MUTATION IN BRIEF

Identification of Novel Mutations in Spanish Patients with Muscle Carnitine Palmitoyltransferase II Deficiency

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Carnitine palmitoyltransferase II (CPT II) deficiency is the most common recessively inherited disorder of lipid metabolism affecting skeletal muscle and the most frequent cause of hereditary myoglobinuria. We studied 5 Spanish patients with CPT II deficiency from four unrelated families. Four patients had the typical clinical phenotype of muscle CPT II deficiency with recurrent episodes of myoglobinuria, triggered by prolonged exercise, fasting, or fever, and marked elevation of creatine kinase values during metabolic crisis. One patient had exercise-related myalgia, cramps and moderate elevation of serum CK values, but had never had myoglobinuria. Molecular analysis showed that three patients were heterozygous for the S113L mutation and one patient heterozygous for the P50H substitution. To identify the mutations in the other alleles of our patients we amplified and sequenced genomic DNA fragments encompassing the entire coding region and intron/exon boundaries of the *CPT2* gene. We found the recently reported 178 insT/del 25 bp in one patient. Three novel mutations were identified: a Y120C substitution that leads to a nonconservative amino acid replacement; a 36-38 insGC mutation that results in premature termination of the translation; and an I502T substitution that affects a conserved amino acid residue in the CPT II protein. Our data confirm the molecular heterogeneity of patients with CPT II deficiency, and suggest that the ethnic origin has to be taken into account before performing mutation analysis in these patients.

KEY WORDS: carnitine palmitoyltransferase II deficiency; CPT II; CPT2; exercise intolerance; molecular analysis; myoglobinuria; muscle disease

INTRODUCTION

Mitochondrial fatty acid oxidation is a complex process that requires the concerted action of several enzymes located in the mitochondrial membranes and in the mitochondrial matrix [Guzmán and Geelen, 1993]. The

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carnitine palmitoyltransferase (CPT; palmitoyl-CoA:L-carnitine O-palmitoyltransferase, EC 2.3.1.21) enzyme system plays a key role in the transfer of long-chain fatty acids from the cytosolic compartment to the mitochondrial matrix [Bieber, 1988]. The CPT system consists of two distinct mitochondrial membrane-bound enzymes: CPT I, located on the inner side of the outer membrane, and CPT II, situated on the inner membrane [McGarry and Brown, 1997]. While CPT I exists as tissue-specific isoforms, CPT II does not [McGarry and Brown, 1997].

CPT II is a homotetrameric mitochondrial enzyme of 68 kDa subunits [Finocchiaro et al 1990] and a single ubiquitously expressed isoform [Roe and Coates, 1995]. CPT II shows remarkable homology with CPT I, although it is a smaller protein (658 vs 773 aminoacid residues) that does not have the transmembrane domains found in CPT I and is not inhibited by malonyl CoA [McGarry and Brown, 1997].

The human *CPT2* gene is located at chromosome 1p32 [Gellera el al 1994], is approximately 20 kb in size, and is composed of five exons, ranging from 1.5 kb to 8 kb in length [Verderio et al, 1995]. CPT II deficiency (MIM# 600650) is the most common recessively inherited disorder of lipid metabolism affecting skeletal muscle and the most frequent cause of hereditary myoglobinuria. Typically, it presents in young adults with recurrent episodes of exercise-induced myoglobinuria [DiMauro and Melis-DiMauro, 1973; Zierz, 1994]. Atypical phenotypes of CPT II deficiency include hypoketotic hypoglycemia (MIM# 600649), cardiomyopathy and sudden death in newborns and children [Bonnefont et al, 1996; Hug et al, 1991], recurrent pancreatitis [Tein et al, 1994], brain and kidney dysplasia [North et al, 1995], and malignant hypertermia-like disorder [Vladutiu et al, 1993]. Several mutations have been detected, but the most common in the adult form is the S113L substitution, accounting for some 60% of mutant alleles [Taroni et al, 1993; Zierz et al, 1994b, Kaufmann et al, 1997; Martín et al, 1999]. The R631C mutation was initially described in an infant with hypoketotic hypoglycemia and cardiomyopathy, and was also found in several patients with the adult phenotype of CPT II deficiency [Demaugre et al, 1991;Taroni et al, 1992] . The P50H missense mutation has been observed in several patients with the muscle form of the disease [Verderio et al, 1995]. Other more rarely-ocurring mutations have also been reported [Verderio et al, 1995; Wataya et al, 1998; Yang et al, 1998; Taggart et al 1999] in individual patients. These reports demonstrate the genetic heterogeneity of the disease. In the present study we have identified three novel mutations in three Spanish families with CPT II deficiency, demonstrating that there are “private” mutations in the different ethnic backgrounds.

PATIENTS AND METHODS

Patients

We studied 5 Spanish patients with CPT II deficiency from four unrelated families (families A,B,C, and D. Figure 1). In 4 patients CPT II deficiency was documented biochemically by a muscle biopsy (Table 1). Histologically, their skeletal muscle was normal. Patients 1 and 2 were siblings. Four patients had the typical clinical phenotype of muscle CPT II deficiency with recurrent episodes of myoglobinuria, triggered by prolonged exercise, fasting, or fever, and marked elevation of creatine kinase (CK) values during metabolic crisis. Acute renal failure occurred only in patient 1. Patient 2 had exercise-related myalgia, cramps and moderate elevation of serum CK values, but had never had myoglobinuria. Patients 3 and 4 have been previously reported (patients 14 and 4 respectively in our earlier report) [Martín et al; 1999]. The main clinical findings of our patients are summarized in Table 1.

The activity of CPT II in muscle was measured as described [Norum; 1964; Martín et al; 1999]. Normal values obtained from 25 individuals deemed to be free of neuromuscular disorders were 0.42 ± 0.10 nmol min^{-1} mg non-collagen $protein^{-1}$ (mean ± standard deviation). Residual CPT II activities in muscle ranged from 10 % to 26 % of mean control value (Table 1). Moreover, to better discriminate between a defect in CPT II and CPT I, we measured muscle CPT II activity as the malonyl CoA insensitive fraction as described [Demaugre et al; 1991]. Enzyme data showed negligible activities in the four patients.

We studied DNA from muscle biopsy specimen in 4 patients. In patient 2, who was the sibling of patient 1, as muscle biopsy and biochemical studies were not performed, DNA from peripheral leukocytes was studied. We also examined blood DNA from four siblings of patients 1 and 2 (family A), from 3 healthy relatives of patient 3 (family B), and from three healthy relatives of patient 5 (family D). DNA samples from relatives of patient 4 (family C) were not available for analysis (Figure 1).

To determine whether newly identified mutations were polymorphisms, we studied DNA from 100 unrelated control subjects.

Mutation analysis

Genomic DNA was extracted according to standard procedures and screened for the missense mutations S113L, R631C, P50H, and Y628S by PCR amplification and restriction enzyme digestion (PCR-RFLP) using primer sets and protocols described elsewhere. [Bonnefont et al, 1996; Taroni et al, 1992; Taroni et al, 1993; Verderio et al, 1995].

The coding sequence of the entire *CPT2* gene (5 exons) and exon/intron boundaries was amplified by PCR from genomic DNA as described [Verderio et al, 1995], except for the exon 4, which was amplified in four overlapping fragments using intronic and exonic primers. PCR reactions were performed as described and the fragments so obtained purified using spin columns (GFX PCR DNA and gel band purification kit; Amersham Pharmacia Biotech, Piscataway, NJ), and sequenced directly with the ABIDyeDeoxy Terminator Cycle Sequencing Kit on an ABI-PRISM 310 Genetic Analyzer (PE-Applied Biosystems, Foster City, CA) according to the manufacturer´s specifications. Each PCR product was sequenced on both strands, and each analysis was performed at least twice independently. Sequences were compared with the genomic structure of the *CPT2* gene [Verderio et al, 1995]

To confirm the mutations and to simplify their detection we designed PCR-RFLP methods (figure 2).

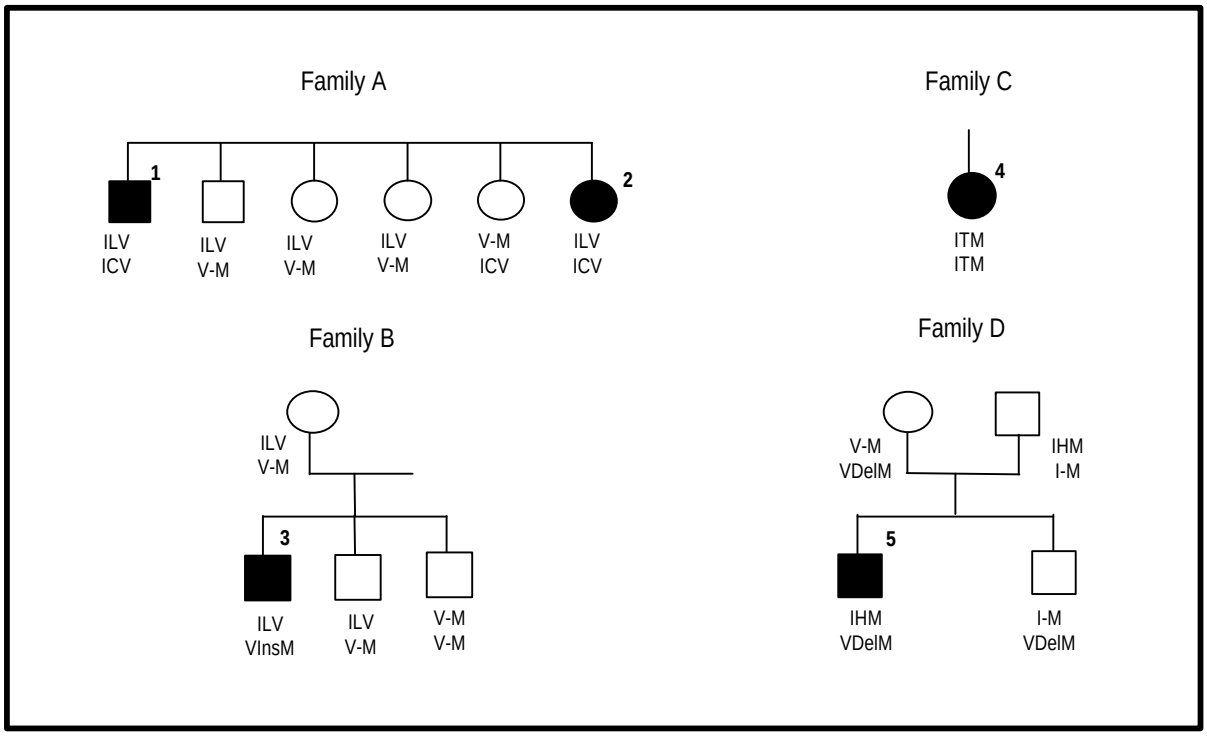

Figure 1. Family pedigrees.

Filled symbols and arabical numbers denote the probands. ILV: allele with the S113L substitution and the polymorphisms V368I and M647V; ICV: allele with the Y120C mutation and the polymorphisms V368I and M647V; V-M: normal allele without the polymorphisms V368I and M647V; VInsM: allele with the 36-38 insGC not harboring the polymorphisms V368I and M647V; ITM: allele with I502T substitution and the polymorphism V368I but not with the M647V; IHM: allele with the P50H mutation and the polymorphism V368I, but not with the M647V; I-M: normal allele with the polymorphism V368I, but not with the M647V; VDelM: allele with the 178 insT/del 25 bp mutation without the polymorphisms V368I and M647V.

RESULTS

Screening by RFLP showed that in family A patients 1 and 2 as well as three of their siblings, and in family B patient 3 and two of his relatives were heterozygous for the S113L mutation. In family D patient 5 and two of his relatives were heterozygous for the P50H substitution (Figure 1). To identify the mutations in the other alleles of our patients we amplified and sequenced genomic DNA fragments encompassing the entire coding region and intron/exon boundaries of the *CPT2* gene. Three novel mutations were identified. In addition, we found the recently reported 178 insT/del 25 bp in patient 5. Moreover, the S113L alleles were associated with both the V368I and M647V polymorphims, and the P50H allele with the V368I polymorphism. Data on distribution of mutations and polymorphisms in alleles of patients and family relatives are shown in Figure 1.

In patients 1 and 2 a heterozygous A-to-G transition at nt 875 that changed the TAC codon 120 for tyrosine to a TGC codon for cysteine in exon 4 (Y120C) was identified. This allele also carried the V368I and M647V polymorphisms. Both patients were also heterozygous for the S113L mutation.

In patient 3 a heterozygous GC insertion at codon 36-38 (nt 624-629) in exon 1 was observed (36-38 insGC). This allele did not carry the V368I or the M647V polymorphisms. The patient was also heterozygous for the S113L mutation.

In patient 4 we identified a homozygous T-to-C transition at nt 2021 that changed the ATC codon 502 for isoleucine to a ACC codon for threonine in exon 4 (I502T). These alleles also carried the V368I polymorphism.

Table 1. Clinical, biochemical, and molecular findings in patients with CPT II deficiency.

	Patient nº				
	1	2	3	4	5
Sex	*M*	*F*	*M*	*F*	*M*
Age at onset	*12*	*14*	*14*	*24*	*8*
Age at biopsy	*37*	*ND*	*21*	*41*	*13*
Myoglobinuria	+	-	+	+	+
Muscle CPT residual activity (nmol min^{-1} mg NCP^{-1})	*0.10*	*ND*	*0.11*	*0.08*	*0.04*
Molecular analysis					
S113L	+ / -	+ / -	+ / -	- / -	- / -
P50H	- / -	- / -	- / -	- / -	+ / -
R631C	- / -	- / -	- / -	- / -	- / -
Y628S	- / -	- / -	- / -	- / -	- / -
Y120C	- / +	- / +	- / -	- / -	- / -
36-38 insGC	- / -	- / -	- / +	- / -	- / -
I502T	- / -	- / -	- / -	+ / +	- / -
178 insT/del 25 bp	- / -	- / -	- / -	- / -	- / +

Normal values for CPT: see text. M, male; F, female; ND, not done; NCP, non-collagen protein.

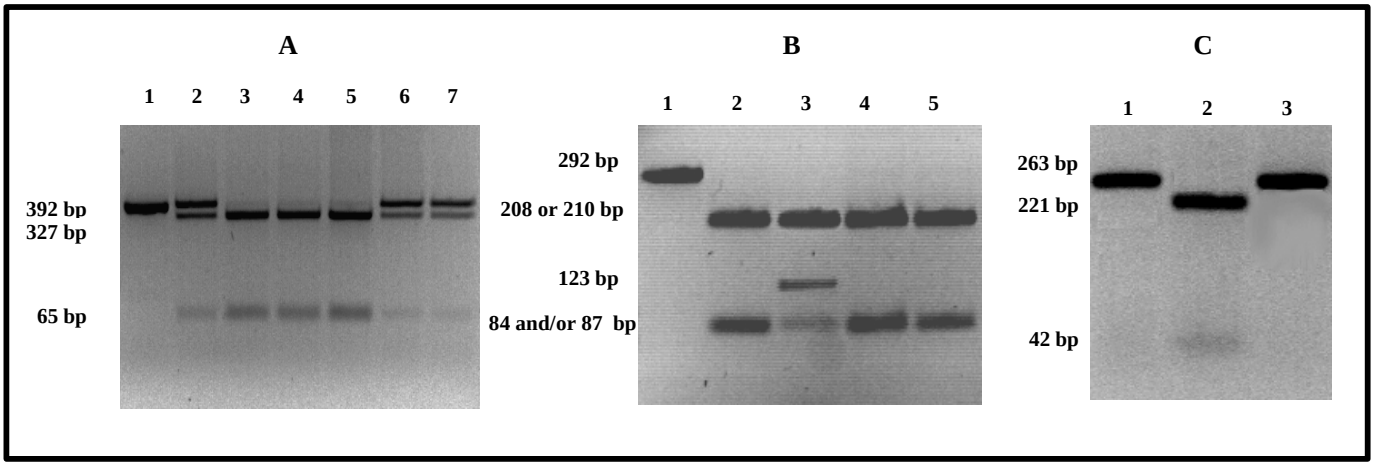

Figure 2. Polymerase chain reaction-restriction fragment length polymorphisms (PCR-RFLP) analysis.

A) PCR-RFLP analysis for the Y120C mutation with the forward primer AI3S [Verderio et al, 1995] and a reverse primer 5'-GTT TGG GTA AAC GAG TTG AGT T-3', and *Rsa* I digestion. Lane 1: undigested 392 bp fragment; lanes 2 and 7: heterozygous probands (patients 1 and 2); lane 3, 4, and 5: relatives not harboring the mutation; lane 6: relative being heterozygous for the mutation. B) PCR-RFLP analysis for the 36-38 insGC mutation with primers A1 and A2 [Verderio et al, 1995] and *Bss*H II digestion. Lane 1: undigested 292 bp fragment; lanes 2, 4 and 5: relatives not carrying the mutation; lane 3: heterozygous proband (patient 3). C) PCR-RFLP analysis for the I502T substitution with a forward primer 5'- CTG CTA AGG AAA AGT TTG ATG CCA C-3' and a modified reverse primer 5'-AAA GGC CTC AGA GCA CCT CTT TGT ATA GAC GGA GGC CGG CCG-3' (mismatched nucleotide underlined) that creates a restriction site for *Bcn* I in presence of the I502T mutation. Lane 1: undigested 263 bp fragment; lane 2: homozygous proband (patient 4); lane 3: control subject.

DISCUSSION

CPT II deficiency is the most common recessively inherited disorder of lipid metabolism affecting skeletal muscle. Typically, it is present in young adults with recurrent episodes of exercise-induced myoglobinuria [DiMauro and Melis-DiMauro, 1973; Zierz, 1994]. Atypical phenotypes of CPT II deficiency include hypoketotic hypoglycemia, cardiomyopathy and sudden death in newlyborns and children [Bonnefont et al, 1996; Hug et al, 1991], recurrent pancreatitis [Tein et al, 1994] , brain and kidney dysplasia [North et al, 1995] and malignant hypertermia-like disorder [Vladutiu et al, 1993].

Several CPT II mutations have been detected in patients from several ethnic backgrounds, establishing the genetic heterogeneity of the disease. The most common mutation in adult CPT II deficiency is a missense mutation that replaces a leucine for a serine residue at amino acid position 113 of the CPT II protein (S113L) [Taroni et al, 1993; Zierz et al, 1994; Kaufmann et al, 1997; Martín et al, 1999]. Most other mutations have been found in individual patients.

We identified three novel mutations in the coding region of the CPT II gene: two missense substitutions Y120C and I502T, and a 36-38 insGC mutation. The Y120C and 36-38 insGC mutations were observed in compound heterozygotes in combination with the S113L and thus seem to be recessive alleles. Consistent with previous reports [Taroni et al, 1993; Verderio et al, 1995] the S113L mutant allele carried the well-known polymorphisms V368I and M647V. Moreover, the Y120C mutant allele also harbored both polymorphisms, and the I502T mutant allele carried the substitution V368I alone. Each of these three novel mutations would be expected to cause the disease for several reasons: (i) the missense mutations cause non-conservative amino acid substitutions in the CPT II primary structure and the 36-38 insGC mutation results in the synthesis of a severely truncated protein; (ii) they were the only nucleotide alteration, besides the S113L mutation found in compound heterozygotes, in the coding region and splice junctions of the CPT 2 gene; and (iii) they were not detected in 100 controls of the same ethnic background.

The Y120C substitution replaces a large aromatic amino acid, tyrosine, with a small polar one, cysteine. The tyrosine 120 residue is strictly conserved in all the acyltransferases of different species and occurs in a 19-amino

acid stretch (103-121) that is completely conserved in the rat, mouse and human enzymes [Mc Garry and Brown, 1997; Finocchiaro et al, 1991]. This is consistent with a crucial role of this amino acid in the normal function of CPT II. This substitution is close to the S113L mutation commonly found in the majority of patients with CPT II deficiency.

The 36-38 insGC mutation predicts a frameshift and a premature termination of the protein 36 amino acids after the site of the mutation, resulting in a protein of 72 amino acids instead of 658 amino acids [Mc Garry and Brown, 1997]. This severely truncated protein is likely to be unstable and, since it misses the catalytic domain, is expected to be enzymatically inactive.

The I502T mutation leads to the replacement of a hydrophobic residue, isoleucine, with a polar one, threonine, within a stretch of amino acids completely conserved in both the rat and human enzymes [McGarry and Brown, 1997; Finocchiaro et al, 1991]. In M- and L-CPT I this particular amino acid residue is valine, whose structure and hydrophobicity are similar to those of isoleucine [McGarry and Brown, 1997]. Furthermore, this substitution is very close to the R503C mutation recently reported in three patients with adult CPT II deficiency [Taggart et al, 1999]. Interestingly, the patients reported by Taggar et al appeared to be simple heterozygotes, suggesting that the R503C mutation could be a dominant one. Since it produces a dramatic change, replacing a basic polar arginine residue for a disulfide donor cysteine, the R503C mutation could probably have deleterious structural/functional consequences even if present in a single allele. Compared with the R503C mutation, the I502T substitution seems to produce a less drastic change in the primary CPT II structure, and it is thus likely that overt muscle symptoms become apparent only if both alleles carry the mutation.

Molecular analysis also revealed that one patient was a compound heterozygote for the P50H mutation and for the recently reported 178 insT/del 25 bp mutation [Yang et al, 1998]. The latter results in a leucine to phenylalanine replacement followed by an in-frame deletion of amino acid residues 179-186. Six of these residues are completely conserved in the human and rat CPT II, and the lysine at position 182 is strictly conserved in mitochondrial CPTs of different species, indicating their importance for the structure /function of the enzyme [McGarry and Brown, 1997; Finocchiaro et al, 1991]. The fact that this mutation has been found in a patient of different ethnic background from that reported before supports its pathogenicity.

The Y120C and the I502T substitutions lie in exon 4, the largest among the five exons of *CPT2* gene [Verderio et al 1995], which harbors the majority of mutations reported so far in this gene.

The 36-38 insGC mutation is the most N-terminal mutation documented in the *CPT2* gene and the second one (the first was the P50H mutation) to be located in exon 1.

The presence of “private” mutations, as the ones described here, confirms the molecular heterogeneity of patients with CPT II deficiency, and suggests that the ethnic origin has to be taken into account before performing mutation analysis in these patients.

REFERENCES

Bieber LL. 1988. Carnitine. Annu Rev Biochem 57:261-283.

Bonnefont JO, Taroni F, Cavadini P. 1996. Molecular analysis of carnitine palmitoyl transferase II deficiency with hepatomuscular expression. Am J Hum Genet 58:971-978.

Demaugre F, Bonnefont JP, Collonna M, Cepanec C, Leroux JP, Saudubray JM. 1991. Infantile form of carnitine palmitoyltransferase II deficiency with hepatomuscular symptoms and sudden death. Physiopathological approach to carnitine palmitoyl transferase II deficiencies. J Clin Invest 87: 859-864.

DiMauro S, Melis-DiMauro PM. 1973. Muscle carnitine palmitoyltransferase deficiency and myoglobinuria. Science 182:929-931.

Finocchiaro G, Colombo I, DiDonato S. 1990. Purification, characterization and partial amino acid sequences of carnitine palmitoyltransferase from human liver. FEBS Lett 274: 163-166.

Finocchiaro G, Taroni F, Rocchi M, Liras A, Colombo I, Tarelli GT, DiDonato S. 1991. CDNA cloning, sequence analysis, and chromosomal localization of the gene for human carnitine palmitoyltransferase. Proc Natl Acad Sci USA 88: 661-665 (erratum: 10: 981).

Gellera C, Verderio E, Floridia G, Finochiaro G, Montermini L, Cavadini P, Zuffardi O, Taroni F. 1994. Assignment of the human carnitine palmitoyltransferase II gene (CPT II) to chromosome 1p32. Genomics 24:195-197.

Guzmán M, Geelen MJ. 1993. Regulation of fatty acid oxidation in mammalian liver. Biochim Biophys Acta 1167: 227-241.

Hug G, Bove KE, Soukoup S. 1991. Lethal neonatal multiorgan deficiency of carnitine palmitoyltransferase II. N Engl J Med 325: 1862-1864.

Kaufmann P, El-Schahawi M, DiMauro S. 1997. Carnitine palmitoyltransferase II deficiency: diagnosis by molecular analysis of blood. Mol Cell Biochem 174:237-239.

Martín MA, Rubio JC, De Bustos F, Del Hoyo P, Campos Y, García A, Börnstein B, Cabello A, Arenas J. 1999. Molecular analysis in Spanish patients with muscle carnitine palmitoyltransferase deficiency. Muscle Nerve 22: 941-943.

Mc Garry J D, Brown NF. 1997. The mitochondrial carnitine palmitoyltransferase system: from concept to molecular analysis. Eur J Biochem 244:1-14.

North KN, Hoppel CL, De Girolami U, Kozakewich HP,Korson MS. 1995. Lethal neonatal deficiency of carnitine palmitoyltransferase II associated with disgenesis of the brain and kidneys. J Pediatr 127:414-420.

Norum K. 1964. Palmityl CoA: carnitine palmityltransferase. Biochim Biophys Acta 89: 95-108.

Roe CR, Coates PM. 1995. Mitochondrial fatty acid oxidation disorders. In: Scriver C, Sly WS, Valle D, editors. Metabolic and molecular basis of inherited disease. 7 th edition. New York: McGraw Hill. P 1501-1533.

Taggart RT, Smail D, Apolito C, Vladutiu GD. 1999. Novel mutations associated with carnitine palmitoyltransferase II deficiency. Hum Mutat 13: 210-220.

Taroni F, Verderio E, Fiorucci S, Cavadini P,Finochiaro G, Uziel G, Lamantea E, Gellera C, DiDonato S. 1992. Molecular characterization of inherited carnitine palmitoyltransferase II deficiency. Proc Natl Acad Sci USA 89: 8429-8433.

Taroni F, Verderio E, Dworzak F, Willems PJ, Cavadini P, DiDonato S. 1993. Identification of a common mutation in the carnitine palmitoyltransferase II gene in familial recurrent myoglobinuria patients. Nat Genet 4:314-320.

Tein Y, Christodoulou J, Donner E, McInnes RR. 1994. Carnitine palmitoyltransferase II deficiency: a new cause of recurrent pancreatitis. J Pediatr 124:938-940.

Verderio E, Cavadini P, Montermini L, Wang H, Lamantea E, Finochiaro G, DiDonato S, Gellera C, Taroni F. 1995. Carnitine palmitoyltransferase II deficiency: structure of the gene and characterization of two novel disease-causing mutations. Hum Mol Gen 4:19-29.

Vladutiu GD, Hogan K, Saponara I, Tassini L, Conroy J. 1993. Carnitine palmitoyltransferase deficiency in malignant hyperthermia. Muscle Nerve 16: 485-491.

Wataya K, Akanuma J, Cavadini P, Aoki Y, Kure S, Invernizzi F, Yoshida Y, Kira J, Taroni F, Matsubara Y, Narisawa K. 1998. Two CPT 2 mutations in three Japanese patients with carnitine palmitoyltransferase deficiency: functional analysis and association wiyh polymorphic haplotypes and two clinical phenotypes. Hum Mut 11:377-386.

Yang BZ, Ding JH, Roe D,Dewese T, Day DW, Roe CR. 1998. A novel mutation identified in carnitine palmitoyltransferase deficiency. Mol Genet Metab 63:110-115.

Zierz S. 1994a. Carnitine palmitoyltransferase deficiency, In Engel AG, Franzini-Armstrong C, editors. Myology, 2nd edition, New York: McGraw Hill.1994 p 1577-1586.

Zierz S, Engel AG, Olek K. 1994b. The Ser113Leu mutation in the carnitine palmitoyltransferase II gene in patients with muscle carnitine palmitoyltranferase deficiency. Muscle Nerve 19 Suppl 1: S129.