Aus der Universitatsklinik und Poliklinik fur Neurologie
der Martin-Luther-Universitat Halle-Wittenberg
(Direktor: Prof. Dr. Stephan Zierz)

Clinical, biochemical and genetic characterization of muscle carnitine palmitoyltransferase II (CPT II) deficiency

Habilitation
zur Erlangung des akademischen Grades
Dr. rer. medic. habil.

vorgelegt
der Medizinischen Fakultat
der Martin-Luther-Universitat Halle-Wittenberg

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Datum der Vorlesung: 2. Mai 2019
Datum der Verteidigung: 21. Mai 2019

Referat und bibliographische Beschreibung

Carnitine Palmitoyltransferase II (CPT II) katalysiert den Transfer von langkettigen Fettsäuren aus dem Zytoplasma in die Mitochondrien während der Oxidation von Fettsäuren. CPT II Defekt ist als die häufigste autosomal-rezessiv vererbte Erkrankung des Lipidstoffwechsels und die häufigste Ursache der erblichen Myoglobinurie angesehen.

In der vorliegenden Arbeit wurden klinische, biochemische und molekulargenetische Daten in einer Kohorte von 59 Patienten mit Muskel-CPT-II-Mangel analysiert. Episoden von Myoglobinurie traten bei 80% der Patienten auf. Bei 95% war der auslösende Faktor die Körperliche Belastung. Obwohl die myopathische Form oft als Erwachsener Typ bezeichnet wird, war das Alter bei 61% der Patienten in der Kindheit (1-12 Jahre). Alle biochemisch untersuchten Patienten (n=42) zeigten im Muskelhomogenat eine normale Enzymaktivität von Gesamt-CPT I+II, aber die Aktivität wurde signifikant durch Malonyl-CoA und Triton inhibiert. FGF-21Konzentration in Attacken freien Intervallen war normal bei den Patienten, bei denen dieser Mitochondrialer Biomarker gemessen wurde (n=13).

Die p.Ser113Leu-Mutation wurde bei 46/49 Indexpatienten (94%) in mindestens einem Allel nachgewiesen. Deeizehn andere Mutationen wurden ebenfalls identifiziert. Es gab keinen bemerkenswerten Unterschied im klinischen und biochemischen Phänotyp von Patienten mit p.Ser113Leu-Mutation in homozygoter oder Compound heterozygoter Form. Die Ausnahme war eine Tendenz von etwas höherer restlicher Enzymaktivität bei der Inhibierung von Malonyl-CoA in Ser113Leu Compound Heterozygoten. Obwohl der CPT-II-Mangel als autosomal-rezessiv betrachtet wird, können heterozygote Patienten mit nur einem Mutanten-Allel auch die typischen Symptome zeigen.

Die klinischen Daten, die durch Fragebogenerhebung in einer Untergruppe mit 13 Patienten erhalten wurden, hinweisen ebenfalls, dass die Häufigkeiten der Attacken von Symptomen beim myopathischen CPT II-Mangel sehr unterschiedlich sind. Fragebogen Studie zeigt, dass übergewichtige oder adipöse Patienten ein erhöhtes Risiko für häufigere Attacken haben können. Darüber hinaus brauchten die Patienten im Durchschnitt fast drei Jahrzehnte für die Diagnose eines CPT-II-Mangels, an dem mehrere Ärzte beteiligt waren. Dies zeigt, dass das mangelnde Bewusstsein für diese Erkrankungen bei vielen Ärzten.

Bibiliographie

Joshi, Pushpa Raj: Clinical, biochemical and genetic characterization of muscle carnitine palmitoyltransferase II (CPT II) deficiency. Universität Halle-Wittenberg, Medizinische Fakultät, Habilitation, 73 Seiten, 2018

Contents

Abbreviations

ATP: Adenosine triphosphate

BAT: brown adipose tissue

BMI: Body mass index

CACT: Carnitine-acylcarnitine translocase

cDNA: Complimentary Deoxyribonucleic acid

CI: Confidence interval

CK: Creatine kinase

CoA: Coenzyme A

CoASH: Coenzyme A

CPT: Carnitine Palmitoyltransferase

CTD: Carnitine transporter disorder

DNA: Deoxyribonucleic acid

dNTP: Deoxyribonucleotide triphosphate

EDTA: Ethylenediaminetetraacetic acid

ELISA: Enzyme linked immunosorbent assay

F: Female

FGF-21: Fibroblast growth factor 21

GSDII: Glycogen storage disease type II

GSDV: Glycogen storage disease type V

KCl: Potassium chloride

KCN: Potassium cyanide

kDa: Kilo Dalton

l: Liter

lcFA: Long chain fatty acid

lcFAOD: Long cjain fatty acid oxidation defect

M. Pompe: Morbus Pompe

M: male

MAD: Multiple acyl-CoA dehydrogenase

$MgSO_4$: Magnessium sulphate

MHC: Myosine heavy chain

Ml: Mililiter

mM: Milimolar

mRNA: Messenger ribonucleic acid

MTP: Mitochondrial trifunctional protein

N: Number

NLSD: Neutral lipid storage disease

nt: Nucleotide number

OMIM: Online Mendelian inheritance in man

OPD: Out-patient-department

PCR: Polymerase chain reaction

pg: Picogram

RFLP: restriction fragment length polymorphism

RT-PCR: Reverse transcription polymerase chain reaction

SD: Standard deviation

U: Unit

V: Volt

VAS : Visual analogue scale

VLCAD: Very long-chain acyl-CoA dehydrogenase

Yrs.: Years

1. Introduction and Outline

Long-chain fatty acids (lcFA) are an important source of energy, especially for the heart, liver and muscles. They are used as the preferred substrate in the myocardium at rest and during prolonged exercise in skeletal muscle [1]. Additionally, fatty acids also serve as building blocks for membrane lipids and cellular signaling molecules [2–5]. Ketone bodies produced in the liver during the oxidation of long-chain fatty acids can replace glucose in the brain and thus ensure the maintenance of normal content of sugar in the blood. In addition, the ketone bodies prevent the breakdown of muscle protein for the purpose of gluconeogenesis [6].

The mechanism of transport of fatty acids from cytoplasm to mitochondria where they are oxidized and the components that are essential for the transport are described below:

1.1 Long chain fatty acid transport system

A long-chain acyl fatty acid derivative ester of carnitine facilitates the transfer of long-chain fatty acids from cytoplasm into mitochondria during the oxidation of fatty acids [7]. This Carnitine Palmitoyltransferase (CPT) is present in two subforms, CPT I and CPT II that are localized in mitochondria [8,9]. CPT I is transmembrane protein that is located at the outer mitochondrial membrane [10]. There are three tissue specific isoforms of CPT I:

(i) The liver and fibroblast specific isoform (CPT IA or LCPT I) is encoded at chromosome 11q13.1 [9,11]. This protein has 773 amino acids and is about 88 kDa. LCPT I is synthesized in liver, kidneys, lungs, spleen, intestine, ovaries, lymphocytes, fibroblasts and brain [12–14].

(ii) The second isoform is found in skeletal and cardiac muscles (CPT I B or M CPT I) on chromosome 22q13.31 [9,13]. This isoform constitutes of 772 amino acids and the protein is also about 88 kDa. It is predominantly present in the skeletal muscles, heart, adipose tissues and testicles [15]. The L and M isoforms are 63 % identical but they still show varied kinetic properties [14,16].

(iii) The third and common brain isoform is encoded by gene on chromosome 19q13.33 [17]. The gene encoding this isoform was identified in 2002 [17]. It constitutes of 803 amino acids and the protein is about 91 kDa. This form is predominantly present in brain and in lesser amount in testicles, ovaries and intestine [14].

On the other hand, CPT II is encoded on chromosome 1p32.3 and is localized on the inner mitochondrial membrane [18–20]. CPT II has only one isoform. The cDNA of CPT II was

first isolated from rat muscles in 1990 [19] followed by isolation of cDNA in humans 1 year later [21]. The human *CPT2* gene is about 20 kb long and consists of 5 exons [19]. However, coding of more than half of the translating sequence of the gene is in exon 4 [20].

Apart from CPT I and CPT II, Carnitin/Acylcarnitin-Translokase (CACT) also takes part in transport of the long-chain fatty acids across the inner mitochondrial membrane into the mitochondrial matrix for β-oxidation [22,23]. CACT catalyzes the one to one exchange of acyl L-carnitine with L-carnitine and a much slower, unidirectional transport of L-carnitine. This leads to equilibrium of the L-carnitine concentration via the inner mitochondrial membrane [24–27]. CACT is encoded on chromosome 3p21.31. The CACT coding gene has 301 amino acids and the protein is 32.9 kDa [28].

These three components [Carnitine palmitoyltransferases (CPT I and CPT II), Carnitin-Acylcarnitin-Translocase] are the integral part of the transport system for esterification of fatty acids through the mitochondrial membrane (Figure 1).

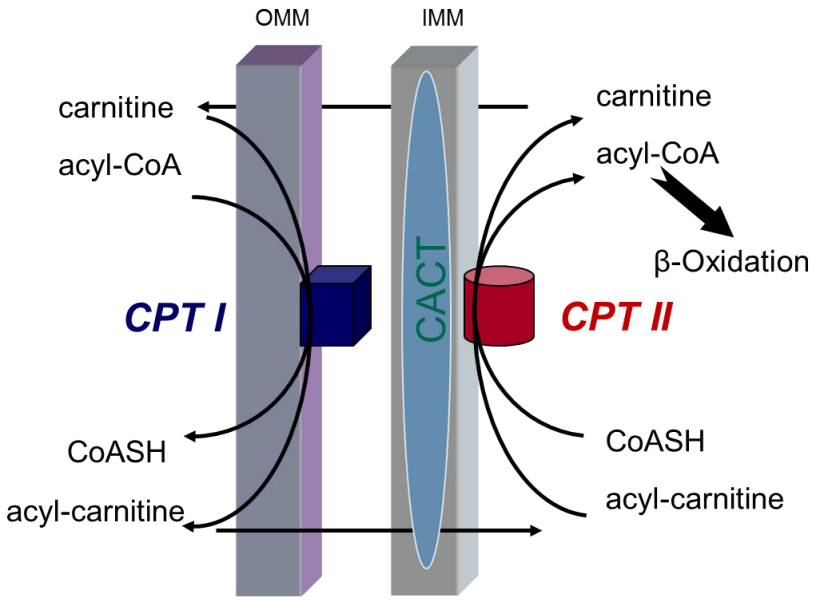

Figure 1: Transport system for esterification of fatty acids through mitochondrial membranes. (*CoASH: free CoEnzyme A. CPT I catalyzes the reaction where long chain Acyl- CoA with Carnitine are converted to long chain Acyl- Carnitine und Coenzym A.The resulting Acyl- Carnitin is transported to the mitochondrial matrix through Carnitine-Acylcarnitine-Translokase. The CPT II on inner mitochondrial membrane transfers the acyl residue of the acyl-carnitine into to coenzyme. Acyl-CoA and carnitine are formed.)*

Carnitin-palmitoyltransferase I at the outer mitochondrial membrane catalyzes the first transport step. At this step, long-chain acyl-CoA together with carnitine is converted into long-chain acyl carnitine and coenzyme A. This catalysis step is rate-limiting for all subsequent reactions including β-oxidation [29]. One of the end products of this reaction, acyl carnitine, can now be transported through the inner mitochondrial membrane by means of the carnitine acylcarnitine translocase. The remaining acyl of the acyl carnitine is transferred back to coenzyme A on the inner mitochondrial membrane by carnitine palmitoyltransferase II. This results in production of acyl-CoA and carnitine. The carnitine released in this way returns through the carnitine-acylcarnitine translocase back into the intermembrane space of the mitochondrion and is available for the re-transport of fatty acids [24–27]. A disorder of the carnitine palmitoyltransferase system may affect both CPT I and CPT II enzyme production. Both of the resulting diseases follow autosomal recessive mode of inheritance.
Figure 1 depicts the schematic representation of transport system for esterification of fatty acids through mitochondrial membranes.

1.2 Carnitine palmitoyltransferase II (CPT II) deficiency

Carnitine palmitoyltransferase II (CPT II) deficiency is the most common inherited disorder of long-chain fatty acid oxidation affecting skeletal muscle [30–32]. During prolonged exercise, fasting, exposure to cold, fever, emotional stress, drugs, and other strenuous conditions production of extra energy demand is met by oxidation of fatty acids. In these conditions, long-chain fatty acids are the main source of energy substrate of muscle. However long-chain fatty acids do not readily diffuse across the mitochondrial membrane and hence require trans-esterification to acylcarnitine. As discussed above, formation of acylcarnitine from carnitine and long-chain fatty acyl-CoA is catalyzed by CPT I at the outer mitochondrial membrane and then crosses the inner mitochondrial membrane. At the inner side of inner mitochondrial membrane formation of acyl-CoA is catalysed by CPT II. Acyl-CoA is then available for ß-oxidation [33].

1.2.1 Phenotypes of CPT II deficiency

There are three phenotypes of CPT II deficiency:

(i) Lethal neonatal form presenting with hypoketotic hypoglycaemia and severe hepatomuscular symptoms [34–37]. The lethal neonatal form is characterized by

reduced CPT II enzyme activity in multiple organs, reduced serum concentrations of total and free carnitine, and increased serum concentrations of long-chain acylcarnitines and lipids. The patients are reported to have liver failure, hypoketotic hypoglycemia, cardiomyopathy, respiratory distress, and/or cardiac arrhythmias. Affected individuals have liver calcifications and cystic dysplastic kidneys [34,35]. An analysis of 19 patients with neonatal form of CPT II deficiency illustrated polycystic kidneys (n=9), hydrocephalus (n=8), cerebellar vermian hypoplasia (n=5), polymycrogyria (n=4), pachygyria (n=4), cerebral calcifications (n=3), cystic dysplasia of the brain (n=2) and agenesis of the corpus callosus (n=1) [37]. So far, about 20 families with the lethal neonatal form [37–41] have been described. CPT II deficiency or other fatty acid oxidation disorders are mostly undetectable during pregnancies due to severe cerebral malformations of the foetus. Hence prevalence of this form seems to be higher than previously suspected.

(ii) Severe infantile hepatocardiomuscular form that is characterized by hypoketotic hypoglycemia, liver failure, cardiomyopathy, and peripheral myopathy [34,42–44]. The main cause of death in this form during infancy could be cardiac arrhythmia [34,45]. Apart from cardiac arrhythmia, hepatomegaly [44] and Dandy-Walker malformation could also turn out to be fatal [43]. Some 30 families with this form of CPT II deficiency are described, so far [34,42–45].

(iii) The classical myopathic form is rather mild and it is clinically characterized by recurrent episodes of muscle pain, muscle weakness, and rhabdomyolysis triggered by prolonged exercise [32,46–49]. Affected individuals are generally asymptomatic with no muscle weakness between attacks. Some individuals have only a few severe attacks and are asymptomatic most of their lives, whereas others have frequent myalgia, even after moderate exercise, such that daily activities are impaired and disease may worsen. End-stage renal disease caused by interstitial nephritis with acute tubular necrosis requiring dialysis occasionally occurs [50].

1.2.2 Myopathic form of CPT II deficiency

Myopathic form can manifest from infancy to adulthood (OMIM 600650). Although it is sometimes also termed as ‘adult form’ [8,11,13], cases with early childhood manifestation have also been reported [38,52–56]. Similarly, adult patients with severe

hepatocardiomuscular infantile form of CPT II deficiency are also sporadically reported [39,57].

Clinical presentations: In myopathic CPT II deficient patients, onset of the disease is seen generally in childhood or early adulthood [58]. Single or multiple attacks of severe myalgia (often with myoglobinuria) or frequent exercise-induced myalgia are more common symptoms in CPT II–deficient patients [30,47,54,55,58]. Frequent exercise-induced myalgia is characteristic for Myoglobinuria and is also known to be the clinical hallmark of muscle CPT II deficiency [30,32]. Severity of the attacks can be highly variable, and life-threatening rhabdomyolysis that required dialysis is not frequent in CPT II deficient patients [30,32,58]. The most important trigger factor for attacks is reported to be exercise [30,47,54,55,58].
Usually no signs of myopathy (weakness, myalgia, elevation of serum creatine kinase [CK] concentration) are seen between attacks. Even during the attacks, the severity of pain is highly variable and some of these attacks may be complicated by acute renal failure. Topçu and co-workers have recently reported a case of a 13-year-old girl with recurrent rhabdomyolysis due to CPT II deficiency whose last attack was complicated by acute renal failure. The patient had homozygous p.Ser113Leu mutation. CK values monitored during before and after the attacks were drastically different (Figure 2).

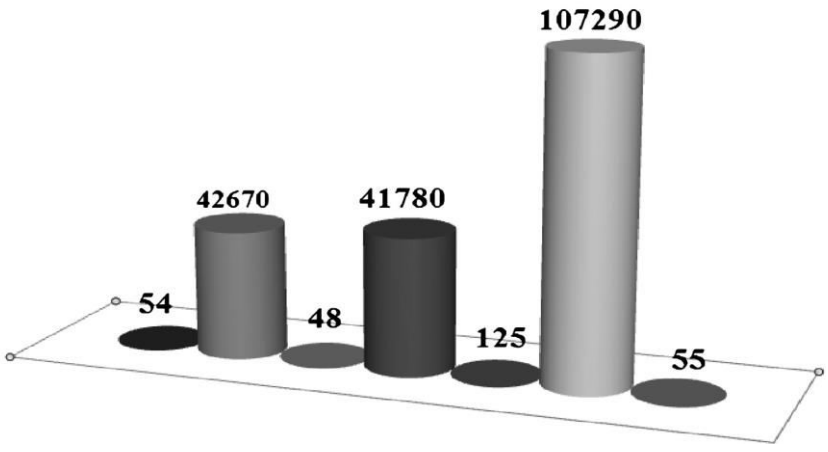

Figure 2: Creatine kinase (CK) values in a CPT II deficient patient monitored before, during and after attacks. The numbers denote level of CK in U/L (Figure adapted from Topçu, et al.; 2018 *[59]*)

The three high peaks in figure 2 are excessively increased CK level during attacks. The last attack (CK: 107290 U/l) was complicated by acute renal failure. Apart from periods during attack, CK levels were normal in this patient. The patient did not have permanent weakness [59]. However, sporadically single cases with permanent weakness are also reported [30,60]. This is in contrast to another common metabolic myopathy, McArdle glycogenosis, which is also associated with exercise-induced myoglobinuria. In McArdle diseases, fixed weakness is more frequent. In a study we conducted with McArdle patients, permanent weakness was reported in one third patients [61]. This was in line with the findings of Martin and co-workers who reported permanent weakness in about one fourth patients [62]. In general, the creatine kinase levels in between the attacks in CPT II deficient patients are within the reference range (< 80 U/L) [20]. However permanent elevation of serum CK concentration (≤ 313 U/L) is observed in approximately 10% of affected individuals [63]. Interestingly, more than three fourth of the CPT II deficient patients reported so far are males. This shows a clear male predominance in CPT II deficiency [32,58,61,62,64].

Pathobiochemical characteristics: In CPT II deficiency, acylcarnitine can be transported across the inner mitochondrial membrane, but conversion into acyl-CoA is insufficient. The result is an accumulation of acylcarnitine in the plasma, which is also used for diagnostic purposes. Long-chain fatty acids are the main source of energy for the human muscle during exercise. The severity of the diseases (whether severe neonatal, infantile or a mild adult form) depends on the underlying mutation. The ubiquitous presence of CPT II- explains multisystemic organ involvement in neonatal and infantile disease.

High-performance liquid chromatography tandem mass spectrometry of dried blood spots (i.e, the acylcarnitine profile) demonstrates an elevation of C12 to C18 acylcarnitines, notably of C16 and C18:1 in CPT II deficiency. Following table compares the Acylcarnitine characteristics for long chain fatty acid oxidation disorders (lcFAOD) (Table 1).

Table 1: Acylcarnitine characteristics for lcFAODs (Table adapted from Knottnerus, et al. 2018 *[65]*)

Deficient enzyme	**Acylcarnitine profile changes**	**Primary marker (NBS)**	**References**
Carnitine transporter (CTD)	C0↓	C0↓	[66]
CPT I	C0↑,C2↓, acylcarnitine↓	C0/(C16 + C18)↑	[67,68]
CACT	C16↑, C18↑, C18:1↑, C18:2↑	(C16 + C18:1)/C2↑	[69]
CPT II	**C16↑, C18↑, C18:1↑, C18:2↑**	**(C16 + C18:1)/C2↑**	[70]
Very long-chain acyl-CoA dehydrogenase (VLCAD)	C12↑, C14↑, C14:1↑, C16↑, C18↑	C14:1/C2↑	[67,71]
Mitochondrial trifunctional protein (MTP)	C18OH↑, C16OH↑, C16↑, C14OH↑	C16OH↑, C18OH↑	[72]

However, CPT II deficiency cannot be excluded based solely on acylcarnitine quantification in dried blood spots alone and investigation of plasma is recommended for reliable diagnosis [73]. In our experience there are numerous false negative results based on dried blood analysis.

As discussed, CPT II deficiency is the disorders of lipid metabolism affecting muscle involving β-oxidation. The pathological hallmark of some of these diseases is an increased neutral lipid content, which may be observed on muscle biopsies specimen with the specific staining of Sudan black or oil red O techniques by optic microscopy. In a normal muscle, lipid content takes the aspect of small droplets which concentration and size are usually higher in type 1 fibres than in type 2 fibres [74].

Comparison of contents of muscle lipid droplets in different forms of lipidosis is illustrated in table 2.

A recent MRI study on long chain fatty acid (lcFAO) disorder patients has shown association of specific patterns of increased T1W and STIR signal intensity in lcFAO patients. These patterns reflect lipid accumulation and inflammation secondary to lcFAO defects and progressive muscle damage. T1W and STIR signal intensities were less prominent in muscle MRIs of CPT II deficient patients. However, the significance of MRI investigation in CPT II deficiency is still unclear. Future studies are needed to investigate whether muscle MRI might be a useful tool to monitor the disease course and to study pathogenesis of CPT II deficiency and of other lcFAO related myopathies [75].

Table 2: Clinical and biological features of metabolic disorders with muscle lipidosis (Table modified from Laforet and Vianey-Saban *[74]*).

Disorder	**Main neuromuscular symptoms**	**Increase in muscle lipid droplets**	**Gene**
Primary carnitine deficiency	Proximal muscle weakness, cardiomyopathy	+++	*SLC22A5*
Neutral lipid storage disease (NLSD)	Proximal or distal muscle weakness, cardiomyopathy	+++	*ABHD5* *PNPLA2*
Multiple acyl-CoA dehydrogenase (MAD) deficiency	Proximal and axial weakness Rhabdomyolysis (rarely)	++ to +++	*ETFDH*
CPT II deficiency	**Rhabdomyolysis episodes**	**0 to +**	***CPT2***
Very-long-chain acyl-CoA dehydrogenase (VLCAD) deficiency	Rhabdomyolysis episodes Cardiomyopathy	0 to +	*ACADVL*
Mitochondrial trifunctional protein (MTP) deficiency	Rhabdomyolysis episodes Cardiomyopathy Axonal peripheral neuropathy	0 to +	*HADHA* *HADHB*
Phosphatidic acid phosphatase deficiency	Rhabdomyolysis episodes	0 to +	*LPIN1*

In CPT II deficiency, normal or very slightly increased content of lipid is seen in biopsy sections under microscope. This is similar in some other lipid metabolism disorders such as, very-long-chain acyl-CoA dehydrogenase (VLCAD) deficiency, mitochondrial trifunctional protein (MTP) deficiency and phosphatidic acid phosphatase deficiency. Table 2 compares the contents of neutral lipid that is found in different metabolic disorders with muscle lipidosis.

Additionally, the histological examination of the muscle biopsy sections of CPT II deficient patients seldom shows accumulation of lipid droplets in Sudan staining. The comparison of Sudan staining of muscle biopsy sections of CPT II deficient patient and patient with primary carnitine deficiency is illustrated in the following figure (Figure 3).

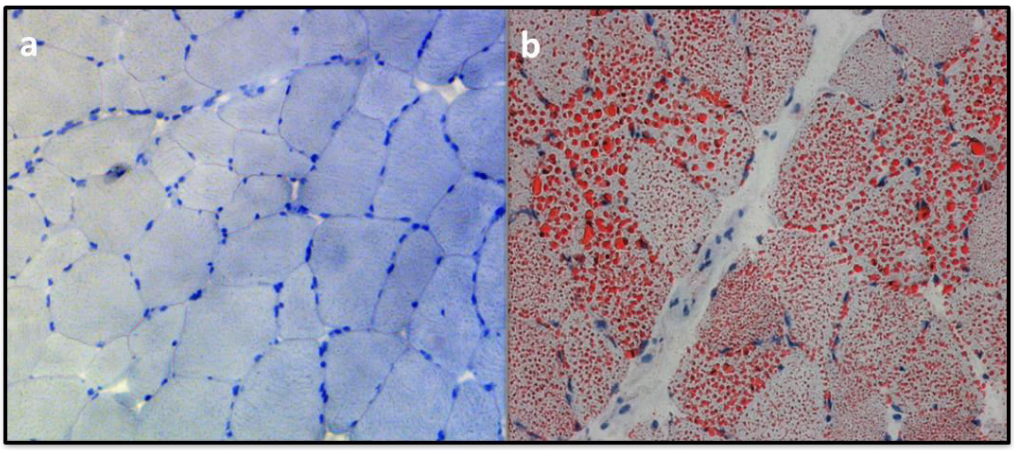

Figure 3: Sudan staining of muscle biopsy sections of (a) CPT II deficient patient compound heterozygous for p.Ser113Leu/p.Arg151Gln mutations and (b) primary carnitine deficient patient.

As depicted by figure 3, there is no or very less lipid accumulation in muscles of CPT II deficient patient (figure 3 a) in comparison to excessively accumulated lipid droplets in carnitine deficient patients (figure 3b). Figure 3a shows the Sudan staining of muscle biopsy section of a 35-years old female CPT II deficient patient compound heterozygous for p.Ser113Leu/p.Arg151Gln mutations. The patient suffered from at least 50 attacks per year and the residual CPT II activities upon malonyl CoA and Triton X were severely reduced. In between the attacks, the patient did not experience severe physical disability. This shows that the symptoms in CPT II deficiency are also only intermittent in comparison to other lipid accumulation deficiencies such as carnitine deficiency or neutral lipid storage disease [74,76]. Hence, the normal protein content and enzyme activity seem to allow a normal function of the CPT system in situations without stress on fatty acid metabolism.

Immunohistochemistry: Immunohistological analysis of muscle sections of CPT II deficient patients using CPT II rabbit polyclonal antibody have demonstrated CPT II in similar intensity as in controls (Figure 4). The immunoreactivity identified by MHC-slow staining was expressed predominantly in type I fibers [77].

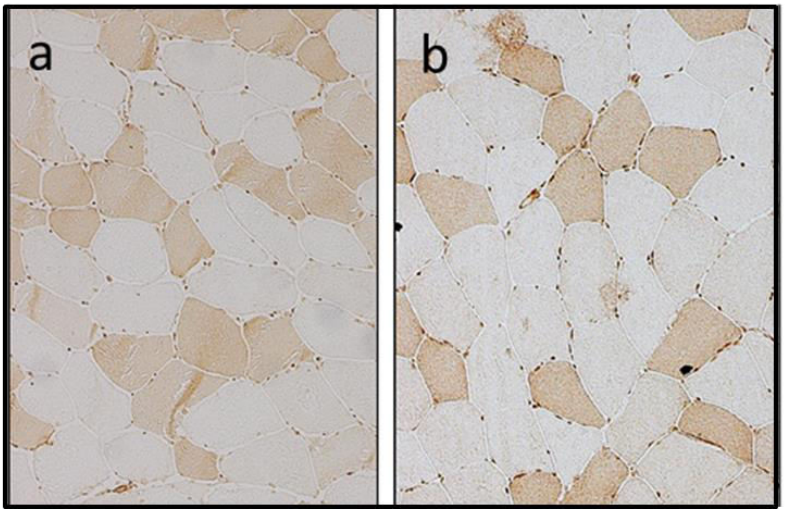

Figure 4: Immunohistochemical staining of CPT II (a) control muscle section and (b) muscle section of CPT II deficient patient (figure adapted and modified from Lehmann & Zierz; 2014 *[77]*)

Molecular genetic aspects: In patients with the myopathic form of CPT II deficiency, a common p.Ser113Leu mutation (Figure 5) is identified in about 70% of mutant alleles [32,47,48,55]. This missense mutation is located in exon 3 of the *CPT2* gene and results in exchange of amino acid serine at position 113 to leucine. The phenotypes of this mutation are generally mild. This mutation is exclusively associated with muscle form of CPT II deficiency.

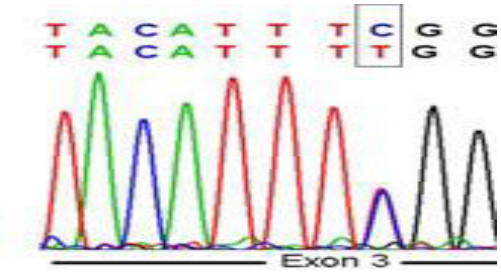

Figure 5: Sequencing electropherogram showing the 'common' p.Ser113Leu mutation in heterozygote state in exon 3 of *CPT2* gene

Furthermore, several other rare disease causing mutations that are associated with muscle form of CPT II deficiency have been reported [32,78]. The complete list of mutations identified in CPT2 gene is included in Appendix 1. The biochemical consequences of these mutations are still controversial [79]. Hypotheses include lack of enzymatically active protein, partial enzyme deficiency and abnormally regulated enzyme [80]. In previous studies, CPT

activities in muscles of patients with CPT II deficiency are reported to be undetectable [46,81,82], reduced [83–87] or normal [88].

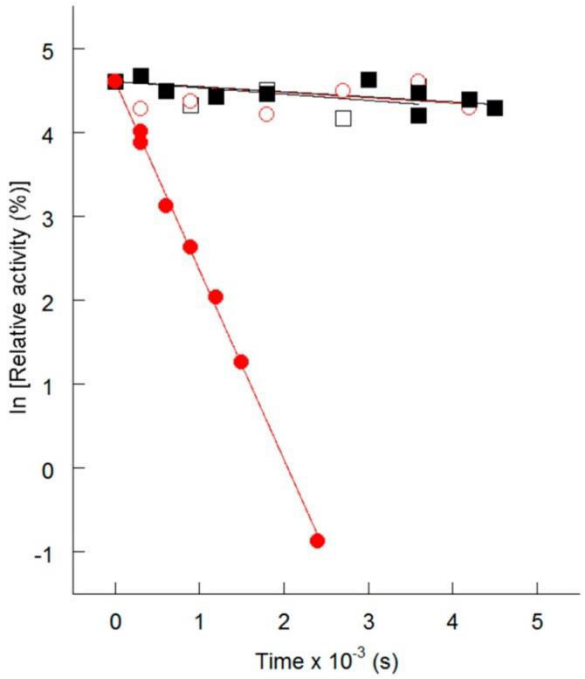

Figure 6: Thermal inactivation of His6-N-hCPT2 (open symbols) and His6-N-hCPT2/Ser113Leu (closed symbols) at 30 and 40 °C. Black squares show thermal inactivation at 30 °C, red circles represent values at 40 °C. The data is presented as time-dependent changes of natural-log-transformed relative activities. (Figure adapted from Lehmann et al.; 2016 *[79]*)

A recent study conducted in our laboratory revealed the phenomenon of impaired kinetic stability of human CPT II by p.Ser113Leu mutation at increased temperatures (Figure 6) [80]. This was consistent with the lower heat resistance of the mutated enzyme in cultured fibroblasts [89]. The biochemical consequences of other CPT II mutations in our laboratory are still under investigation.

1.3 FGF-21 Activity in CPT II deficiency

FGF-21 (Fibroblast growth factor 21) has been established as a biomarker for diagnosis of mitochondrial diseases [90–96]. Suomalainen and coworkers have for the first time reportrd excessively increased concentration of FGF-21 in serum of patients with mitochondrial disorders (Figure 7) [92].

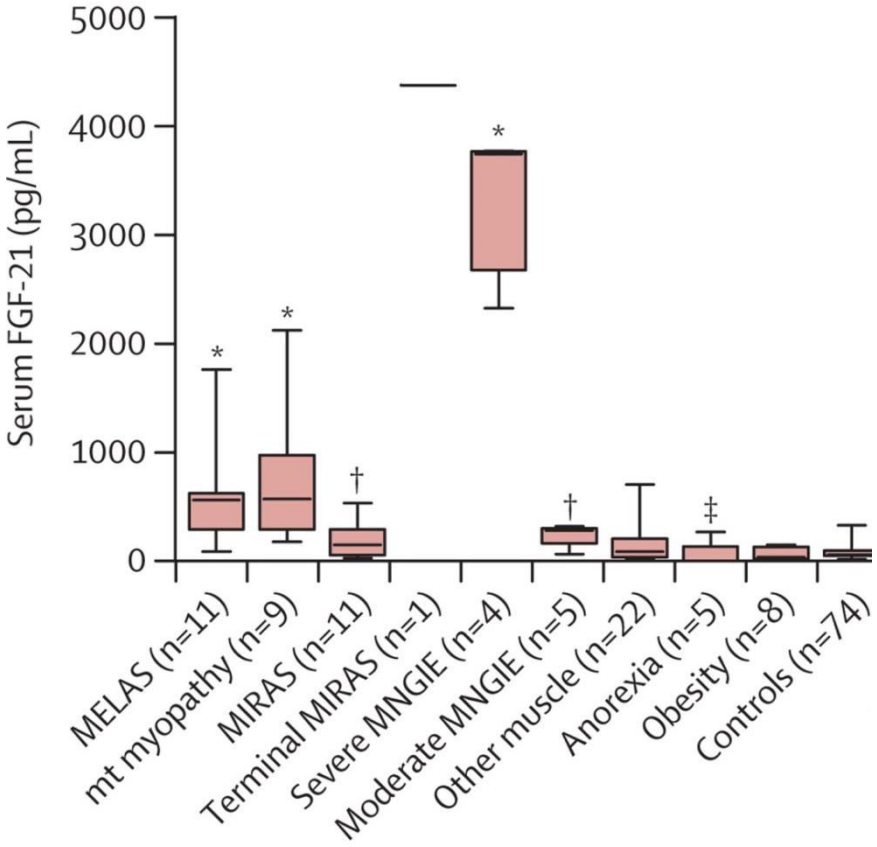

Figure 7: FGF-21 concentrations in serum in patients with mitochondrial disorders and healthy controls (Figure adapted from Suomalainen, et al., 2011 *[92]*)

FGF-21 level in humans with CPT II deficiency is not known of yet. However, studies on high fat-fed mice with a liver-specific knockout of CPT II (Cpt2L−/−) showed increased serum FGF-21 levels. This reflected an adopting role of hepatokinase in response to a loss of mitochondrial fatty acid oxidation [97,98]. Another group of mice with an adipose-specific knockout of CPT II (CPT2A−/−) [99], showed an increased expression of mRNA of FGF-21 in brown adipose tissue (BAT) following cold exposure [100]. Based on these observations, FGF-21 measurement in human subjects would show us whether the level of FGF-21 is also increased in them during attack free condition.

2. Aims of the study

There is a wide spectrum of symptoms in muscle CPT II deficiency. In general, different triggering factors and varied muscles involvement associated with CPT II deficiency are reported. In addition, CPT II deficiency presents with diverse biochemical and molecular genetic features. However, there are only few systematic studies on a considerable number of CPT II deficient patients (>20 patient) that have characterized the clinical, biochemical and molecular aspects of this disorder. A genotype-phenotype correlation based on diverse clinical, biochemical and molecular genetic aspects has not been established in CPT II deficiency. Present study addresses following aspects of CPT II deficiency:

1. A systematic clinical characterization of CPT II deficient patients from the clinical data stored in the department of Neurology since 1994.
2. A retrospective survey based on questionnaires in patients that are available for personal interview. This will enable us to compare the clinical data of the patients taken directly from their history files from our archive and the clinical data of the patients collected through questionnaires. Apart from that, the questionnaire survey will address the issues such as affected muscle locations, intensity of pain during different attacks of symptoms, pain mitigating methods implied by patients, living standard of the patients and diagnosis timeline.
3. Measurement and interpretation of total and residual CPT II enzyme activities in muscle biopsies of patients with the muscle phenotype of muscle CPT II deficiency.
4. Molecular genetic analysis of the patients based on sequencing of complete *CPT2* gene.
5. Measurement of activities of mitochondrial biomarker FGF-21 in muscle CPT II deficient patients. This would identify the suitability of FGF-21 as possible diagnostic tool in CPT II deficiency.
6. Establishing a possible genotype-phenotype correlation in CPT II deficiency.
7. Comparison of clinical data of CPT II deficient patients with that of glycogen metabolic myopathies to identify similarities and differences between glycogen metabolic myopathies and long-chain fatty acid metabolic myopathy.

3. Patients

Clinical, biochemical and molecular genetic data in a cohort of 59 patients (49 index cases) with muscle form of CPT II deficiency are analyzed. The patient data were retrospectively analyzed from the patient files in our department where clinical data of patients since 1994 are archived. The analyzed data include that of patients who personally reported to our neuromuscular out-patient-department (OPD) or the clinical data shared by referring clinicians from external hospitals. Thirty six patients were males and 23 were females. The mean age of patients was 28 years (range: 1-60 years, Median 25.5 years). CPT II activity was measured in muscle biopsies of 42 patients (38 index cases) (Table 3). Biopsies of other 17 patients were not available for biochemical investigation. Diagnosis was directly based on clinical and molecular genetic findings.

Apart from that, 13 patients from this cohort were available for direct interview that accessed their clinical characteristics by means of questionnaire survey. Additionally, mitochondrial biomarker FGF-21 level also measured in serums of these 13 patients and compared with 20 normal controls.

Table 3: Gender and age distribution of CPT II deficient patients included in the study

Gender	**Age (yrs.)** Mean (median, Range)	**Biopsy available (n)**
Male (n=36)	30.1 (36.5, 4-57)	27
Female (n=23)	24.6 (21, 1-60)	15
Total (n=59)	**28 (25.5, 1-60)**	**42**

In the second part of the study, the clinical data of the patients were compared with that of patients with glycogen metabolism diseases, viz. McArdle [(Glycogen storage disease Type V, GSDV), n=40] and Morbus Pompe [(Glycogen storage disease type II, GSDII), n=18]. This would enable us to identify the clinical similarities and differences in patients with long-chain fatty acid metabolism and glycogen metabolism diseases.

4. Methods

Clinical biochemical and molecular genetic analysis of all patients in the cohort was performed. Additionally, mitochondrial biomarker, FGF-21, was measured in serum of patients and compared with that of normal controls. Clinical data of CPT II deficient patients were also compared with data of McArdle and late onset Morbus Pompe patients. Methodical details of the analyses are given below.

4.1 Clinical characterization

The clinical data of patients was collected retrospectively through clinical reports of all patients stored in our archive. The patients included the ones who personally presented themselves in our department and the ones whose clinical reports were sent to us by referring clinicians. Additionally, 13 patients in our cohort were available for personal interview. For this interview, a questionnaire was designed for assessment of clinical aspects (symptoms, triggering factors, affected muscles, diagnosis timeline and modes of disability mitigation) of CPT II deficiency and subsequent living standard of patients. All participating patients were asked to fill in the questionnaire personally aided by an unbiased experienced clinician at our department.

The data of questionnaires were systematically collected and analyzed using Sigmaplot. A priori statistical power calculation was not conducted due to limited number of patients available for the study. Data are presented as [Mean (± SD), Median, 95% confidence interval (CI)] wherever applicable. The P values for identifying possible correlation between age at onset, body mass index (BMI) and intensity of pain during normal attack were calculated by regression analysis. Level of significance was set to $P < 0.05$.

4.2 Biochemical analysis of CPT II in muscle

Muscle biopsies of the patients were frozen immediately using liquid nitrogen. The biopsies were stored further in liquid nitrogen for subsequent analysis. Total CPT activity in muscle homogenate was measured biochemically using the isotope forward assay under optimal conditions and in the presence of malonyl-CoA (0.2 mM) and Triton X (0.4 %) as described previously [101]. In this assay, CPT II enzyme activity is measured as the fraction that is not inhibited by malonyl-CoA and Triton X.

Enzyme Assays: The enzyme assays and subsequent measurements were done according to Zierz and Engel, 1985 [101]. Frozen muscle tissues were homogenized with 19 vol. of a solution containing 50mM Tris Buffer (pH 7.6), 100 mM KCL, 5 mM $MgSO_4$, 1 mM EDTA and 1 mM ATP. The isotope exchange and forward assays were used to measure CPT activity in the homogenates.

Isotope exchange assay: The standard reaction system contained 0.1 M Tris buffer (pH 7.6), 2 mM KCN, 0.1% fatty-acid-free BSA, 1 mM dithiothreitol, 0.5 mM DL-Palmitoylcarnitine, 0.2mM COASH, 0.1 ml of a 5% muscle homogenate and 5 mM DL-carnitine in a final volume of 1.0 ml. The reaction was started by the addition of labelled carnitine and continued for 20 minutes at 37°C. The reaction was stopped and the reaction product [^{14}C]palmitoylcarnitine was extracted using isobutanol and ammonium sulphate saturated water and 0.5 ml of the organic phase was assayed for radioactivity in a liquid scintillation counter.

Isotope forward assays: The reaction mixture was identical to that in thje isotope exchange assay except that 0.08 mM palmitoyl-CoA was present instead of palmitoylcarnitine and no CoASH was added. The reaction was started with 0.01 ml of a 5% muscle homogenate and incubated for 20 minutes at 30°C. The reaction was stopped and the product [^{14}C]palmitoylcarnitine was extracted by the same method as in the isotope exchange assay.
The K_i values for malonyl-CoA in the forward assay was determined by the graphical method using malonyl-CoA concebtrations from 0.05 µM to 1.5 µM in the presence of 0.02 mM, 0.03 mM and 0.08 mM Palmitoyl CoA.

4.3 Molecular genetic analysis

Genomic DNA was extracted from the muscle or blood of all patients using standard protocol. With the help of previously described PCR-RFLP methods [102,103], screening of the mutations p.Ser113Leu, p.Pro50His and c.1238delAG was performed in all the patients. Direct sequencing of the coding regions of the *CPT2* gene including exon–intron boundaries was performed in patients who were heterozygous for one of these three mutations or were negative for these three mutations. Sequencing was done by amplifying the coding region of the *CPT2* gene with six matching primer pairs, as previously described [104]. The exact positions of the primers are listed in table 4.

Table 4: List of primers used to amplify CPT2 gene.

Exons	Forward Primer	Reverse primer
1	nt 39 to 63	nt 76 to 52 (intron 1)
2	nt -65 to -38 (intron 1)	nt 46 to 23 (intron 2)
3	nt -49 to -27 (intron 2)	nt 33 to 6 (intron 3)
4a	nt -47 to -21 (intron 3)	nt 1178 to 1152
4b	nt 1122 to 1144	nt 73 to 48 (intron 4)
5	nt -56 to -32 (intron 4)	nt 2115 to 2091

Nucleotide positions for exonic primers according to cDNA sequence published by Finocchiaro et al.[21]. *Due to large size of exon 4, amplification of this exon was done in two parts by designing two sets of overlapping primers.*

For amplification of the DNA, PCR amplification protocol and reagents were used from Qiagen GmbH (Hilden, Germany). Annealing temperatures for the primers were calculated by using Wallace-rule [105,106] and the exact temperatures were determined by performing multiple gradient PCRs.

The amplified product was separated on 2% agarose gel under 120V. For detection of p.Ser113Leu and p.Pro50His mutations, restriction fragment length polymorphism (RFLP) was performed by digesting the amplified product with restriction enzymes *BST XI* and *Ade I* (both from Fermentas GmbH, Germany), respectively. For the patients that were negative for these two mutations, sequencing of the amplified DNA (whole gene: 5 exons) was done using capillary sequencer and fluorescent labelled dNTPs. The sequencing chromatograms were analyzed by using sequence analysis program Chromas version 2.5.1 (Technelysium GmbH, Austria).

For cDNA analysis in one patient with novel mutation, total RNA was extracted from the blood of the patient using a PAXgene RNA extraction kit (Qiagen GmbH, Germany). The cDNA was obtained by reverse transcriptase polymerase chain reaction (RT-PCR; Qiagen GmbH, Germany) using the following set of primers that amplified a part of exon 1, whole exons 2 and 3 and a part of exon 4 resulting in an amplified product of 502 bp (forward primer: $^{5'}$caccatgcactaccaggaca$^{3'}$ and reverse primer: $^{3'}$attgaccaggtaggccccata$^{5'}$). The cDNA thus obtained was separated on a 2 % agarose gel. DNA was retained from individual upper and lower DNA bands using a gel extraction kit (Qiagen GmbH, Germany) and sequenced using the same set of forward and reverse primers used for RT-PCR.

4.4 FGF-21 mitochondrial biomarker analysis

In 13 patients whose serum was available, FGF-21 serum concentration was measured in duplicate samples by ELISA (BioVendor, Brno, Czech Republic) according to the manufacturer's instructions. The absolute concentration of FGF-21 in all samples was determined according to a linear standard curve. The samples from patients were obtained during attack-free intervals and were stored at -80 °C until analysis. Undetectable level was set at 0 pg/mL. The FGF-21 concentrations of the patients were compared with that of 50 healthy individuals.

Test principle: In the BioVendor Human FGF-21 ELISA, the standards, quality controls and samples are incubated in microtitrate wells pre-coated with polyclonal anti-human FGF-21 antibody. After 60 min incubation and a washing, biotin-labelled polyclonal anti-human FGF-21 antibody is added and incubated with captured FGF-21 for 60 min. After another washing, the streptavidin-HRP conjugate is added. After 30 min incubation and the last washing step, the remaining conjugate is allowed to react with the substrate solution (TMB). The reaction is stopped by addition of acidic solution, and absorbance of the resulting yellow product is measured. The absorbance is proportional to the concentration of FGF-21. A standard curve is constructed by plotting absorbance values against concentrations of standards, and concentrations of unknown samples are determined using this standard curve.

4.5 Comparison of clinical data of CPT II deficient patients with that of McArdle and late onset M. Pompe patients

The clinical data of the patients in this study were systematically compared with already published clinical data of patients with McArdle [61,107] and late onset Morbus Pompe [108]. The compared data included, gender frequency, age of onset and presence/absence of permanent weakness.

The clinical data of patients in all three groups (CPT II, McArdle, M. Pompe) were collected from the archive and then analyzed using Sigmaplot. A priori statistical power calculation was not conducted due to limited number of patients available for the comparison. Data are presented as [Mean (± SD), Median] wherever applicable. The P values for identifying possible correlation between different entities were calculated by regression analysis. Level of significance was set to $P < 0.05$.

5. Results

The results obtained by performing aforementioned analyses described in methods section are listed below. The subsections are separated based on clinical, biochemical and molecular genetic analyses.

5.1 Phenotypes of attacks and triggering factors

The majority of the patients (61%) had an early childhood onset compared to later adolescent or adulthood onsets. Only 3 patients (5%) in our cohort reported first attack in early third decade of life. About one third patients (34%) reported first symptoms in adolescence (13-22 years). Table 5 illustrates the frequencies of ages of onset in our patients compared to the results of other studies with considerable number of patients. A combined total of 94 patients were analyzed on these 5 compared studies.

Table 5: Ages of onset of patients with CPT II deficiency

Onset	p.Ser113Leu Homozygotes (n=32)	p.Ser113Leu Heterozygotes (n=24)	Other Mutations (n=3)	Total (n=59)	Metaanalysis of other studies [30,47,54,55,58] (n=94)	p (total vs. other studies)
Childhood	23 (72%)	11 (46%)	2 (67%)	**36 (61%)**	**39 (41%)**	n.s
Adolescence	8 (25%)	11 (46%)	1 (33%)	**20 (34%)**	**33 (35%)**	n.s
Adult	1 (3%)	2 (8%)	0 (0%)	**3 (5%)**	**22 (23%)**	**0.01**

Childhood: 0–12 years, adolescence: 13–22 years, adult: >22 years, p: two tailed probability coefficient (chi-square test).

In addition, attacks of myalgia were reported in almost all the patients followed by myoglobinuria (86%) and muscle weakness (76%). Exercise was found to be the most common triggering factors in almost all the patients followed by infection (62%). Other triggering factors like fasting and cold were seen in lesser number of patients. Detailed clinical data of the patients is listed in table 6. The results of our study are compared with combined outcomes of other studies. The external studies considered for comparison of our data are same as in table 5.

Table 6: Phenotypes of attacks and triggering factors.

Phenotypes	p.Ser113Leu Homozygotes (n=32)	p.Ser113Leu Heterozygotes (n=24)	Total [a] (n=56)	Other studies [30,47,54,55,58]	p (total vs. other studies)
Attacks with					
Myalgia	31 (97%)	21 (87%)	**52 (93%)**	**22/46 (48%)**	**<0.001**
Myoglobinuria	24 (75%)	21 (87%)	**45 (80%)**	**92/99 (93%)**	n.s.
Weakness	23 (72%)	21 (87%)	**44 (79%)**	**17/46 (37%)**	**<0.001**
Renal failure	8 (25%)	4 (17%)	**12 (21%)**	**15/85 (18%)**	n.s.
Triggering factors					
Exercise	30 (94%)	23 (96%)	**53 (95%)**	**55/63 (87%)**	n.s.
Infection	21 (66%)	14 (58%)	**35 (62%)**	**7/63 (11%)**	**<0.001**
Fasting	21 (66%)	12 (50%)	**33 (59%)**	**26/63 (41%)**	n.s.
Cold	5 (16%)	6 (40%)	**11 (20%)**	**5/63 (8%)**	**0.01**

[a]*Total number represents the number of all patients analyzed including two symptomatic heterozygous p.Ser113Leu carriers and two patients harboring mutations other than the p.Ser113Leu on both alleles. Three patients with other than p.Ser113Leu mutation on both alleles were excluded in the analysis.*

5.2 Clinical characterization based on Questionnaire survey:

Frequencies of myalgia and rhabdomyolysis obtained by the questionnaires in the sub group of 13 patients that were available for personal interview are in line with the clinical history directly taken in our collective (Joshi et al.; J Neurol Sci 2018, submitted). Attacks of myalgia (13/13 patients), weakness (13/13) and rhabdomyolysis (10/13 patients) were most frequently reported. The number of attacks ranged from 1-85/year. Common triggers were exercise (13/13), fasting (13/13), cold (12/13) and infections (12/13). 2/13 patients required dialysis. Mean intensity of pain in visual analogue scale (VAS) during regular attack was 4.77 (±1.36). Increased number of attacks were positively correlated with higher BMI (P=0.05). Body rest, carbohydrate-rich nutrients and fluid-supplement mitigated the pain. After the first attack [Mean: 9.7 (±4.46) years], diagnosis took an average of 26.7 (± 13.06) years. Clinical, molecular and epidemiological data of these patients are listed in table 7.

Table 7: Clinical data of patients analyzed through questionnaire-based survey.

Patient	Gender	Genotype	BMI	Age of first attack (yrs.)	Attacks per year (n)	Intensity of pain*	Age at diagnosis (yrs.)	Time to diagnosis (yrs.)
Onset during childhood (0-12 years)								
1	F	p.Ser113Leu/p.Ser113Leu	23.9	6	5	3	19	13
2	F	p.Ser113Leu/p.Ser113Leu	25.9	6	1	4	34	28
3	M	p.Ser113Leu/p.Ser113Leu	26.6	8	1	4	33	25
4	M	p.Ser113Leu/p.Ser113Leu	27.1	8	7	7	53	45
5	M	p.Ser113Leu/p.Ser113Leu	24.2	10	7	4	21	11
6	M	p.Ser113Leu/c.1238delAG	24.2	5	1	7	54	49
7	M	p.Ser113Leu/c.340+1G>A	30.7	4	85	4	25	21
8	M	p.Ser113Leu/c.340+5G>A	26.6	9	10	7	24	15
9	M	p.Ser113Leu/c.182_203del22	30.0	10	1	4	45	35
10	M	p.Arg231Trp/p.Glu487Lys	26.4	10	1	4	22	12
Onset during adolescence (13-22 years)								
11	M	p.Ser113Leu/p.Ser113Leu	24.9	17	11	5	57	40
12	F	p.Ser113Leu/p.Arg151Gln	27.9	18	50	4	53	35
13	F	p.Ser113Leu/p.Pro50His	20.4	15	6	5	39	18

F: Female, M: Male; BMI: body mass index; *: intensity of pain on a linear scale (0: no pain at all, 10: unbearable severe pain) during regular attack

5.2.1 Frequency and duration of attacks

All patients experienced multiple attacks of myalgia and muscle weakness. The age at first attack ranged from 4 to 18 years [Mean: 9.7 (±4.46), Median: 9.0, 95% CI: 7.00 to 12.39]. The frequency of attacks per year for the immediate previous year ranged from 1 to 85 attacks [Mean: 13.85 (±25.12), Median: 5, 95% CI: 0 to 29.02]. Ten patients (77%) experienced at least one episode of rhabdomyolysis. However, dialysis was required in only 2 patients (15%).

Myalgia and muscle weakness (and sometimes other symptoms) were persistent for less than just 1 hour in only 1 (8%) patient. In another patient the symptoms were persistent for less than 1 hour only after intake of carbohydrate supplement immediately after the attacks. In 4 (31%) patients the symptoms were persistent for 1 to 3 days. In 8 (62%) patients, the symptoms lasted for up to one week after the attack.

5.2.2 Characteristics of attacks

Eight patients (62%) had fatigue/heaviness, 7 patients (54%) had feeling of muscle stiffness/rigidity and 4 patients (31%) complained of muscle soreness. Only 3 patients (23%) reported of cramps. Frequencies of phenotypes of attacks are elucidated in Figure 8.

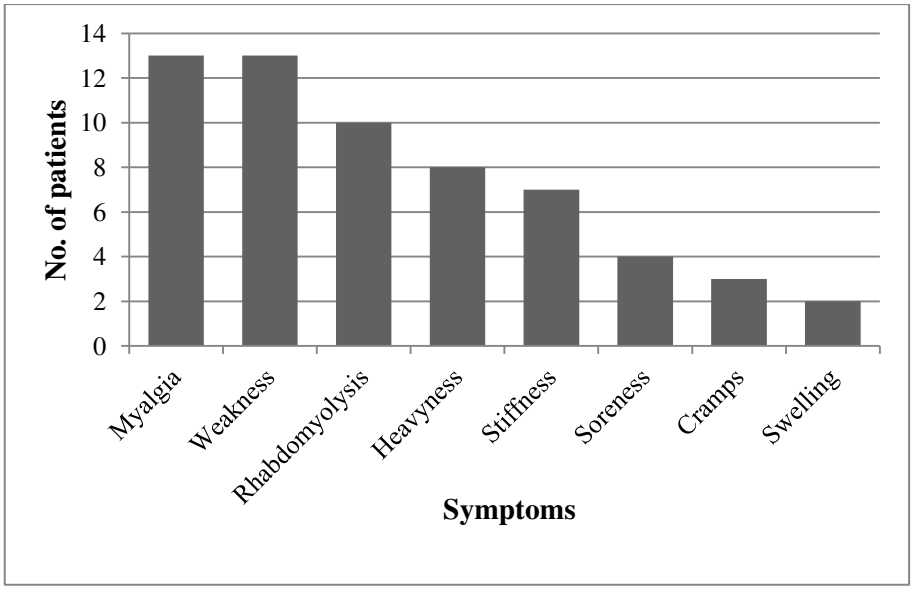

Figure 8: Frequencies of phenotypes of attacks (in all patients more than one symptoms and triggering factors were reported).

5.2.3 Severity of attacks

Mean intensity of pain during regular attacks in visual analogue scale (VAS) (0: no pain at all, 10: severe unbearable pain) was 4.77 (±1.36) [Median: 4.0, 95% CI: 3.95 to 5.59]. The mean intensity during weakest attack was 2.31 (±0.85) [Median: 2.0, 95% CI: 1.79 to 2.82] and during most severe attack was 8.77 (±1.30) [Median: 9.0, 95% CI: 7.98 to 9.56].

The severity of attacks have decreased in 5 patients (38%) and remained unchanged in 5 patients (38%). Other 3 patients (23%) reported increase in severity with time. The attacks have forced 11 patients (85%) unable to work at least few times. However, only 3 patients (23%) were forced to change their profession permanently.

5.2.4 Triggering factors

Exercise and fasting for prolonged periods were triggering factors for attacks in all patients. In 7 patients (54%), the attacks were triggered by an exercise lasting for 15 minutes to 1 hour. In 3 patients (23%), the attacks were initiated by an exercise of 1 to 4 hours, in 2 patients (15%) exercise for 15 minutes and in 1 patient (8%) exercise for a whole. In 12 (92%) patients, the attacks of symptoms seemed to take place immediately during the physical activity that would trigger the attacks. Only in 1 (8%) patient, the attacks would take place after a few hours of the physical activity. In all patients, the attacks were avoidable and/or were less pronounced by intake of sugar and/or carbohydrate supplement (glucose, banana, rice, noodles, etc.).

Exposure to cold [12 patients (92%)] and infections [12 patients (92%)] were also triggering factors in the majority of patients. Low fluid intake [7 patients (54%)], psychological stress [6 patients (46%)], consumption of different medicines including pain killers [3 patients (23%)], lack of sleep [2 patients (15%)] and intake of fatty foods [1 patient (8%)] were also reported to be triggering factor. The frequencies of triggering factors are illustrated in Figure 9.

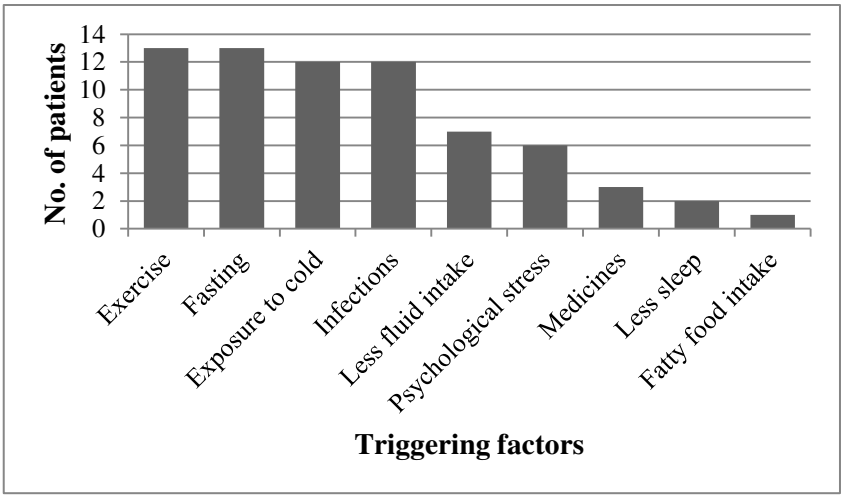

Figure 9: Frequencies of different triggering factors (in all patients more than one symptoms and triggering factors were reported).

Regression analysis showed a significant correlation between frequencies of attacks and increasing BMI (P=0.05, R^2=0.30) (Figure 10). However, intensity of pain was not significantly correlated with BMI. Furthermore, age of onset was also not significantly correlated with frequencies of attacks and intensity of pain (statistically insignificant data not shown).

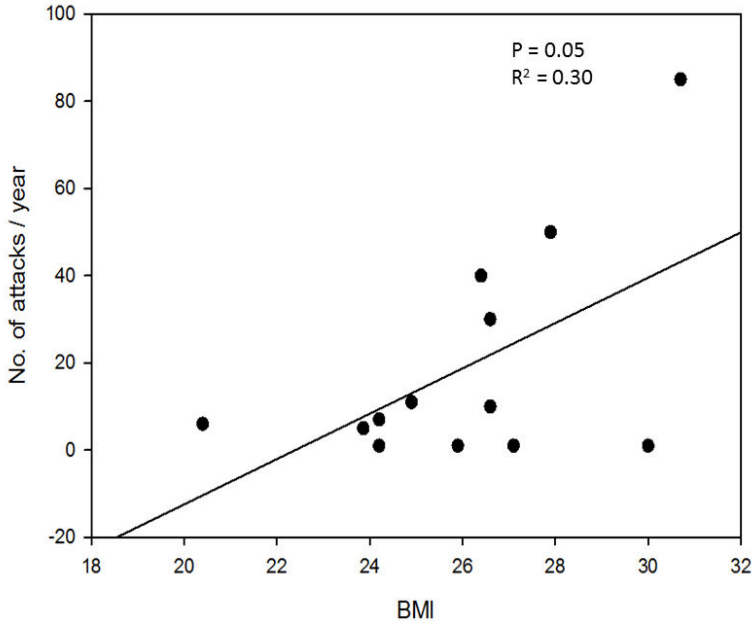

Figure 10: Correlation between body mass index (BMI) and frequencies of attacks

5.2.5 Affected muscles

Mostly leg [thigh: 13 patients (100%), calves: 13 patients (100%)] and arm [upper arm: 12 patients (92%), lower arm: 10 patients (77%)] muscles along with upper and lower limb girdles were affected. In addition, back/trunk [9 patients (69%)] and face muscles [7 patients (54 %)] were also affected in majority of patients. The frequencies of affected locations during attacks are demonstrated in Figure 11.

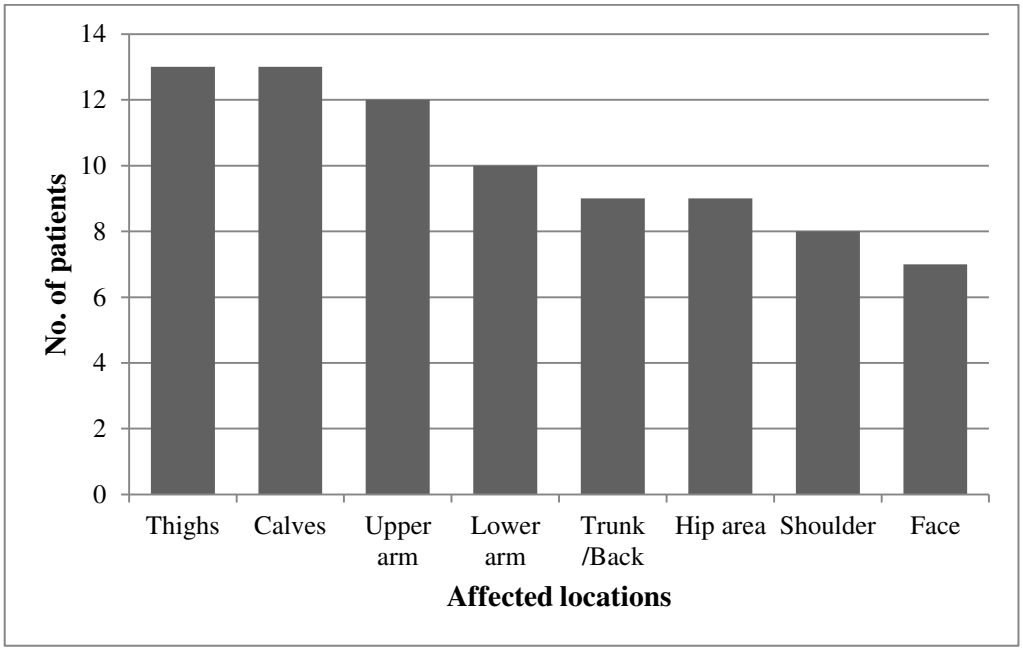

Figure 11: Frequency of locations that are affected during attacks of symptoms.

5.2.6 Living standard

All patients play or used to play some sport ranging from daily to every few weeks. Seven patients (54%) were forced to give up sports completely or partially due to the attacks. Disabilities were persistent irrespective of attacks only in 2 patients (15%). Independently of attacks, 8 patients (62%) complained of headache and out of them 3 patients (23%) were diagnosed with migraine. Out of 4 female patients in the study, three patients had at least one pregnancy and 1 patient (33%) reported of more intense attacks during pregnancy.

5.2.7 Mitigation

All patients communicated that they practice different methods to mitigate pain during attacks. These include body rest, carbohydrate supplement, fluid supplement and warmth. The frequencies of different methods implemented by the patients to mitigate pain are illustrated in Figure 12. Moreover, 8 patients (62%) routinely observed special diet that constitutes mostly carbohydrate rich ingredients.

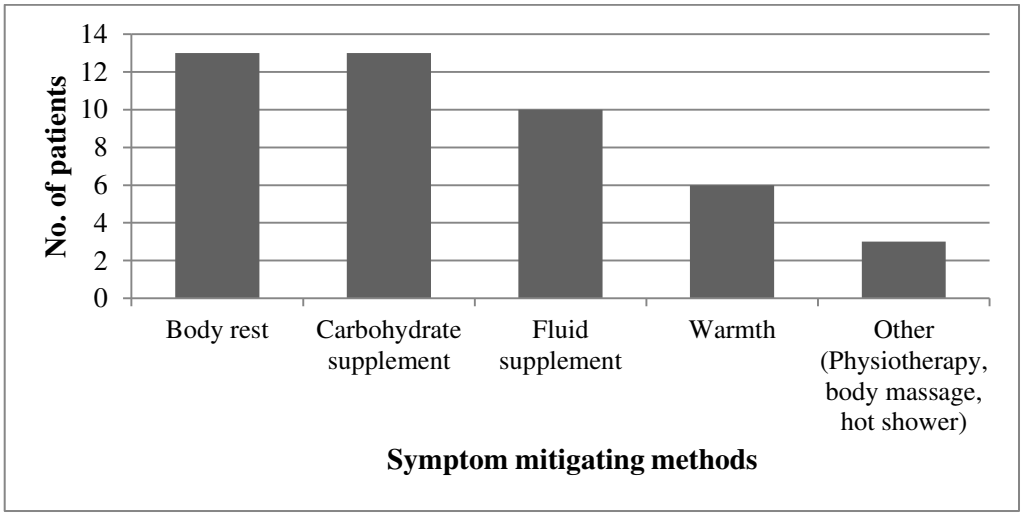

Figure 12: Methods implemented to mitigate pain during attacks of symptoms.

5.2.8 Diagnosis timeline

From the emergence of first symptom [Mean: 9.7 (±4.46) years], it took an average of 26.7 (±13.06) years, (range: 11-49, Median: 25, 95% CI: 18.8 to 34.58) to have a diagnosis of CPT II deficiency. In order to be diagnosed correctly, the patients had visited an average of 4 (range: 2-10) different physicians [Median: 3, 95% CI: 2.61 to 5.55]. Diagnosis was based on both biochemical and genetic investigations in 10 patients (77%). In 3 patients (23%), the diagnosis was established directly by genetic analysis.

5.3 Biochemical activity

Total CPT activity (CPT I+II) was normal in all 42 investigated patients (38 index cases). However, residual CPT II activity was reduced in all these patients (Table 8). In three patients the CPT II activity was mildly reduced in the range between healthy controls and CPT II deficient patients. There was no statistical difference in total and residual (upon malonyl CoA

and Triton X) activities in p.Ser113Leu homozygote patients and p.Ser113leu heterozygotes (i.e., p.Ser113leu on one allele and other mutation on another allele) (Table 8).

Table 8: Total (% of total CPT I and II) and residual CPT activity (after pre-incubation with malonyl-CoA and with addition of Triton-X -100) in muscle homogenates of patients and controls.

Enzyme activity	p.Ser113Leu Homozygotes (n=23)	p.Ser113Leu Heterozygotes (n=19)	Total patients (n=42)	Controls (n=21)	p
Total CPT (nMol /min/mg NCP)	2.02 ± 0.54 (1.7) Range: 1.3-4.6	1.95 ± 0.68 (2.1) Range: 1.0-4.	1.97 ± 0.74 (2.0) Range: 1.0-4.6	1.84 ± 0.67 (1.58) Range: 1.1-3.39	n.s.
Residual (%) malonyl-CoA	7.07± 5.32 (4.5) Range: 1.4-23.8	12.2± 6.3 (7.0) Range: 1.8-23.0	8.2 ± 5.57 (6.5) Range: 1.4-23.8	35.2 ± 9.34 (33.5) Range: 28.0-68.0	<0.001
Residual (%) Triton X	9.16± 8.61 (7.0) Range: 3.8-22.3	7.72± 8.23 (4.0) Range: 0-28.0	7.52 ± 6.6 (5.7) Range: 0-28.0	43.4 ± 8.48 (43.0) Range: 22.0-58.0	<0.001

Numbers indicate mean ± SD, median are given in parentheses (NCP: non-collagen protein; p: correlation coefficient, ns: not significant)

Apart from above observation, three patients in our cohort showed normal total CPT activity but intermediate residual CPT II activities after pre-incubation with malonyl-CoA and Triton-X were reported (Figure 13). All three patients were heterozygous for the common p. Ser113Leu mutation. Sequencing of all exons and of exon–intron boundaries did not identify a second mutation in DNA of these three patients. In all these patients, symptoms were observed in adulthood after extensive physical activities. Two patients are already reported in detail in a previous publication [109].

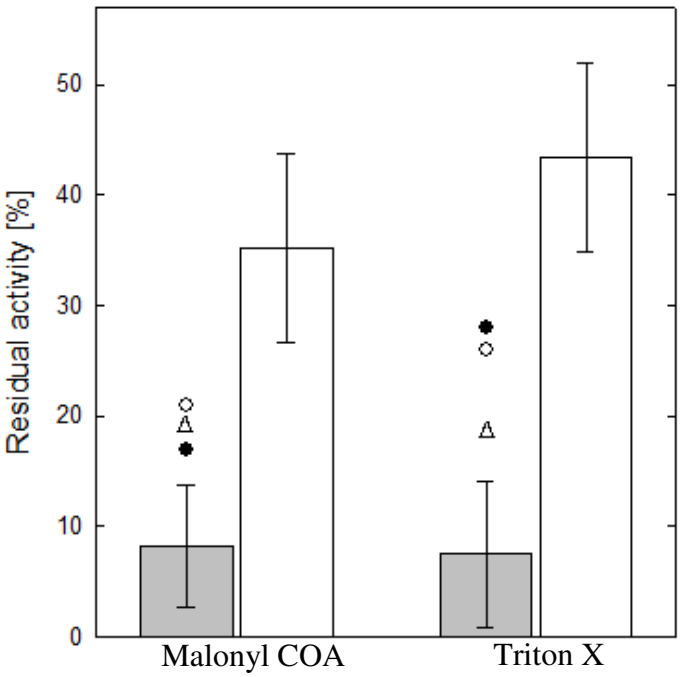

Figure 13: Residual CPT Activity (% of total CPT I and II) after pre-incubation with malonyl-CoA and Triton-X. Grey bars represent patients with mutations on both alleles (n=40) and white bars represent controls (n=21). Three manifesting heterozygotes patients are represented by closed circle, open circle and open triangle, respectively.

5.4 Molecular genetics

Molecular genetic analysis identified the p.Ser113Leu mutation in 46 index patients (94%) in at least one allele with an allele frequency of 0.72. Fifty four percent patients in our collective were homozygote for this common mutation. Allele frequency of the p.Pro50His mutation was 0.06 and that of c.1238delAG mutation was 0.05. Apart from these 3 mutations, 11 other mutations were also identified. Four mutations were novel. All these 11 mutations were in compound heterozygote form and ten mutations were reported in single cases and p.Arg231Trp and p.Glu487Lys mutations were reported in two unrelated cases each. Spectrum of mutations identified in our collective and comparison of frequent mutations in metaanalysis of other studies are illustrated in table 9.

Table 9: Comparison of allele frequencies of different mutations identified in index patients.

Mutations	Our Cohort (n=98)	Other Studies **[54,55,58,102,103]** (n=134)
p.Ser113Leu	71 (72%)	77 (57%)
p.Pro50His	6 (6%)	3 (2%)
c.1238delAG*	5 (5%)	5 (4%)
p.Arg231Trp	2 (2%)	
p.Glu487Lys	2 (2%)	
p.Tyr479Phe	1	
c.1646_49del*	1	
c.340+5C>G	1	
p.Met214Thr p.Arg151Gln	1	
c.340+1G>A*	1	
c.182_203del22*	1	
p.Gly451Glu	1	
p.Ser590Asn	1	

In two patients mutations were identified on only one allele. Potentially truncating Mutations are marked with asterisk. Novel Mutations identified in our collective are in bold cases.

Identification of novel mutations: Four novel mutations were identified in four unrelated patients. All these mutations were in compound heterozygote state with the 'common' p.Ser113Leu mutation. The clinical and genetic features of these patients are listed below in table 10 and the mutations are illustrated in figure 14. These four patients are reported in detain in our previous study [78].

Table 10: Clinical and genetic features of four patients with novel mutations

Patient	1	2	3	4
Gender	Male	Male	Male	Female
Origin	Turkey	Germany	Germany	Germany
Age at diagnosis (years)	25	54	52	60
Age of onset (yrs.)	4	10	childhood	12
No. of attacks	several attacks of muscle weakness & myoglobinuria	7-8 attacks of muscle weakness & myoglobinuria	multiple attacks of myoglobinuria	several attacks of myalgia & myoglobinuria
Dialsyis required	> two times	at least once	at least once	no
Trigger	prolonged exercise, fasting, infections	Exercise, fasting, cold, fever	prolonged exercise	E xercise & fasting
Genotype	p.Ser113Leu / **c.340+1G>A**	p.Ser113Leu / **c.182_203del22**	p.Ser113Leu / **p.Gly451Glu**	p.Ser113Leu / **p.Ser590Asn**

Novel mutations are marked in bold cases

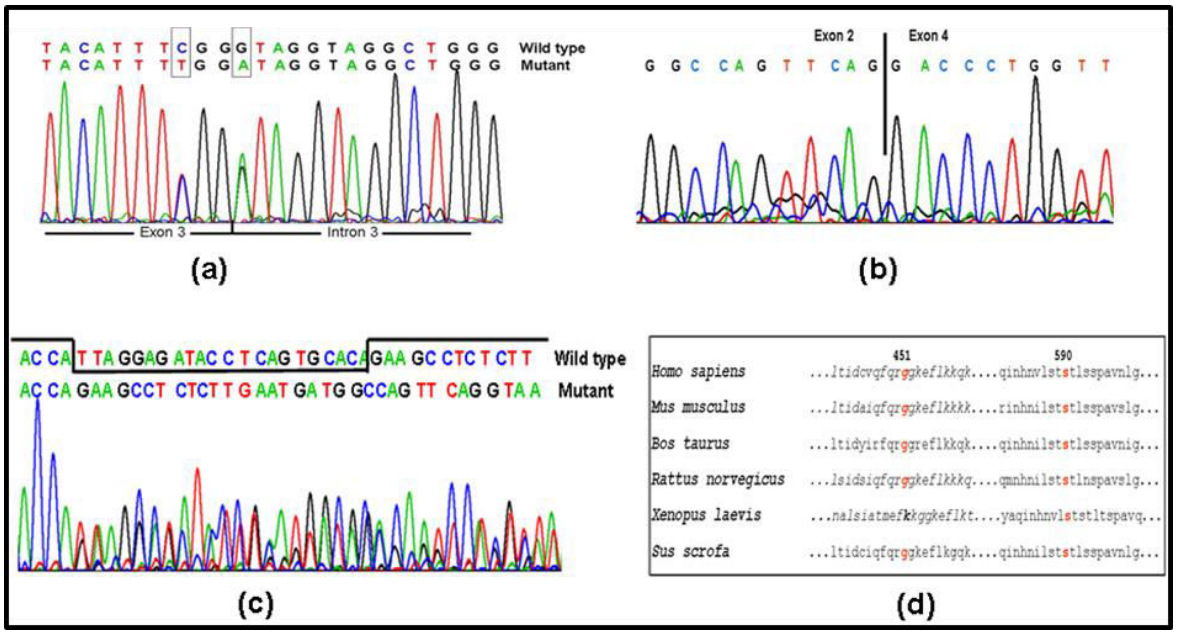

Figure 14: (a) Electropherogram showing a novel splice-site c.340+1G>A mutation in intron 3 compound heterozygous with the common p.S113L mutation (patient 1), (b) Electropherogram showing skipping of exon 3 in cDNA sequencing, (c) Electropherogram showing a novel 22 bp deletion, c.182_203del22, in exon 2 (patient 2) and (d) Alignment of novel mutations p.G451E (patient 3) and p.S590N (patient 4) in different species

5.5 FGF-21 activity

The mean FGF-21 concentration in the plasma of patients was 68.5 pg/ml (Range: 0-157.5 pg/ml). The mean FGF-21 concentration measured separately in male patients was 59.22 pg/ml and in female patients was 82.0 pg/ml. (Table 11). The cutoff 190 pg/ml for FGF-21 was used based on the results of the 95th percentile of controls.

Table 11: FGF-21 levels in serum of CPT II deficient patients and in normal controls

Patient	Gender	Genotype	FGF-21 (pg/ml)
1	F	p.Ser113Leu/p.Ser113Leu	47.0
2	F	p.Ser113Leu/p.Ser113Leu	66.5
3	M	p.Ser113Leu/p.Ser113Leu	152.5
4	M	p.Ser113Leu/p.Ser113Leu	82.0
5	M	p.Ser113Leu/p.Ser113Leu	57.0
6	M	p.Ser113Leu/c.1238delAG	37.0
7	M	p.Ser113Leu/c.340+1G>A	110.5
8	M	p.Ser113Leu/c.340+5G>A	25.5
9	M	p.Ser113Leu/c.182_203del22	56.5
10	M	p.Arg231Trp/p.Glu487Lys	12.0
11	M	p.Ser113Leu/p.Ser113Leu	0
12	F	p.Ser113Leu/p.Arg151Gln	157.5
13	F	p.Ser113Leu/p.Pro50His	57.0
		Mean (Range)	**68.5 (0-157.5)**

	FGF-21 (pg/ml) in Controls: Mean (Range)
M (n=23)	87.17 (0-148.5)
F (n=27)	64.82 (0-132.3)
Total (50)	**66.23 (0-148.5)**

M: Male, F: Female

5.6 Comparison of clinical data of CPT II deficient patients with that of McArdle and late onset M. Pompe patients

A clear male predominance was seen in patients with CPT II deficiency and Morbus Pompe patients (both 61%) in comparison to balanced gender ratio in McArdle disease (50%). On the other hand, onset in most of Morbus Pompe patients began comparatively late in adult age (61%) in comparison to early (childhood and adolescence) onset in CPT II deficiency (81%) and McArdle disease (90%) [CPT II vs M. Pompe; $p<0.01$]. Permanent weakness was observed in lesser number of CPT II deficient patients (12%) compared to McArdle (33%) and Morbus Pompe (100%) patiemts [CPT II vs McArdle; $p<0.01$, CPT II vs M. Pompe; $p<0.001$]. Comparisons of clinical data (only relevant partial data are compared) of these three forms of metabolic disorders are tabulated in table 12.

Table 12: Comparison of clinical data of patients with CPT II, McArdle and late onset M. Pompe diseases.

	Gender		Age at Onset			Permanent weakness
	Male	Female	Childhood	Adolescence	Adult	
CPT II (n=59)	36 (61%)	23 (39%)	36 (61%)	20 (34%)	3 (5%)	7 (12%)
McArdle **[61,107]** (n=40)	20 (50%)	20 (50%)	32 (80%)	4 (10%)	4 (10%)	13 (33%)
M. Pompe **[108]** (n=18)	11 (61%)	7 (39%)	3 (17%)	4 (22%)	11 (61%)	18 (100%)

Childhood: 0–12 years, adolescence: 13–22 years, adult: >22 years

Data of the patients used in this comparison were taken from our previously published reports on McArdle patients [61,107] and Morbus Pompe patients [108]. The data of 50 CPT II deficient patients are also previously reported in detail [32,78,109].

6. Discussion

6.1 Clinical features

So far, there are only few reports with clinical characterization of CPT II deficient patients [30,38,47,48,54,55,58]. The muscle form CPT II deficiency is extensively reported with adult or late onset [47,58,102,110]. However, few cases with early childhood manifestation with muscle form are also reported [38,52–56]. In present study, early or childhood (1-12 years) onset was reported more frequently than adolescence (13-22 years) and adulthood (>22 years) onset compared to metaanalysis of four previous studies [30,47,55,58] (Table 5).

Clinical symptoms such as myoglobinuria and attacks leading to renal failure and triggering factors such as exercise and fasting were reported almost similarly frequently with no statistical significance in present cohort and metaanalysis of previous studies. However, in present series, symptoms such as exercise induced myalgia and attacks of muscle weakness and inter-current infections and exposure to cold as triggering factor were observed in higher frequencies compared to meta-analysis of five previous studies [30,47,54,55,58] (Table 6).

Serum CK and myoglobin could be monitored during an attack in a manifesting heterozygous patient. The patient in his late twenties was admitted to our intensive station after suffering from a severe attack of myalgia. Before the attack, the patient had completed a 10 kilometer race on a very hot sunny afternoon. The previous evening, he had worked multiple hours in his garden. At the time of admission, the patient was almost unconscious and his CK was slightly elevated but myoglobin during admission was, however, immensely increased. Increase of CK and myoglobin showed different kinetics. CK gradually increased during the first 7 days while myoglobin was already maximally elevated during the first few hours (Figure 15). This is consistent with previous studies on healthy half-marathon runners [93]. However, in this patient, it remains still open, whether the almost-normal myoglobin at day 2 and then subsequent mild re-increase was due to the initially excessive fluid substitution or due to other reasons. The initial disseminated intravascular coagulation was most likely due to the release of various prothrombotic substances from the damaged muscle fibers activating the coagulation cascade [94].

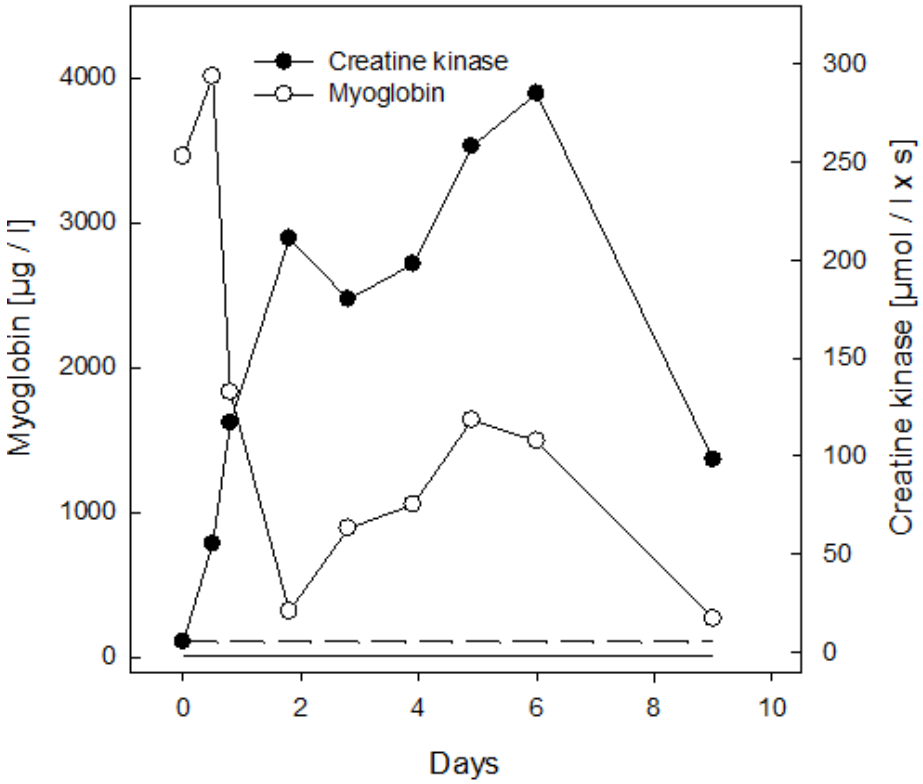

Figure 15: Levels of CK and myoglobin in a manifesting heterozygote patient. Day 0 indicates the time of treatment by the emergency physician previous to admission. The broken horizontal line represents normal value of CK (2.85 µmol/l*s) and the solid horizontal line represents the normal value of myoglobin (106 µg/l). CK and myoglobin levels were normal during re-examination after 6 months.

6.1.1 Questionnaire survey (Joshi, et al., J Clin Neuroscience 2018, paper in press)

Thirteen patients took part in the questionnaire survey. The outcome of this sub-study has been submitted as a clinical paper in Journal of clinical neuroscience. The manuscript is accepted for publication and is currently in press. The detailed discussion on results of this sub-study is documented as following.

6.1.1.1 Symptoms and triggers

Frequencies of myalgia and rhabdomyolysis obtained by the questionnaires in the sub group of 13 patients that were available for questionnaire survey are in line with the history directly taken in our large cohort of patients [32]. Our survey strengthens the hypothesis that prolonged physical stress, infections and exposure to cold might be related to a changed thermal stability of the mutated enzyme [80]. During these extreme conditions the extra muscular energy is required which is met by compensatory mitochondrial heat production

which ultimately provokes the attacks. Although, cramps were previously excluded as symptoms of CPT II deficiency [30], occurrence of cramps in 3/13 patients (23%) in our cases suggest that cramps are not rare in CPT II deficiency. However, our study also emphasizes that muscle cramps are not that frequent in comparison to McArdle patients [61,62]. Our findings are supported by other studies where patients with cramps were sporadically reported [59,82,111]. Medicines, mainly painkillers such as Ibuprofen initiated the attacks in three patients. This is also in line with identification of transiently decreased carnitine content in CPT II deficient patients upon ibuprofen therapy [112]. There are a couple of reports that show that rhabdomyolysis could be induced by side-effects of other medicines such as Valproic acid [113] and high doses of Diazepam [31].

Significant correlation between body mass index and frequency of attacks/year shows that patients with high fat content in the body are in risk of having more frequently attacks than patients with normal fat content (Figure 10). This can be explained by increased lipoprotein lipase activity and free fatty acid utilization in muscle tissue of obese patients [114,115].

6.1.1.2 Affected muscles

In CPT II deficiency, entire regions of skeletal muscle can be affected. However, in the patients in our questionnaire survey, arms and legs were affected more frequently (Figure 11). These are the muscle groups that are most active during exercise and other strenuous day-to-day activities. Consistently there are also reports on jaw musculature involvement while chewing in CPT II deficient patients [116,117]. In more than half of the patients on the present study, facial musculature involvement was reported (Figure 11).

6.1.1.3 Living standard

Myopathic CPT II deficiency is not considered a life threatening severe disease. Generally, CPT II deficient patients could live a rather normal life. The notion that myopathic CPT II deficiency is a ‘mild’ disease is strengthened by (1) no symptoms were reported in our patients in between attacks, (2) the disabilities have remained the same or even decreased during disease progression in 10 (77%) patients, (3) persistent disabilities irrespective of attacks occurred only in 2 (15%) patients, (4) the average intensity of pain was found to be 4.77 on visual analogue scale (VAS) during regular attacks, (5) attacks of rhabdomyolysis were reported in 10 (77%) patients but dialysis was required in only 2 (15%) patients, (6) only

3 (23%) patients were forced to change their profession due to disabilities, and (7) all patients regularly played some sports where symptoms were triggered during or after sports. This shows that only strenuous physical activity is responsible for emergence of symptoms.

6.1.1.4 Mitigation

There is no approved drug for treatment of CPT II deficiency. Complete body rest and carbohydrate supplement seem to mitigate the symptoms during and after the attacks in all patients (Figure 12). Hence the CPT II deficient patient should be advised to avoid strenuous physical activities and to shift their nutrient towards carbohydrate rich nutrients and low in fat most of which should be medium chain triglycerides, and supplemented with carnitine. This therapy improves the acylcarnitine profile and prevents further attacks of hypoglycemia and arrhythmia [118].

Complication during general anesthesia (including rhabdomyolysis and renal post-anesthetic failure) has been reported in CPT II deficient patients [119,120]. Hence it is advised to evaluate the asymptomatic at-risk relatives as well. Due to early diagnosis and treatment fatality caused by CPT II deficiency in the family could be reduced immensely.

6.1.1.5 Diagnosis Timeline

Diagnosis of CPT II deficiency seemed difficult as disabilities were seen only during attacks. Between the attacks, patients were normal. An average of 26.7 years for diagnosis and involvement of several physicians for the diagnosis reflects the diagnostic impediment associated with this disease. This shows the lack of awareness of this special field of neuromuscular disorders in many physicians.

Due to recent advancement in diagnosis of neuromuscular disorders, steps are being taken forward in fast and reliable diagnosis of CPT II deficiency. For that, newborn screening programs to identify disorders of fatty acid oxidation and the carnitine cycle are globally expanding. However, it poses new challenges for the medical practitioner and for the clinical geneticist as CPT II deficiency have the abnormal acylcarnitine profile at birth consisting of low C0 with increased C16-C18 species. In many cases, children will be symptomatic before the results of newborn screening become available. The neonatal form of CPT II deficiency is very severe and responds poorly to therapy. Milder forms of CPT II deficiency benefit from the therapy discussed in mitigation section above.

6.2 Biochemical features

In all patients where CPT activity was measured in muscle homogenates, total activity (CPT I+II) was not significantly different from the total activities in normal controls. However, the residual CPT II activity upon inhibition by malonyl-CoA and Triton X-100 was significantly decreased in patients compared to controls (Table 8). This observation confirms the previous notion that CPT II deficiency is not exclusively due to loss of total enzyme activity but rather due to abnormal regulation of the enzyme. Interestingly, in three patients with normal total activity, the residual CPT II activity was mildly reduced in the range between healthy controls and CPT II deficient patients. Out of these three patients, two patients are already described in detail [109]. Not only through radioactive method, immunohistological analysis of muscle sections of CPT II deficient patients demonstrated same intensity of CPT II in patients as in controls. In Western blot studies, COX (cytochrome c oxidase) was used as a mitochondrial marker for the quantification of CPT II protein. Patients and controls all showed the same staining intensity [77]. These observations could further explain the phenomenon behind occurrence of only intermittent symptoms in muscle CPT II deficiency.

6.3 Molecular genetic features

The 'common' p.Ser113Leu mutation was identified in at least one allele in 96% patients. Our results are in line with previous findings as the p.Ser113Leu mutation was reported frequently (allele frequency: 0.58-0.76) in patients with muscle CPT II deficiency [38,55,58,102,103] (Table 2). Inheritance of CPT II deficiency is considered to be autosomal recessive. However, two of the patients harbored only the p.Ser113Leu mutation on one allele [109] indicating that although CPT II deficiency is an autosomal recessive disorder, rare symptomatic heterozygous cases with intermediate residual enzyme activity are possible as suggested by others [38,55,58,102,103]. Hence, in heterozygotes, additional epigenetic or environmental factors might further compromise the only intermediately reduced biochemical parameters. . In CPT II deficiency, through sequence analysis variants that are benign, likely benign, of uncertain significance, likely pathogenic, or pathogenic could be identified. Pathogenic variants may include small intragenic deletions/insertions and missense, nonsense, and splice site variants; typically, exon or whole-gene deletions/duplications are not detected.

Moreover, in most of autosomal disorders, the silent mutations are considered to be nonpathogenic and are easily overlooked during genetic analysis. Most of the silent mutations

are polymorphisms as they do not alter the resulting protein but not all silent mutations are harmless. A silent mutation can create an ectopic splice site which is then used in preference to the natural splice site, shortening the exon. Analysis comparing silent mutations resulting in exon-skipping and common SNPs showed that silent mutations responsible for abnormal splicing are commonly located in regulatory elements within the exon that are important for exon definition [121]. A silent mutation in exon 4 of *CPT2* gene p.Gly310Gly has been reported as a pathogenic mutation [58]. This mutation initiates abnormal splicing of exon 4 and subsequently modification of the resulting enzyme. This is consistent with identification of pathogenic silent mutations in other autosomal disorders as well [122–125]. Therefore, pathogenicity of the silent mutations identified in *CPT2* gene should be evaluated in order to increase specificity of genetic analysis.

Identification of four different novel mutations in our cohort extended the heterogeneity of CPT II gene mutations [78]. The four novel mutations are considered pathogenic because (1) sequencing of whole coding region (including intron-exon boundaries) of the CPT II gene identified no other mutant changes, (2) the splice site c.340+1G>A mutation was not identified in 40 normal controls and resulted in skipping of exon 3 (Figure 14b), (3) the 22 bp deletion (figure 1c) introduces a stop codon 6 amino acids downstream resulting in premature termination of protein synthesis, (4) the mutations were identified in different alleles (5) all these mutations are not listed as SNP (single nucleotide polymorphism) [11], (6) the prediction program 'polyphen-2' suggests the missense mutations p.Gly451Glu as mildly pathogenic (score:0.206) and p.Ser590Asn as severely pathogenic (score: 1.00) [12] and (6) both the missense mutation are located at residues that are conserved among different species (Figure 14d). Our study shows that screening for second mutations in patients that are heterozygote for the common p.Ser113Leu is justified although rare symptomatic heterozygotes might be possible [109]. In general, muscle biopsy is not required for diagnosis of CPT II deficiency. However, if a muscle biopsy has been performed in p.Ser113Leu heterozygotes, enzyme activity can be characterized as described [101]. If there are intermediate levels of enzyme inhibition by malonyl CoA and Triton X-100 [109], further mutation screening is generally not necessary.

6.4 FGF-21 mitochondrial biomarker

FGF-21 has been established as a biomarker for diagnosis of mitochondrial diseases [90,91,126–128]. However, FGF-21 concentrations in CPT II deficient patients are not known. In our 13 patients, FGF-21 serum concentration was not significantly different from those of controls. This was independent of the underlying mutation, gender, BMI, number & severity of attacks. None of the patients showed a FGF-21 concentration above the cutoff value. However, the induction of FGF-21 in mitochondrial myopathies is proposed to be a transcriptional response, which is a part of an integrated mitochondrial stress response (ISRmt). It should be controlled by mTORC1 in muscle [129]. In a mouse model with a heart and muscle CPT II deficiency (Cpt2M−/−) the mTOR-regulated genes were significantly upregulated [130]. Morover, FGF-21 level was also significantly increased above normal level in Serums on CPTII knock out mice [97–100,131]. Based on these findings, a higher FGF-21 serum level in CPT II deficiency patients compared to controls can be expected. However, this was not the case in our patients. It has to be noted that the serum samples in our patients were collected during attack-free intervals. This strengthens the notion that in CPT II deficient patients, there is no permanent lack of active enzyme but rather an abnormal regulation and thermostability of the mutant enzyme [79,80]. The only attack-like impairment of fatty acid utilization in these patients might not be sufficient to cause an increased TGRF.

6.5 Genotype-Phenotype analysis

Previous studies suggested a phenotype/genotype correlation in CPT II deficiency. Severe mutations such as p.Pro227Leu, c.1923_1935del, Asp328Gly on both alleles are associated with neonatal or severe infantile form [38,39]. These mutations result in severe symptoms leading to death. These severe mutations are generally not found in patients with mild muscle form of CPT II deficiency. However, severe infantile form of CPT II deficiency is also described in compound heterozygous states in combination with a severe pathogenic variant and a variant usually associated with mild phenotype ([p.Gly520Ala) [40]. Apart from that, the p.Arg503Cys pathogenic variant identified in a family presented with a slowly progressive mild myopathy characterized by progressive muscle weakness and myopathic symptoms [34]. Moreover, isolated cases of myopathy related to *CPT2* mutations are also reported [132]. The diagnosis of myopathy as well as that of CPT II deficiency in reported patients cannot be explicitly validated. The diagnosis process in these cases is still questionable [132]. Not only

pathogenic mutations but the polymorphisms have also been identified to be detrimental. The polymorphism p.Phe352Cys, found only in East Asians, has been reported to be associated with acute encephalopathy during infectious disease and sudden unexpected death in infancy. This polymorphism is thermolabile and reduces enzyme activity during high temperatures [15,133].

On the other hand, the most frequent p.Ser113Leu mutation associated with mild muscle form is not found in other severe forms of CPT II deficiency. The p.Ser113Leu is frequently found in northern Europeans while p.Phe383Tyr appears to have the highest prevalence in Japanese population [134].

In the present series, a genotype-phenotype correlation was analyzed based on patients with homozygous p.Ser113Leu mutation (n=34) and patients with heterozygous p.Ser113Leu mutation (n=25). In these two groups, the age of onset, frequencies of symptom of attacks and triggering factors were similar (Table 5 and Table 6). This was in line with findings of previous studies [38,55,58,102,103]. There was no significant difference in the residual activity upon inhibition with Triton X-100 in these two groups. However, there was a tendency of higher residual enzyme activity upon malonyl-CoA inhibition in heterozygotes than in homozygotes with a slight statistical difference (p=0.054, Mann-Whitney rank sum test).

The comparison of patients with missense mutations on both alleles (n=48) and patients with possible truncating mutation (deletions and splice-site mutations) on at least one allele (n=11) revealed no significant difference regarding clinical symptoms. However, fasting as triggering factor was reported in 10/11 patients with truncating mutations and in 16/48 patients with missense mutations on both alleles (p=0.0034, Chi-square test). There was no significant difference in the residual activities upon inhibition with malonyl-CoA and Triton X-100 in these two groups of patients.

Present study shows that screening of p.Ser113Leu mutation in suspected muscle CPT II deficiency is justified as this so called ‘common’ mutation was identified in 96% patients. In more than half of the patients, age of onset was in childhood, suggesting that the often used term ‘adult’ onset for muscle CPT II deficiency is rather misleading. Within the muscle form of CPT II deficiency, different genotypes have only a marginal influence in clinical and biochemical phenotypes.

Apart from these observations, three manifesting heterozygote patients were also reported. All three patients showed typical symptoms of CPT II deficiency with exercise-induced attacks of

muscle weakness with onset in adulthood. The intermediate residual enzyme activities after preincubation with Triton-X-100 and upon addition of malonyl- CoA (Figure 13) are consistent with the molecular finding of only one mutation in these patients. The possibility of a large deletion or a second mutation in the intronic region cannot be entirely excluded in these patients. However, large deletions have not been reported in patients with CPT II deficiency, so farAlthough CPT II deficiency is considered as an autosomal recessive disorder, both cases indicate that heterozygotes with only one mutant allele might also show the typical attacks of symptoms. However, all three patients were professional athletes and the attacks took place after strenuous physical activities (such as after a game of tennis, running 10 Kilometer distance on a hot sunny day, after working hard the whole afternoon in garden). These are the typical triggers for inhibition of the enzyme that would triggers symptoms. Hence, it would be really interesting to document physical activities of further heterozygotes to identify a possible cut-off (duration of physical activity, severity of the activity, CK, myoglobin, etc.) for emergence of attacks. These observations stress that despite the recessive mode of inheritance of this disorder; the heterozygotes could not only be regarded as carriers but could also occasionally show typical symptoms. Hence, if CPT II deficiency is identified in any member in the family, the immediate family members are also in risk. A genetic counselling would be helpful to them.

6.6 Comparison with McArdle and adult M. Pompe

McArdle and late onset Morbus Pompe are two most common metabolic myopathies [61,62,107,108,135] that are characterized by exercise-induced myalgia, weakness and myoglobinuria. Both these disorders are glycogen storage diseases. However the general clinical characteristics of these metabolic myopathies are similar to that of CPT II deficiency. There are two forms of Morbus Pompe; multi-systemic early and myopathic late onset. In our cohort, the late onset was seen frequently (61%) in contrast to early (childhood and adolescence) onset in CPT II deficiency (95%) [CPT II vs M. Pompe; $p<0.01$]. However, there was no difference in ages on onset between CPT II deficiency and McArdle (early onset in 90% patients). This shows that the progression of CPT II and McArdle start similarly in early stage of life in contrast to delayed progression in Morbus Pompe. In late onset Morbus Pompe severe additional symptoms (such as liver and cardiac involvement) are frequently reported [136–138]. However, in contrast, myopathic form of CPT II deficiency showed no

different organ involvement. This reflects the higher severity of symptoms in Morbus Pompe in comparison to milder symptoms in CPT II deficiency. Additionally, a clear male predominance was seen in patients with CPT II deficiency and Morbus Pompe patients (both 61%). This was consistent with other CPT II deficiency studies as well [54,55,58]. The male dominance in these metabolic disorders could not be explained explicitly. Hence, the question remains open, whether the male predominance is due to sex-related differences in exercise activities, an X-chromosomal modifier gene, or hormonal factors such as estrogen that seem to be a regulator of CPT [45,139]. Moreover, less frequently observed permanent weakness in CPT II deficient patients in comparison to glycogen metabolic myopathies (McArdle and late onset M. Pompe) further strengthens that in CPT II deficiency, there is no permanent lack of active enzyme but rather an abnormal regulation and thermostability of the mutant enzyme [79,80].

To sum up, our study emphasizes that CPT II deficiency the most common disorders of long-chain fatty acids. However, only around 400 unrelated families with different three forms of CPT II deficiency are reported globally. It can be predicted that there should be lot more undiagnosed cases of CPT II deficiency as the patients are suffered by occasional attacks of symptoms. Between the attacks, the patients are more or less normal and can perform their daily activities without much problem. Additionally, females may be less likely to develop myoglobinuria and therefore remain undetected. The diagnostic facility of CPT II deficiency is not readily available and lots of patients have to wait for multiple decades just to get the proper diagnosis. Molecular genetic investigation is regarded as the gold standard in diagnosis of CPT II deficiency. Due to introduction of multi-panel gene sequencing of muscle patient and exome sequencing in recent times, new cases of CPT II deficiency are emerging more frequently. However, even during molecular genetic analysis, the potentially pathogenic silent mutations seem to be overlooked not only in CPT II deficiency but in a lot of autosomal disorders.In addition, biochemical analysis of muscle homogenates also potentially identifies CPT II deficient patients with high specificity. Biochemical activity can be measured by isotope forward assay [101] or by ELISA [77]. The MRI analysis of muscles of CPT II deficient patients or histological or immunihistological analysis are not conclusive and cannot be accepted as potential diagnostic measures in CPT II deficiency. The MRI of affected muscle of CPT II deficient patients during an attack would be really interesting to analyze. However, this does not seem to be feasible as the patients suffer from unexpected attacks of symptoms. Unlike enzyme replacement therapy in late onset Morbus Pompe [108,140,141],

there is no therapy available for CPT II deficiency. Complete body rest, supplement of carbohydrate rich nutrition and substitution of fluids seem to decrease the frequency and intensity of attacks. A couple of clinical studies are underway towards finding potential therapy for CPT II deficiency. Roe and colleagues were able to get rid of rhabdomyolysis or hospitalization of seven CPT II deficient patients while on the triheptanoin (anaplerotic) diet [142]. These individuals returned to normal physical activity including strenuous sports after anaplerotic diet. Moreover, another study demonstrated the increase of CPT2 mRNA and normalization of enzyme activity in mild forms of CPT II-deficient cultured fibroblasts and myoblasts by bezafibrate treatment [143]. Furthermore, a trial on six CPT II deficient patients that were treated with bezafibrate showed elevation of fatty acid oxidation levels in muscle biopsies as well as a significant increase in palmitoyl-L-carnitine oxidation, increased CPT2 mRNA, and increased translated protein. In these patients, episodes of rhabdomyolysis were considerably decreased and the quality of life (measured by SF-36) analysis showed increase in physical activity and a decline in intensity of muscle pain [143]. Despite these steps put forward towards finding diagnosis and management strategies of CPT II deficiency, this muscle disorder is still a mystery for many medical doctors, clinical geneticists and even for the patients. However, the knowledge and awareness of CPT II deficiency is gradually increasing among patients and clinicians and in due time more and more cases of CPT II deficiency are bound to emerge. This will also pave the path towards finding a potential therapy in this metabolic disorder.

7. Summary

Carnitine palmitoyltransferase II (CPT II) facilitates the transfer of long-chain fatty acids from cytoplasm into mitochondria during the oxidation of fatty acids. Deficiency this enzyme results in the most common inherited disorder of long-chain fatty acid oxidation affecting skeletal muscle. This disorder presents in three forms; (i) lethal neonatal form presenting with hypoketotic hypoglycaemia and severe hepatomuscular symptoms, (ii) severe infantile hepatocardiomuscular form characterized by hypoketotic hypoglycemia, liver failure, cardiomyopathy, and peripheral myopathy and (iii) the classical rather mild myopathic form characterized by recurrent episodes of muscle pain, muscle weakness, and rhabdomyolysis triggered by prolonged exercise.

Clinical, biochemical and molecular genetic data in a cohort of 59 patients with muscle CPT II deficiency were analyzed. Attacks of myoglobinuria occurred in 80% patients. In 95% patients triggering factor was exercise. Although the myopathic form is often called the adult from, in 61% patients, the age of onset was in childhood (1-12 years). Although, cramps were previously excluded as symptoms of CPT II deficiency, occurrence of cramps in 3/13 patients (23%) in our cases (questionnaire survey) suggest that cramps are not rare in CPT II deficiency. A clear male predominance (61% males) was seen in our cohort that was in line with the observations of other studies. All the patients in whom biochemical activity was measured (n=42) had normal enzyme activity of total CPT I+II but the activity was significantly inhibited by malonyl-CoA and Triton. Despite identification of high levels of mitochondrial biomarker FGF-21 in CPT II knock mice, the FGF-21 concentration in humans during attack-free intervals is normal. This strengthens the fact that there is no permanent lack of active enzyme in CPT II deficient patients but rather an abnormal regulation and thermostability of the mutant enzyme.

The p.Ser113Leu mutation was detected in 46/49 index patients (94%) in at least one allele. More than 52% index patients were homozygous for this mutation. Thirteen other mutations were also identified. There was no notable difference in clinical and biochemical phenotype of patients with p.Ser113Leu mutation in homozygous or compound heterozygous form. The exception was a tendency of slightly higher residual enzyme activity upon malonyl-CoA inhibition in Ser113Leu compound heterozygotes. Phenotype was also not significantly different in patients with missense mutations on both alleles and patients with truncating mutation on one allele and missense mutation on the other allele. However, only exception

was that, attacks were triggered by fasting in almost all the patients with truncating mutations. In contrast, fasting triggered the attacks only in one third of patients with missense mutations on both alleles. The data indicates that within the muscle form of CPT II deficiency, the various genotypes have only marginal influence on the clinical and biochemical phenotype.

Although CPT II deficiency is considered as an autosomal recessive disorder, present study shows that heterozygote patients with only one mutant allele might also show the typical attacks of symptoms. However, all symptomatic heterozygotes were professional athletes and the attacks took place after strenuous physical activity.

In comparison to frequently suffered permanent weakness in glycogen metabolism diseases (late onset GSD II and GSD V), permanent weakness in CPT II deficient patients is rare. This further strengthens that in CPT II deficiency, there is no permanent lack of active enzyme but rather an abnormal regulation and thermostability of the mutant enzyme. The ages of onset were also early in childhood or in adolescent in CPT II deficient patients. That was in contrast to later onset in glycogen metabolism diseases. There was a clear male predominance in CPT II deficiency and late onset GSD II. However, male dominance in these metabolic disorders could not be explained explicitly. Hence, the question remains open, whether the male predominance is due to sex-related differences in exercise activities, an X-chromosomal modifier gene, or hormonal factors such as estrogen that seem to be a regulator of CPT.

The clinical data obtained by questionnaire survey in a sub group including 13 patients also emphasizes that frequencies of attacks of symptoms are highly variable in myopathic CPT II deficiency. It further stresses that despite the fact that the myopathic CPT II deficiency is a mild form, severe patients requiring dialysis due to kidney failure could be present. There was a significant correlation between body mass index and frequency of attacks/year showing that patients with high fat content in the body are in risk of having more frequently attacks than patients with normal fat content. This can be explained by increased lipoprotein lipase activity and free fatty acid utilization in muscle tissue of obese patients. Moreover, patients needed an average of almost three decades for diagnosis of CPT II deficiency that involved several physicians. This reflects the diagnostic impediment associated with this disease. This shows the lack of awareness of this special field of neuromuscular disorders in many physicians.

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9. Theses

1. In myopathic CPT II deficiency attacks of myalgia, myoglobinuria and muscle weakness were frequently observed. These attacks were triggered predominantly by physical exhaustion. Other triggers were infection, prolonged fasting and cold exposure.

2. Although myopathic CPT II deficiency was previously also referred as adult onset, the majority of patients (95%) had early onset (childhood or adolescence).

3. Despite cramps being previously excluded as symptoms of CPT II deficiency occurrence of cramps in about one fourth patients in our collective suggest that cramps are not rare in CPT II deficiency.

4. Painkillers such as Ibuprofen could frequently initiate the attacks by transiently decreasing the carnitine content in CPT II deficient patients.

5. Severe cases with kidney failure needing dialysis are not that rare in ‘mild’ form of CPT II deficiency.

6. Patients with high fat content in the body are in risk of having more frequently attacks than patients with normal fat content.

7. Total CPT activity (CPT I + CPT II) is normal in CPT deficient patients but residual activity upon malonyl COA and Triton X seems to be considerably reduced.

8. Prolonged physical stress, infections and exposure to cold might be related to a changed thermal stability of the mutated enzyme.

9. Apart from other skeletal muscles that are consistently in use during physical activities and exercise (legs, arms, and trunk), facial musculature involvement could also be observed in CPT II deficiency.

10. Manifesting heterozygotes with only one mutation and intermediate CPT II residual activity also show typical symptoms upon physical exercise. Mainly, person involved in physical works and athletes are more prone to such attacks.

11. Myopathic CPT II deficiency is not considered a life threatening severe disease and the patients could live a rather normal life. In between the attacks, the patients generally do not suffer any difficulty.

12. Complete body rest and carbohydrate supplement seem to mitigate the symptoms during and after the attacks

13. Unlike increased mitochondrial biomarker FGF-21 levels in CPT II deficient knockout mice, levels of FGF-21 in serums of CPT II patients is within normal range.

14. A genotype-phenotype correlation could not be established in CPT II deficiency. However, fasting as triggering factor was reported more frequently in patients with truncating mutations on at least one allele.

15. In comparison to frequently suffered permanent weakness in glycogen metabolism diseases (late onset GSD II and GSD V), permanent weakness in CPT II deficient patients is rare.

10. List of figures and tables

10.1 Figures

10.2 Tables

11. Appendix

Appendix 1: List of known Mutations in CPT2 Gene. Mutations identified in our cohort are marked red, novel mutations are in bold cases. Intronic mutations are marked with asterisk. Intronic mutations other than those identified in our cohort are marked blue.

Exon1	Exon2	Exon3	Exon4	Exon5
1.p.Pro41Leu	1.p.Pro55Arg	1. p.Cys84Arg	1. p.Tyr120Cys	1. p.Arg560Gln
2.p.Pro50His	2. p.Ala67Gly	2. p.Ala101Val	2. p.Leu121Gln	2. p.Leu575Pro
3.c.36-38 insGC	**3.c.182_203del 22**	3. p.Ser113Leu	3. p.Arg124Gln	3. p.Asp576Gly
4.c.36_43dupGGGCCC		4.c.256_257delAG	4. p.Arg124Ter	4. p.Ser588Cys
5.c.113_114dupGC	4. c.153-1G>A (Intron 2)*		5. p.Asn146Thr	**5. p.Ser590Asn**
		5. c.232+1G>A (Intron 3)*	6. p.Arg151Gln	6. p.Gly600Arg
			7. p.Arg151Trp	7. p.Pro604Ser
			8. p.Arg161Trp	8. p.Val605Leu
			9. p.Lys164Ter	9. p.Asp608His
			10. p.Arg167Gln	10. p.Tyr628Ser
			11. p.Pro173Ser	11. p.Arg631Cys
			12. p.Glu174Lys	12. p.Leu644Ser
			13. p.Tyr210Asp	13. c.1816_1817delGT
			14. p.Asp213Gly	
			15. p.Met214Thr	14. c.1645+5G>A (Intron 5)
			16. p.Gln216Arg	
			17. p.Pro227Leu	
			18. p.Arg231Trp	
			19. p.Arg247Trp	
			20. p.Lys274Met	
			21. p.Arg296Gln	
			22. p.Arg296Leu	
			23. p.Arg296Ter	
			24. p.Gly310Gly	
			25. p.Cys326Tyr	
			26. p.Asp328Gly	
			27. p.Met342Thr	
			28. p.Phe352Cys	
			29. p.Val368Ile	
			30. p.His369Gln	
			31. p.Arg382Lys	
			32. p.Phe383Tyr	
			33. p.Gln413Gln	
			34. p.Phe448Leu	
			35. p.Arg450Ter	
			36. p.Gly451Glu	
			37. p.Glu454Ter	
			38. p.Lys457Ter	
			39. p.Tyr479Phe	
			40. p.Tyr479Cys	
			41. p.Gly480Arg	
			42. p.Glu487Lys	
			43. p.Gly497Ser	
			44. p.Ile502Thr	
			45. p.Arg503Cys	
			46. p.Pro504Leu	
			47. p.Phe516Ser	
			48. p.Glu545Ala	
			49. c.1569_1570delCA	
			50. c.1444_1447delACAG	
			51. c.1634_1636delAAG	
			52. 1646_49del	
			53. c.1273_1274delAC	
			54. c.1238_1239delAG	
			55. c.1543_1546delGCCT	
			56. c.907_918ins11	
			57. c.533insT, c.534-558del25	
			58. c.340+1G>A (Intron 4)*	
			59. c.340+5G>A (Intron 4)*	

Appendix 2: Questionnaire for assessment of clinical aspects in sub group of 13 patients that participated in a questionnaire based retrospective survey

Questionnaire for Patients with CPT II-deficiency

Date: _____

Patient: _____ **Date of Birth: _____**

Height: _____ **Weight: _____** **BMI: _____**

Serum triglyceride **Normal □** **Elevated □ (if elevated, how high?) _____**

Serum Cholesterol **Normal □** **Elevated □**

(if elevated, how high?) _____

1. **Did you experience attacks involving muscle difficulties?** Yes □ No □

2. **If yes, when did you experience the first attack?** _____

3. **How frequently do the attacks occur?**

Only once □
Multiple attacks □
Total number of attacks: _____
When multiple attacks in a year, number of attacks in last year: _____
in last month: _____

4. **Which of the following disabilities occur during attacks?**

	always	sometimes	never
Muscle pain	□	□	□
Cramps	□	□	□
Muscle weakness	□	□	□
Heaviness	□	□	□
Swelling	□	□	□
Stiffness	□	□	□
Tenderness	□	□	□
Brown colored Urine	□	□	□
Dialysis	□	□	□
Other disabilities	□	□	□

If yes, which? _____

5. **Which of these symptoms resulted in severe disability?**
_____

6. **Are the disabilities predictable?**

Yes, exactly	□
Yes, somewhat	□
Less predictable	□

7. **If muscle pain, muscle cramps or muscle weaknesses occur, which region of the body is affected by these symptoms?**

Face	□	Thighs	□
Shoulder	□	Calves	□
Upper arm	□	Trunk /Back	□
Lower arm	□	Hip area	□

8. **Please allocate the intensity of pain in an ascending scale of 1 to 10.**
(1: lowest intensity, 10: maximal intensity)

- During regular attack 1□ 2□ 3□ 4□ 5□ 6□ 7□ 8□ 9□ 10□
- During weakest attack 1□ 2□ 3□ 4□ 5□ 6□ 7□ 8□ 9□ 10□
- During most severe attack 1□ 2□ 3□ 4□ 5□ 6□ 7□ 8□ 9□ 10□

9. **For how long are the muscle pain, cramps, and weakness persistent?**

Less than 1 hour	□
1-4 hours	□
4-12 hours	□
12-24 hours	□
1-3 days	□
1 week	□
1 month	□
More than 1 month	□

10. **If brown colored urine was observed, for how long did it persist?**

Few hours	□
One whole day long	□
Up to 3 days	□
Up to 1 week	□

Longer than 1 week □

11. What were the triggers for attacks?

	Yes	No
Physical exertion for less than 15 minutes Example: _____	□	□
Physical exertion for 15-60 minutes Example: _____	□	□
Physical exertion for 1-4 hours Example: _____	□	□
Physical exertion for a whole day Example: _____	□	□
Physical exertion for multiple days Example: _____	□	□
Infection Which: _____	□	□
Exposure to cold	□	□
Fasting/ skipped meal	□	□
Substantially fatty meals	□	□
Less Fluid intake	□	□
Menstruation	□	□
Medicines If yes, which medicine(s)? _____	□	□
Psychological stress If yes, which? _____	□	□

Other triggers □ □
If yes, which?
_____

12. Estimated number of attacks, they were triggered by

- One trigger _____
- More triggers _____
- Without any apparent trigger _____

13. At what time interval of physical activities do the attacks take place?

During the activity □
Few hours afterwards □
Next day □
After few days □

14. Are the attacks avoidable or are they less pronounced by taking food just before physical activity?

Yes □ No □
If yes, is there any specific food that you preferably take before physical activity?

_____

15. How frequently do you take meals?

2x daily □ 3x daily □ 4x daily □ minimum 5x daily □

16. Have you noticed a threshold physical activity that you should not exceed to avoid attacks? Could this reduce the frequency of attacks?

Yes □ No □

17. Do you play sport? Yes □ No □
If yes, how frequently?

Daily □
Multiple times per week □
Once per week □
Every few weeks □

Which sport(s)? _____

18. Did you give up sports due to difficulties /ailment? Yes □ No □

If yes, which sport(s)? _____

19. Are difficulties or symptoms persistent outside of attacks?

Yes □ since when? _____
No □

If yes, which difficulties?

Muscle pain □

Muscle cramps □

Muscle weakness □

Others □ which? _____

20. Do you regularly take medicines? Yes □ No □
If yes, which medicine(s)? _____

21. Do you take Ibuprofen, Valproin acid or Diazepam? Yes □ No □
If yes, only during attacks □ or permanently □

22. Do you suffer from headache? Yes □ No □
If yes, is migraine diagnosed? Yes □ No □

23. Do you suffer from any other diseases (e.g. Diabetes)? Yes □ No □
If yes, which disease(s)? _____

24. Have you got general anesthesia, at least once? Yes □ No □
If yes, were specific difficulties experienced during anesthesia?
Yes □ No □
If yes, which specific difficulty? _____

25. Were more intense attacks seen during pregnancy? Yes □ No □

26. Were any complications seen during delivery or did you experience any attacks?

What type of complication? _____

27. During the course of the disease, the disabilities during attacks have:

Decreased □ Increased □ Remained same □

28. Have the disabilities led you unable to work?

Never □
Few times □
Frequently □

29. Were you forced to change your profession due to the disease?

Yes □ No □

30. When did you first start looking for the diagnosis of your disease?

_____

31. Date of final diagnosis? _____

32. How many physicians did you visit to get the final diagnosis done? _____

33. What was the specialization of those physicians?

_____

34. Was a muscle biopsy done? Yes □ No □

If yes, where and when was it done? _____

35. How was the diagnosis done?

Genetic investigation □
Biochemical investigation □
Unknown □

Are your other family members also affected? Yes □ No □

If yes, who else is affected? _____

36. Are your parents Consanguine (blood relatives)? Yes □ No □

37. Are you in a regular supervision of a neurologist? Yes □ No □

Who is your supervising neurologist? _____

38. Are you in a regular supervision of a General practitioner?

Yes □ No □

Who is your supervising General practitioner? _____

39. Do you take pain killers against the muscle pain during the attacks?

Yes □ No □

If yes, how frequently? _____

Which pain killer? _____

40. Do you observe a different treatment for the symptoms? Yes □ No □

If yes, which? _____

41. Do you practice other methods to pacify disabilities during attacks? Yes □ No □

Which methods?

Complete body rest □

Warmth □

Carbohydrate supplement □

Fluids supplement □

Other _____

42. Do you observe special diet? Yes □ No □

If yes, what kind of diet? _____

12. Lebenslauf

16.04.1977	in Baitadi, Nepal geboren
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13. Danksagung

Herrn Prof. Dr. S. Zierz, Direktor der Klinik und Poliklinik für Neurologie der Martin-Luther-Universität Halle-Wittenberg möchte ich herzlich danken, sowohl für die Möglichkeit, die Forschungstätigkeit in Halle fortzusetzen als auch für die beständige Unterstützung dieser Arbeit durch anregende Diskussionen.

Ohne die Unterstützung durch die Mitarbeiter aus dem Muskellabor der Neurologischen Universitätsklinik Halle wäre die Arbeit nicht möglich geworden. Ich bin allen Mitarbeitern dafür sehr dankbar. Besonderer Dank gilt dabei Thekla Wangemann und Kathleen Zietz sowie Dr. Leila Scholle, Beate Meinhardt und Lisa Wieland, mit denen ich im Muskellabor zusammenarbeitete.

Aber auch allen klinisch tätigen Kollegen danke ich für die Untersuchung und klinische Klassifizierung von Patienten. Dabei denke ich nicht nur an meine Kollegen in Halle, sondern auch an Ärzte aus auswärtigen Kliniken, die Proben eingeschickt haben.

Besonders danke ich unser ehemaliger Kollege Herrn Prof. Deschauer für seine umfassende Unterstützung der Forschungstätigkeit in Halle sowie die fundierte Ausbildung auf dem Gebiet der Molekularbiologie.

Meinen Eltern danke ich, dass sie mir mein Studium ermöglicht haben. Meine Frau Reshma und meine Tochter Ronya bin ich sehr dankbar dafür, dass die beiden mir immer hilfreich zur Seite standen.

14. Selbständigkeitserklärung

Hiermit erkläre ich an Eides statt, dass ich die vorliegende Habilitation selbständig und nur unter Verwendung der angegebenen Literatur und Hilfsmittel angefertigt habe.

Halle, den 06.11.2018 Pushpa Raj Joshi