	90 (100%)
No consanguinity		57 (93%)	12 (92%)	3 (100%)	11 (85%)	84 (93%)
Age at onset	≤1 y.o.	54 (89%)	10 (77%)	3 (100%)	9 (69%)	76 (84%)
Status	Alive	33 (54%)	7 (54%)	1 (33%)	11 (85%)	53 (59%)
	Dead	28 (46%)	6 (46%)	2 (67%)	2 (15%)	37 (41%)
Sex	Female	30 (49%)	3 (23%)	2 (67%)	6 (46%)	41 (46%)
	Male	31 (51%)	10 (77%)	1 (33%)	7 (54%)	49 (54%)

y.o., years old.