

# **Study of plasma carnitine ester profiles and genotype analysis in carnitine deficiency syndromes**

PhD thesis

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## ABBREVIATIONS

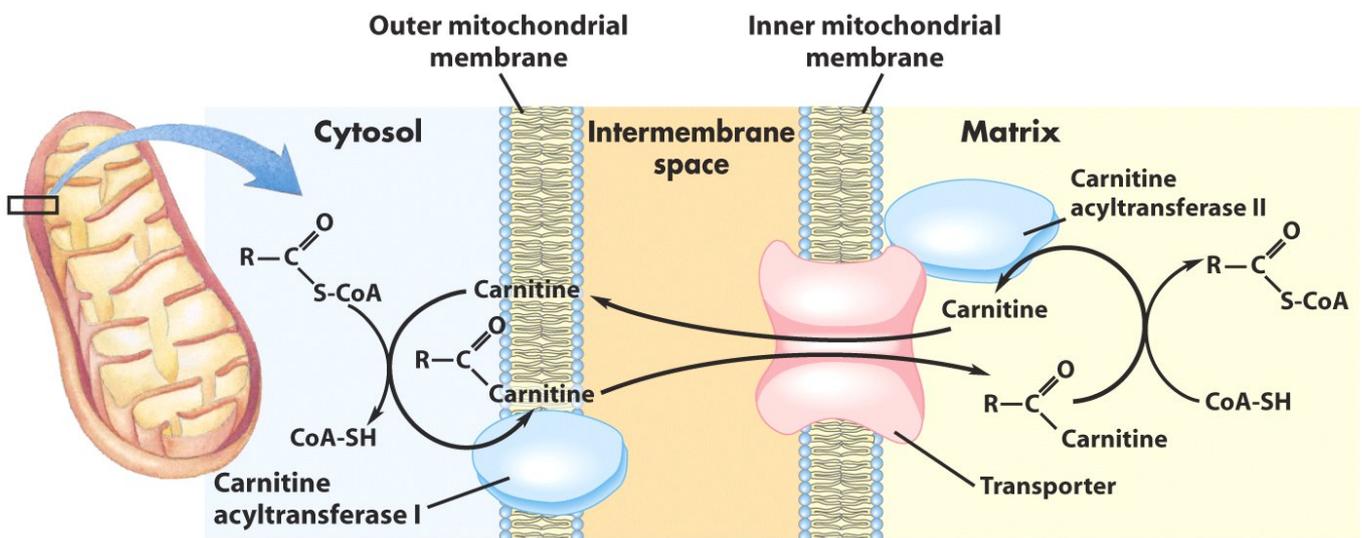
TML	$\epsilon$ -N-trimethyllysine
HTML	$\beta$ -hydroxy- $\epsilon$ -N- trimethyllysine
TMABA	$\gamma$ -trimethylamino-butyraldehyde
TMLD	$\epsilon$ -N- trimethyllysine-dioxygenase
HTMLA	$\beta$ - hydroxy - $\epsilon$ -N- trimethyllysine -aldolase
TMABA-DH	$\gamma$ -trimethylamino-butyraldehyde-dehydrogenase
BBD	$\gamma$ -butyrobetaine-dioxygenase
OCTN1	organic cation transporter 1
OCTN2	organic cation transporter 2
SLC22A4	solute carrier family 22, member 4
SLC22A5	solute carrier family 22, member 5
ESI/MS/MS	electrospray tandem mass spectrometry
IBD	inflammatory bowel disease
CD	Crohn disease
UC	ulcerative colitis
AC	acylcarnitine
FC	free carnitine
PCR	polymerase chain reaction
RFLP	restriction fragment length polymorphism
SNP	single nucleotide polymorphism
CARD15	Caspase recruitment domain-containing protein 15
SCFA	short chain fatty acid

# 1. INTRODUCTION

Carnitine ( $\beta$ -hydroxy- $\gamma$ -N-trimethylaminobutyric acid) is a principal metabolite, it can be found in almost any animal, several microorganisms and plants.

## 1.1.1. The role of carnitine in metabolism

The primary physiological role of carnitine ( $\beta$ -hydroxy- $\gamma$ -N-trimethylaminobutyric acid) is the transport of long chain fatty acids from the cytosol into the mitochondrial matrix where they undergo  $\beta$ -oxidation. This role is essential since the long chain fatty acids cannot cross the inner mitochondrial membrane neither themselves nor in CoA thioester form; the transport takes place in carnitine ester form. These transport processes are catalyzed by carnitine palmitoyl transferase I (CPT I), carnitine acylcarnitine translocase (CT or CACT – „Transporter” in Figure 1) and carnitine palmitoyl transferase II (CPT II) enzymes (Figure 1.).



**Figure 1.** Role of carnitine in the transport of long chain fatty acids from the cytosol into the mitochondrial matrix (Lehninger Principles of Biochemistry, 4th ed., © W. H. Freeman, 2005)

The other crucial role of carnitine is the transport of the products of the peroxysomal  $\beta$  oxidation (e. g. acetyl CoA) from the peroxysomes into the mitochondria where they are completely decomposed in the tricarboxylic acid cycle into  $\text{CO}_2$  and  $\text{H}_2\text{O}$ . This is important because the partial decomposition of the very long chain fatty acids ( $>\text{C}_{22}$ ) and certain branched chain fatty acids takes place in the peroxysomes.

## 1.1.2. Further functions of carnitine

Beyond its classical physiological function, carnitine plays further important roles in the body. Among others, it modulates the acyl-CoA/CoA ratio, which in turn regulates many mitochondrial enzymes involved in TCA cycle, gluconeogenesis, urea cycle and fatty acid oxidation. It contributes to energy storage in the form of acetyl carnitine and modulates the toxicity of partially metabolized acyl groups by promoting their excretion in carnitine ester form. Furthermore, it is involved in the reesterification of triacylglycerol in ER before its excretion in the form of VLDL, in the stimulation of the oxidative metabolism of pyruvate and branched chain amino acids, in the deacylation and reacylation processes during the transformation of membrane phospholipids in erythrocytes, in the transmission of fatty acids between phospholipids and triglycerides in neurons, in the synthesis and elongation of multiply unsaturated fatty acids as well as stabilization of proteins and membranes.

### 1.1.3. Carnitine homeostasis in humans

Carnitine homeostasis is maintained by three main factors: endogenous synthesis, intake from nutrients and reabsorption in kidney tubules. Nutritional sources of carnitine are mainly of animal origin (meat, poultry, fish, dairy products), smaller amounts can be found in cereals, fruits and vegetables.

The total carnitine content of human body is in a fairly dynamic state. Carnitine and acyl carnitines flow from and to the gastrointestinal tract (after absorption), liver (after biosynthesis), kidney (for excretion) and carnitine dependent tissues (e. g. heart or skeletal muscle). Any metabolic change is followed by a redistribution between the carnitine and acyl carnitine pools of the tissues involved. Since carnitine and acyl carnitines cannot cross plasma membranes directly and carnitine is transported through the membranes by tissue specific transport systems (Kerner et al., 1998), total carnitine content and the kinetics of the carnitine homeostasis can vary significantly from tissue to tissue. Dramatic changes can take place in carnitine homeostasis of certain tissues simultaneously with no observable change in others.

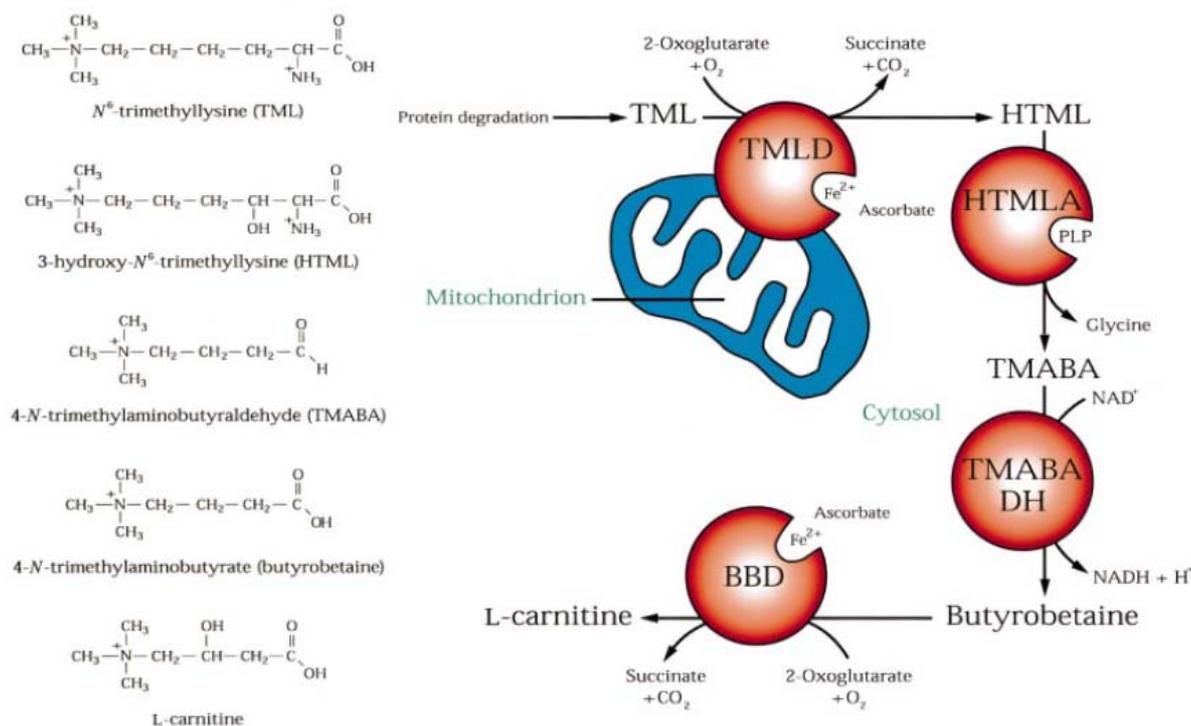
Plasma is only a transporter of carnitine and acyl carnitines, so their concentrations are relatively low. In healthy adults free L-carnitine concentration of plasma is 40-50  $\mu\text{mol/l}$ , that of acetyl carnitine (the most abundant ester) is typically 3-6  $\mu\text{mol/l}$ . Total L-carnitine concentration is approx. 50-60  $\mu\text{mol/l}$  (Evans et al., 2003). Since carnitine has no known metabolic function in plasma, changes in plasma carnitine concentrations can be understood only in relationships with other metabolic or tissue specific information. Kidney, liver and skeletal muscle contains carnitine in much higher concentrations than plasma. Due to the large mass of the skeletal muscle, the majority of the total carnitine content of the body is present in muscles and only very small amounts can be found in plasma or extracellular compartments.

Although 99% of the carnitine amount is intracellular, the relationship between serum acylcarnitine (AC) and free carnitine (FC) is highly sensitive to intramitochondrial alterations. Such alterations occur in different situations, both normal and abnormal. The normal situation is accompanied with changes in the relationship between AC and FC is fasting where increased ketogenesis leads to reduction in plasma free carnitine and the corresponding increase in AC. Other normal conditions include aging and pregnancy. Pathological situations with abnormal AC/FC ratio include several inborn errors of metabolism mainly organic acidurias, cirrhosis, chronic renal failure and iatrogenic situations such as treatment with valproate and zidovudine. Other situations with disturbed ratio AC/FC include all types of diabetes, heart failure and Alzheimer disease.

Under normal circumstances, about 80 % of serum carnitine is free carnitine and normal AC/FC ratio is 0.25; AC/FC > 0.4 are considered as abnormal (carnitine insufficiency).

### 1.2. Carnitine biosynthesis in humans

Carnitine is an essential compound thought to be synthesized in humans to some extent, however, humans do need (or under certain circumstances *may* need) external source of carnitine. Rat tissues were used as models of mammalian carnitine biosynthesis (Figure 2.).



**Figure 2.** L-Carnitine biosynthetic pathway with corresponding structural formulas (Vaz et al., 2002)

Carnitine is synthesized ultimately from the amino acids lysine and methionine. Lysine provides the carbon backbone of carnitine and the 4- $N$ -methyl groups originate from methionine. In mammals certain proteins contain  $N^6$ -trimethyllysine (TML) residues.  $N$ -methylation of these lysine residues occurs as a posttranslational event in proteins such as calmodulin, myosin, actin, cytochrome c and histones. Lysosomal hydrolysis results in the release of the TML, which is the first metabolite of carnitine biosynthesis. TML is first hydroxylated on the 3-position by TML dioxygenase to yield 3-hydroxy-TML (HTML). Aldolytic cleavage of HTML yields 4-trimethylaminobutyraldehyde (TMABA) and glycine, reaction catalyzed by HTML-aldolase. Dehydrogenation of TMABA by TMABA-dehydrogenase results in the formation of 4- $N$ -trimethylaminobutyrate (butyrobetaine). In the last step, butyrobetaine is hydroxylated on the 3-position by  $\gamma$ -butyrobetaine dioxygenase. In humans the rate of carnitine biosynthesis is determined by the availability of trimethyllysine at the intramitochondrial site of trimethyllysine hydroxylase activity. The regulatory effect of trimethyllysine was demonstrated by Rebouche et al., when excess trimethyllysine was given to humans and an 8-fold increase in carnitine biosynthesis occurred. Humans synthesize approx. 1-2  $\mu$ mol of carnitine /kg/day.

### 1.3. Carnitine deficiency

Carnitine deficiency represents a heterogeneous group of diseases with widely varying clinical symptoms. Given that carnitine not only is required for fatty acid oxidation but also plays a crucial role in cellular homeostasis of free and acyl-CoA, the wide variety of clinical manifestation of carnitine deficiency is not unexpected. This is further complicated by the fact that the carnitine requirement depends on several other factors, such as age, diet, metabolic conditions (stress, fed versus fasting, and rest versus exercise) and tissue dependence on fatty acid oxidation. Carnitine deficiency is defined as decreased tissue carnitine concentration that is below the requirement for normal cellular metabolism. Clinical and biochemical data suggest that tissue carnitine levels may have to fall to less than 10% to 20% of normal before the biologic effects can be clinically significant. Carnitine deficiency can be primary and secondary. In primary systemic carnitine deficiency the cellular uptake of carnitine is mainly disturbed,

while in secondary carnitine deficiency the carnitine depletion is the secondary cause of other conditions or disease.

## **2. AIMS OF THE STUDY**

1. In order to achieve a better understanding of carnitine biosynthesis in humans, appearance of supposed intermediates was studied in urine samples of healthy full-term newborns after administration of deuterium labeled TML.
2. Molecular genetic study of *SLC22A5* gene in primary carnitine deficient patients, their family members and siblings died in SIDS.
3. Study of efficacy of carnitine therapy in Leigh syndrome patients.
4. Mass spectrometric study of plasma carnitine ester profiles influenced by malabsorption in celiac patients held on gluten free diet on.
5. Molecular genetic study of possible role of *CARD15* gene variants and polymorphisms of carnitine transporter genes in the pathogenesis of Crohn's disease in child and adult Crohn's disease patients.
6. Study of the effect of *SLC22A4* C1672T and *SLC22A5* G-207C polymorphisms on carnitine ester profiles in adult Crohn's patients.
7. Mass spectrometric study of carnitine metabolism in ulcerative colitis patients.

## **3. PATIENTS AND METHODS**

### **3.1. Patients**

#### *3.1.1. Study of carnitine biosynthesis*

Seven orally fed male infants were studied. The mean (SD) gestational age was 38.5 (0.87) weeks (range, 38-41 weeks). Postnatal age at the beginning of the study was 8 (range, 5-11) days, and body weight was 3360 (100) g (range, 3100-4300 g). The infants were fed 70 g of formula (Aptamil) seven times daily. The study period lasted 5 consecutive days, with 24-h urines collected on the day before administration of N<sup>6</sup>[Me-<sup>2</sup>H<sub>9</sub>]TML (day 0) and on the last day (day 5) of the study. After the preloading control day, the newborns received 0.5 mmol of N<sup>6</sup>[Me-<sup>2</sup>H<sub>9</sub>]TML daily in their formula during the 5-day study period.

The 24-h urines used for determination of the carnitine biosynthesis intermediates in controls were obtained from 40 healthy individuals (23 males and 17 females) with a mean age of 21 years (SD, 17; range, 2-66 years).

#### *3.1.2. Patients with primary carnitine deficiency*

During the study of primary systemic carnitine deficiency the members of two unrelated Hungarian Roma (Gypsy) families were investigated.

#### *3.1.3. A patient with Leigh disease*

In a patient with Leigh disease, presenting with severe cerebellar ataxia, mild mental retardation and muscular myopathy, molecular genetic studies revealed a maternally inherited heteroplasmic 8993 T>C mutation (95 % and 88 % mutant form in skeletal muscle and blood cells, respectively) of the mitochondrial DNA. In addition, a homoplasmic 8860 A>G transition (threonine → alanine change) was also detected in the sequence of the ATPase 6 gene of the patient and his mother.

### 3.1.4. Adult celiac disease

We examined 33 patients with classic form of celiac disease and 35 carefully selected clinically healthy control subjects.

The diagnostic criteria of established celiac disease in our patients included: verification of the specific histological features in small intestinal biopsy specimens, according to the modified Marsh classification, positive serological results (antiendomysial antibody and tissue transglutaminase), unequivocally favorable clinical response to the administration of gluten free diet. Patients with any of the rare manifestations of the disease were excluded. All of the celiac disease patients were at least 17 years old upon diagnosis, and adhered to gluten free diet for at least one year. All patients received long-term oral iron replacement therapy.

### 3.1.5. Crohn's disease

#### Pediatric patients with Crohn's disease

We examined 19 pediatric and 55 adult patients with CD. This cohort was compared with 49 healthy controls. Both the pediatric and adult CD patients exhibited different clinical manifestations, therefore they represented mixed clinical CD populations. The diagnosis was confirmed by clinical, radiological, endoscopic and histological findings.

#### Adult patients with Crohn's disease

For the genetic study we investigated the DNA of 100 patients with CD and 94 carefully selected healthy controls. The circulating plasma carnitine ester profile was determined in 85 patients and 48 healthy controls.

### 3.1.6. Patients with ulcerative colitis

We examined 44 patients with UC, and 44 carefully selected clinically healthy control subjects. The control subjects did not receive any drug medication, while the UC patients were treated with either sulfasalazine or 5-aminosalicylic acid. Diagnosis of the disease relied upon the history of the patients, clinical symptoms, negative stool examination for bacteria and parasites, and histologic results of rectal and/ or colonic biopsy.

Informed consent was obtained from each participant of the study and the study design was approved by the Departmental Ethics Committee.

## 3.2. Analytical methods

### 3.2.1. Analysis of carnitine biosynthesis metabolites in urine by HPLC-ESI tandem mass spectrometry

The analysis of carnitine biosynthesis metabolites in newborns and healthy controls were carried out from 24-h urine samples. After derivatization with methyl chloroformate the analytes were separated on Phenomenex Aqua analytical column using linear gradient elution techniques (mobile phase consist of: 11 ml/l heptafluorobutyric acid and 900 ml/l methanol). A Quattro II tandem mass spectrometer (Micromass) was used in the positive electrospray ionization mode. Nitrogen was used as both the nebulizing and the drying gas. The collision gas was argon, and the cell pressure was 0.25 Pa. The source temperature was set at 80 °C, and the capillary voltage was maintained at 3.5 kV. The detector was used in tandem MS mode with multiple-reaction monitoring to detect the transition of a specific precursor ion to a fragment for each analyte.

### 3.2.2. Determination of plasma and serum carnitine ester profile with ESI tandem mass spectrometry

Acylcarnitines were analyzed as butyl esters using a Micromass Quattro Ultima ESI triple-quadrupole mass spectrometer, combined with a Waters 2795 HPLC system for sample introduction. After derivatization with 3M butanolic HCl, samples were redissolved in the mobile phase (acetonitrile:water 80:20). With the help of the autosampler 10  $\mu$ l of sample aliquots were injected into the mass spectrometer. During the ESI-MS/MS analysis the free carnitine and acylcarnitines were measured by positive precursor ion scan of m/z 85, with a scan range of m/z: 200-550. The applied capillary voltage, cone voltage and collision energy were 2.52kV, 55V and 26eV, respectively. The flow rate was 100  $\mu$ l/min and the total analysis time was 4 minutes per sample. For each sample the measurements were performed in triplicates beginning with the injection step and the means of the three determinations were used for further calculations.

### 3.3. Molecular genetic methods

*Analysis of mitochondrial ATPase 6 gene mutations, SLC22A5, CARD15 gene mutations and SLC22A4 and SLC22A5 gene polymorphism with automated sequencer.*

For the analysis of mitochondrial ATPase6 gene and detection of CARD 15 gene and SLC22A4 and SLC22A5 polymorphism, a gene specific primer pair were designed. For the amplification of the 10 exons of SLC22A5 genes a intron-based specific primer pairs were used. The PCR products were sequenced in both directions in an ABI Prism 3100 Genetic Analyzer with a dye termination method. We also developed a RFLP method for quick genotyping of the samples for the SLC22A5 mutation. After digestion of the PCR product with Bsl I enzyme the restriction fragments were separated in agarose gel.

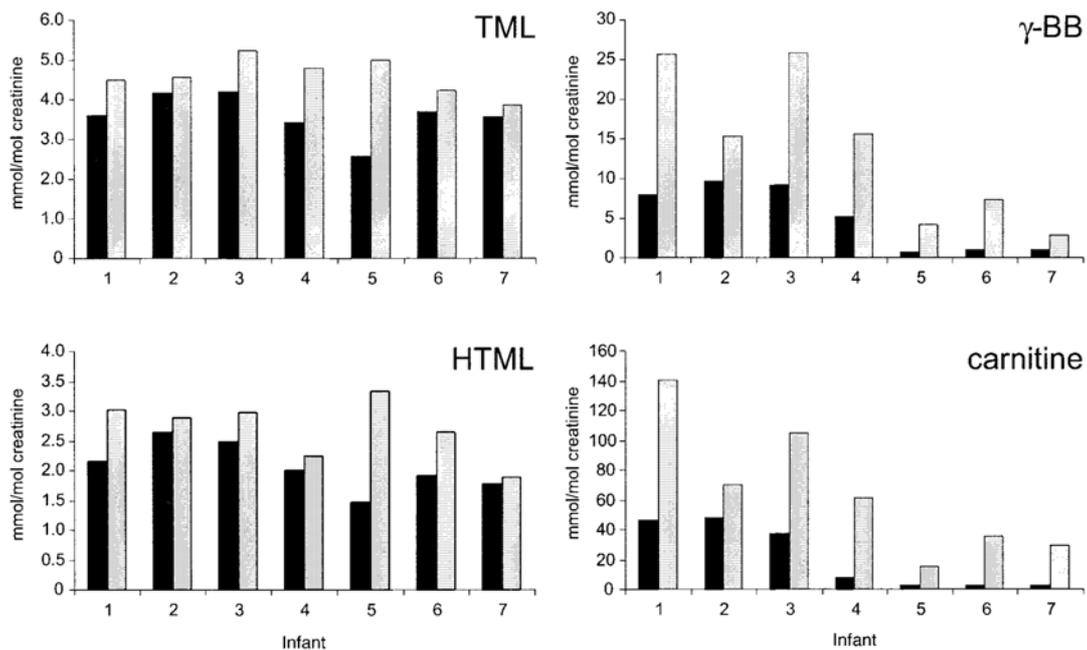
### 3.4. Statistics

For statistics the Student's t-test for unpaired samples was used. The values are expressed as means and their standard errors, in three decimals for the carnitine esters with respect to the low levels in the case of the long-chain carnitine esters. Chi-square test was applied for the determination of the distribution of different genotypes.

## 4. RESULTS

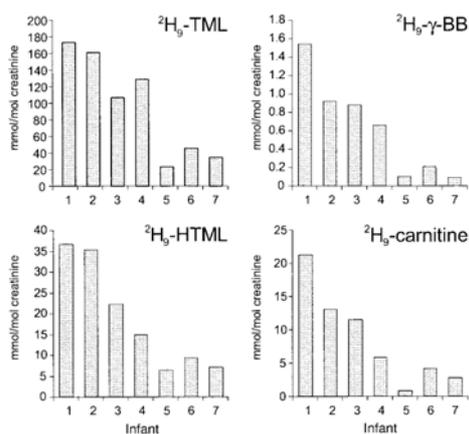
### 4.1. Study of carnitine biosynthesis intermediates

To determine whether full-term newborns can utilize exogenous TML for carnitine biosynthesis, we performed a loading test with deuterium-labeled TML in seven healthy newborns. Figs. 3 and 4 show the urinary concentrations of endogenous and  $^2\text{H}_9$ -labeled TML, HTML,  $\gamma$ -BB, and carnitine before and after [ $^2\text{H}_9$ ]TML administration. Both before and after loading, endogenous concentrations of TML and HTML appeared to be proportional to creatinine (Fig. 3). After [ $^2\text{H}_9$ ]TML administration, the mean concentrations of endogenous TML and HTML increased moderately but significantly ( $P < 0,001$ ). The concentration of endogenous  $\gamma$ -BB before [ $^2\text{H}_9$ ]TML administration varied greatly, ranging from 0.7 to 9.7 mmol/mol creatinine. After loading, the  $\gamma$ -BB excretion increased considerably, ranging from 1.5 to 6.7 times the original concentration (Fig. 3). Carnitine concentrations showed a similar behavior, ranging from 2.5 to 48 mmol/mol creatinine before [ $^2\text{H}_9$ ]TML administration and increasing 1.5- to 12-fold after [ $^2\text{H}_9$ ]TML administration (Fig. 3). The urine samples obtained after [ $^2\text{H}_9$ ]TML administration (Fig. 4) all contained [ $^2\text{H}_9$ ]TML, [ $^2\text{H}_9$ ]HTML, [ $^2\text{H}_9$ ]- $\gamma$ -BB, and [ $^2\text{H}_9$ ]-carnitine, demonstrating that newborns are capable of synthesizing carnitine. [ $^2\text{H}_9$ ]-TMABA was below the detection limit in these urines.



**Figure 3.** Urinary concentrations of endogenous carnitine biosynthesis metabolites (except TMABA) in seven newborns before (black column) and after (grey column) loading with  $[^2\text{H}_9]\text{TML}$ .

For all seven full-term newborns, the products of one or more enzymatic steps of the carnitine biosynthetic pathway accounted for 23 % (SD, 4 %) of the total amount of excreted  $[^2\text{H}_9]$ -labeled compounds, and 10 % (SD, 3 %) of the free carnitine in urine was  $[^2\text{H}_9]$ -labeled carnitine (range, 5-16 %). The concentrations of endogenous and  $[^2\text{H}_9]$ -labeled carnitine measured in the urine samples were in agreement with the results obtained with the established procedure for the analysis of acylcarnitines by tandem MS. Acylcarnitine analysis also revealed the presence of acetyl- $[^2\text{H}_9]$ -carnitine, propionyl- $[^2\text{H}_9]$ -carnitine, and butyryl- $[^2\text{H}_9]$ -carnitine in most of the samples after  $[^2\text{H}_9]\text{TML}$  loading.



**Figure 4.**  $^2\text{H}_9$ -labeled carnitine biosynthesis metabolites (except TMABA) in seven newborns after loading with  $[^2\text{H}_9]\text{TML}$ .

#### **4.2. Molecular genetic study of patients with primary carnitine deficiency**

In two non-consanguineous Hungarian Roma (Gypsy) children who presented with cardiomyopathy and decreased plasma carnitine levels, we identified homozygous single base deletion (844delC in the cDNA and 17081delC in the genomic sequence) of the SLC22A5 gene that results in a frameshift at R282D and leads ultimately to a premature stop codon (V295X) in the OCTN2 carnitine transporter. No other sequence change in any exons could be observed as compared to AB016625 used as reference sequence.

Mutation analysis were carried out for DNA samples from 24 members of the first family and paraffin embedded blocks from two of the fatal cases. The two sudden death patients were also homozygous for the mutation, and a total of 12 carriers were verified.

The molecular genetic analysis of the second family revealed a homozygous single base deletion in the case of the index patient and the sudden death patient. Both parents carries the mutation in heterozygous form.

#### **4.3. Molecular genetic study of the mitochondrial DNA and determination of plasma carnitine ester profile of a patient with Leigh disease**

In a male child patient the molecular genetic studies revealed a maternally inherited heteroplasmic 8993 T>C mutation (95 % and 88 % mutant form in skeletal muscle and blood cells, respectively) of the mitochondrial DNA. In addition, a homoplasmic 8860 A>G transition (threonine → alanine change) was also detected in the sequence of the ATPase 6 gene of the patient and his mother.

In contrast to the normal pre-supplementation pattern, the plasma carnitine ester profile determined during carnitine treatment showed significantly elevated concentrations of free carnitine (117  $\mu\text{mol/l}$ ), butyrylcarnitine (5.29 vs  $0.35 \pm 0.20 \mu\text{mol/l}$ ) and hexanoylcarnitine (1.68 vs  $0.08 \pm 0.04 \mu\text{mol/l}$ ), with normal concentrations of all other acylcarnitines.

#### 4.4. Determination of plasma carnitine ester profile in adult celiac disease patients

The plasma circulating carnitine ester profiles are shown in Table 1. The plasma level of free carnitine did not differ between celiac disease patients and controls. By contrast, a marked decrease was found in the acetylcarnitine level in the patients, which corresponded to 46% of the control value. A significant decrease was also found in the levels of propionyl- (61.5%), butyryl- (56.9%), hexanoyl- (75%), octanoyl- (71.1%), octenoyl- (52.1%), decanoyl- (73.1%) cecenoyl- (58.3%), lauroyl- (61.5%), miristoyl- (66.7%), miristoleyl- (62.5%) and oleylcarnitine (81.1%) in the patients as compared with the controls (the rates of decrease are expressed throughout as percent in parentheses taking the controls as 100%). As a result of the decrease of individual carnitine esters, the plasma level of total esters was lower in patients with celiac disease than in controls ( $6.087 \pm 0.571$  vs.  $12.166 \pm 0.978$ ,  $P < 0.001$ ). The ratio of acetylcarnitine/total carnitine esters was 0.773 in the patients and was 0.841 in the controls.

**Table 1.** Plasma carnitine ester profiles in celiac disease patients and controls (mean  $\pm$  SEM,  $\mu\text{mol/l}$ ).

	PATIENTS n=33	CONTROLS n=35
Free carnitine (C0)	27.191 $\pm$ 1.194	30.029 $\pm$ 1.902
<b>Short-chain acylcarnitines</b>		
Acetylcarnitine (C2)	4.703 $\pm$ 0.205*	10.227 $\pm$ 0.368
Propionylcarnitine (C3)	0.247 $\pm$ 0.014*	0.400 $\pm$ 0.021
Butyrylcarnitine (C4)	0.152 $\pm$ 0.011*	0.267 $\pm$ 0.013
Isovalerylcarnitine (C5)	0.111 $\pm$ 0.010	0.138 $\pm$ 0.010
Tiglylcarnitine (C5:1)	0.034 $\pm$ 0.002	0.033 $\pm$ 0.003
<b>Medium-chain acylcarnitines</b>		
Hexanoylcarnitine (C6)	0.060 $\pm$ 0.004*	0.080 $\pm$ 0.006
Octanoylcarnitine (C8)	0.086 $\pm$ 0.006*	0.121 $\pm$ 0.009
Octenoylcarnitine (C8:1)	0.037 $\pm$ 0.003*	0.071 $\pm$ 0.008
Decanoylcarnitine (C10)	0.103 $\pm$ 0.008*	0.141 $\pm$ 0.009
Decenoylcarnitine (C10:1)	0.063 $\pm$ 0.005*	0.108 $\pm$ 0.010
Lauroylcarnitine (C12)	0.032 $\pm$ 0.002*	0.052 $\pm$ 0.004
<b>Long-chain acylcarnitines</b>		
Myristoylcarnitine (C14)	0.016 $\pm$ 0.001*	0.024 $\pm$ 0.001
Myristoleylcarnitine (C14:1)	0.025 $\pm$ 0.002*	0.040 $\pm$ 0.004
Palmitoylcarnitine (C16)	0.097 $\pm$ 0.006	0.113 $\pm$ 0.006
Palmitoleylcarnitine (C16:1)	0.037 $\pm$ 0.003	0.032 $\pm$ 0.002
Stearoylcarnitine (C18)	0.076 $\pm$ 0.004	0.080 $\pm$ 0.004
Oleylcarnitine (C18:1)	0.137 $\pm$ 0.007*	0.169 $\pm$ 0.008
Hydroxymyristoylcarnitine (C14OH)	0.007 $\pm$ 0.001	0.005 $\pm$ 0.001
Hydroxypalmitoylcarnitine (C16OH)	0.022 $\pm$ 0.001	0.023 $\pm$ 0.002
Hydroxypalmitoleylcarnitine (C16:1OH)	0.026 $\pm$ 0.002	0.029 $\pm$ 0.002
Hydroxyoleylcarnitine (C18:1OH)	0.016 $\pm$ 0.002	0.013 $\pm$ 0.002

\*  $p < 0.01$

#### 4.5. Investigation of polymorphisms of CARD15 gene and the carnitine transporter genes (SLC22A4, SLC22A5) in Crohn's disease patients

A total of 52.6% of pediatric patients with Crohn's disease carried at least one NOD2 mutation compared to 34.5% of adult patients and to 14.3% of the controls (pediatric patients *vs.* controls  $P < 0.05$ ).

While the T allele frequency, leading to heterozygous and homozygous R702W mutation, was increased approximately two-fold in the adult CD population (9.09%) compared to the controls (4.08%), it was only 2.63% in the pediatric CD patients (Table x;  $P < 0.05$  comparing the pediatric susceptibility and/or normal variants *versus* the same values of the adult patients or the controls). By contrast, the C allele frequency, encoding the G908R variant, was found highly elevated (18.4%) in pediatric patients, and was only 1.82% in adult CD patients, and 1.02% in the controls ( $P < 0.05$ ). For the 1007finsC variant, a significantly increased prevalence was found both in pediatric (21.1%) and in adult CD (11.8%) patients as compared with the controls (3.06%) ( $P < 0.05$ ). There were no significant differences in the allele frequencies of SLC22A4 C1672T and SLC22A5 G-207C mutations when compared either the results of the pediatric or the adult CD populations to the results of the controls (Table 2).

**Table 2.** Comparison of the alleles of OCTN cation transporters and NOD2/CARD 15 genes in pediatric and adult Crohn's disease patients with controls

		Pediatric patients <i>n</i> =19 (%)	Adult patients <i>n</i> =55 (%)	Controls children <i>n</i> =49 (%)
CARD15 genotype				
R702W	CC	18 (94.7)	46 (83.6)	46 (93.9)
	CT	1 (5.3)	8 (14.6)	2 (4.1)
	TT	-	1 (1.8)	1 (2.0)
	T allele frequency	2.63 %	9.09 %	4.08 %
G908R	GG	14 (73.7)	53 (96.4)	48 (98.0)
	GC	3 (15.8)	2 (3.6)	1 (2.0)
	CC	2 (10.5)	-	-
	C allele frequency	18.4 %	1.82 %	1.02 %
1007finsC	--	13 (68.5)	44 (80.0)	46 (93.9)
	- insC	4 (21.0)	9 (16.4)	3 (6.1)
	insC insC	2 (10.5)	2 (3.6)	-
	Cins allele frequency	21.1 %	11.8 %	3.06 %
SLC22A4 genotype				
C1672T	CC	4 (21.0)	18 (32.7)	12 (24.5)
	CT	11 (58.0)	30 (54.5)	25 (51.0)
	TT	4 (21.0)	7 (12.8)	12 (24.5)
	T allele frequency	50.0 %	40.0 %	50.0 %
SLC22A5 genotype				
G-207C	GG	3 (15.8)	14 (25.5)	10 (20.4)
	GC	7 (36.8)	31 (56.4)	26 (53.1)
	CC	9 (47.4)	10 (18.1)	13 (26.5)
	C allele frequency	65.8 %	46.4 %	53.1 %

#### 4.6. Plasma carnitine ester profiles in Crohn's disease patients characterized for SLC22A4 C1672T and SLC22A5 G-207C genotypes

The plasma circulating carnitine ester profiles in the mixed CD population are shown in Table 3. The plasma level of free carnitine and acetyl carnitine did not differ between the CD patients and the controls. By contrast, significant decreases were found in fasting propionyl-, butyryl- and isovalerylcarnitine ester levels in CD patients as compared with the controls. There was no significant difference in the level of total short-chain carnitine esters (C2–C5:1) between the CD patients ( $17.301 \pm 0.155$ )  $\mu\text{mol/l}$ ) and the healthy controls ( $16.966 \pm 0.146$ )  $\mu\text{mol/l}$ ).

**Table 3.** Plasma carnitine ester profiles in the mixed group of Crohn's disease patients and controls (mean  $\pm$  SEM,  $\mu\text{mol/l}$ ).

	PATIENTS n=85	CONTROLS n=48
Free carnitine (C0)	28.670 $\pm$ 0.0529	30.639 $\pm$ 0.927
<b>Short-chain acylcarnitines</b>		
Acetylcarnitine (C2)	16.598 $\pm$ 0.402	16.160 $\pm$ 0.548
Propionylcarnitine (C3)	0.243 $\pm$ 0.008*	0.283 $\pm$ 0.014
Butyrylcarnitine (C4)	0.274 $\pm$ 0.009*	0.301 $\pm$ 0.013
Isovalerylcarnitine (C5)	0.147 $\pm$ 0.006*	0.185 $\pm$ 0.009
Tiglylcarnitine (C5:1)	0.039 $\pm$ 0.002	0.037 $\pm$ 0.002
<b>Medium-chain acylcarnitines</b>		
Hexanoylcarnitine (C6)	0.103 $\pm$ 0.004	0.092 $\pm$ 0.004
Octanoylcarnitine (C8)	0.155 $\pm$ 0.007	0.144 $\pm$ 0.009
Octenoylcarnitine (C8:1)	0.086 $\pm$ 0.006*	0.069 $\pm$ 0.005
Decanoylcarnitine (C10)	0.160 $\pm$ 0.008	0.152 $\pm$ 0.009
Decenoylcarnitine (C10:1)	0.119 $\pm$ 0.006	0.125 $\pm$ 0.008
Lauroylcarnitine (C12)	0.049 $\pm$ 0.003	0.045 $\pm$ 0.003
<b>Long-chain acylcarnitines</b>		
Myristoylcarnitine (C14)	0.023 $\pm$ 0.001	0.021 $\pm$ 0.001
Myristoleylcarnitine (C14:1)	0.048 $\pm$ 0.003*	0.037 $\pm$ 0.003
Palmitoylcarnitine (C16)	0.140 $\pm$ 0.005 *	0.122 $\pm$ 0.004
Palmitoleylcarnitine (C16:1)	0.048 $\pm$ 0.002	0.043 $\pm$ 0.003
Stearoylcarnitine (C18)	0.082 $\pm$ 0.003	0.077 $\pm$ 0.004
Oleylcarnitine (C18:1)	0.172 $\pm$ 0.006*	0.156 $\pm$ 0.008
Hydroxymyristoylcarnitine (C14OH)	0.008 $\pm$ 0.001	0.005 $\pm$ 0.001
Hydroxypalmitoylcarnitine (C16OH)	0.033 $\pm$ 0.002	0.033 $\pm$ 0.002
Hydroxypalmitoleylcarnitine (C16:1OH)	0.038 $\pm$ 0.001	0.037 $\pm$ 0.002
Hydroxyoleylcarnitine (C18:1OH)	0.020 $\pm$ 0.001	0.016 $\pm$ 0.001

\*  $p < 0.05$

The level of octenoylcarnitine was decreased in the healthy subjects; and the level of total medium-chain acylcarnitines (C6–C12) was  $0.672 \pm 0.007$  in the CD patients vs  $0.627 \pm 0.007$   $\mu\text{mol/l}$  in the control subjects. In the long-chain acylcarnitine group the plasma levels of myristoleyl-, palmitoyl- and oleylcarnitine were significantly decreased in the healthy group. The level of total longchain carnitine esters (C14–C18:1-OH) was  $0.612 \pm 0.006$  in the patients with CD vs  $0.547 \pm 0.005$   $\mu\text{mol/l}$  in the controls. The plasma carnitine ester profiles with different SLC22A4 1672 C and T genotypes and with different SLC22A5 -207G and C genotypes were determined. Albeit there were some trends for change for some individual esters (C4 and C5 carnitines), there were no statistically significant differences in the levels of any carnitine esters for any genetic variants when the heterozygous or the homozygous

populations were compared. Similarly, TC haplotype did not modify the carnitine ester profile either in the CD group or in the controls.

#### 4.7. Study of plasma fasting carnitine ester profile in patients with ulcerative colitis

The plasma circulating carnitine ester profiles are shown in Table 4. The plasma level of free carnitine and acetyl carnitine did not differ between the UC patients and the controls. By contrast, significant decreases were observed in fasting propionyl-, butyryl-, and isovalerylcarnitine ester levels in UC patients as compared with the controls. The level of total short-chain carnitine esters was markedly lower in the patients with UC ( $9.855 \pm 0.094 \mu\text{mol/L}$ ) than in the healthy controls ( $11.003 \pm 0.100 \mu\text{mol/L}$ ,  $P < 0.01$ ). The levels of octanoyl-, and decanoylcarnitine were decreased in the healthy subjects. The levels of total medium-chain acylcarnitines were obviously higher in the UC patients ( $0.629 \pm 0.007 \mu\text{mol/L}$ ) than in the control subjects ( $0.548 \pm 0.007 \mu\text{mol/L}$ ,  $P < 0.01$ ). In the long-chain acylcarnitine group, the plasma levels of myristoyl-, palmitoyl-, palmitoleyl- and oleylcarnitine were significantly decreased in the healthy group. The levels of total long-chain carnitine esters were markedly higher in the patients with UC ( $0.596 \pm 0.005 \mu\text{mol/L}$ ) than in the controls ( $0.515 \pm 0.009 \mu\text{mol/L}$ ,  $P < 0.01$ ). In addition, the level of total carnitine esters was significantly decreased in the UC patients ( $11.080 \pm 0.035 \mu\text{mol/L}$ ) as compared with the healthy controls ( $12.066 \pm 0.037 \mu\text{mol/L}$ ,  $P < 0.01$ ).

**Table 4.** Plasma carnitine ester profiles in ulcerative colitis and controls (mean  $\pm$  SEM,  $\mu\text{mol/l}$ ).

	PATIENTS n=44	CONTROLS n=44
Free carnitine (C0)	31.595 $\pm$ 1.454	31.431 $\pm$ 1.042
<b>Short-chain acylcarnitines</b>		
Acetylcarnitine (C2)	9.164 $\pm$ 0.426	10.179 $\pm$ 0.461
Propionylcarnitine (C3)	0.331 $\pm$ 0.019*	0.392 $\pm$ 0.017
Butyrylcarnitine (C4)	0.219 $\pm$ 0.014*	0.265 $\pm$ 0.012
Isovalerylcarnitine (C5)	0.111 $\pm$ 0.008*	0.134 $\pm$ 0.008
Tiglylcarnitine (C5:1)	0.030 $\pm$ 0.004	0.033 $\pm$ 0.002
<b>Medium-chain acylcarnitines</b>		
Hexanoylcarnitine (C6)	0.071 $\pm$ 0.006	0.081 $\pm$ 0.005
Octanoylcarnitine (C8)	0.147 $\pm$ 0.009*	0.114 $\pm$ 0.008
Octenoylcarnitine (C8:1)	0.064 $\pm$ 0.005	0.062 $\pm$ 0.007
Decanoylcarnitine (C10)	0.180 $\pm$ 0.012*	0.137 $\pm$ 0.008
Decenoylcarnitine (C10:1)	0.113 $\pm$ 0.006	0.104 $\pm$ 0.008
Lauroylcarnitine (C12)	0.054 $\pm$ 0.003	0.050 $\pm$ 0.003
<b>Long-chain acylcarnitines</b>		
Myristoylcarnitine (C14)	0.026 $\pm$ 0.001	0.024 $\pm$ 0.001
Myristoleylcarnitine (C14:1)	0.048 $\pm$ 0.003*	0.039 $\pm$ 0.003
Palmitoylcarnitine (C16)	0.128 $\pm$ 0.006*	0.109 $\pm$ 0.004
Palmitoleylcarnitine (C16:1)	0.042 $\pm$ 0.003*	0.031 $\pm$ 0.002
Stearoylcarnitine (C18)	0.085 $\pm$ 0.003	0.079 $\pm$ 0.003
Oleylcarnitine (C18:1)	0.183 $\pm$ 0.007*	0.163 $\pm$ 0.007
Hydroxymyristoylcarnitine (C14OH)	0.007 $\pm$ 0.001	0.006 $\pm$ 0.001
Hydroxypalmitoylcarnitine (C16OH)	0.026 $\pm$ 0.002	0.023 $\pm$ 0.001
Hydroxypalmitoleylcarnitine (C16:1OH)	0.033 $\pm$ 0.002	0.029 $\pm$ 0.002
Hydroxyoleylcarnitine (C18:1OH)	0.018 $\pm$ 0.002*	0.012 $\pm$ 0.001

\*  $p < 0,05$

## 5. SUMMARY OF THE RESULTS AND CONCLUSIONS

1. After [<sup>2</sup>H<sub>9</sub>]-TML administration, all the metabolites of carnitine biosynthesis could be detected in the urine of full-term newborns in the [<sup>2</sup>H<sub>9</sub>]-labeled form, except for [<sup>2</sup>H<sub>9</sub>]TMABA. Most of the [<sup>2</sup>H<sub>9</sub>]-TML, however, was excreted unchanged in the urine. Our observations opposite to the results obtained from the animal model suggest that free TML yield carnitine in an extremely poor way.

2. In two patients with primary carnitine deficiency, we detected a homozygous single base deletion (844delC in the cDNA and 17081 delC in the genomic sequence) of the SLC22A5 gene that result a frameshift at R282D and leads ultimately to a premature stop codon in the OCTN2 carnitine transporter. We identified this mutation for the first time in the literature in sudden infant death cases.

3. In a patient with Leigh disease, molecular genetic studies revealed a maternally inherited heteroplasmic 8993 T>C mutation of the mitochondrial DNA. After introduction of carnitine therapy an impressive clinical response was observed.

4. Measuring the carnitine ester profile of celiac patients on long-term gluten free diet a significant decrease was observed in the level of the following esters: C2-, C3-, C4-, C6-, C8-, C8:1-, C10-, C10:1-, C12-, C14-, C14:1- és C18:1-carnitine ester. The changes observed could be the result of impaired carnitine homeostasis, consequence of influenced metabolism of the acyl groups derived mainly from the fatty acid metabolism, and combination of thereof.

5. The molecular genetic analysis of the CARD15, SLC22A4 and SLC22A5 gene polymorphisms showed the accumulation of the CARD15 variants in the Crohn's disease patients. We could not detect the accumulation of either the SLC22A4, SLC22A5 susceptibility variants or the TC haplotype. Our results do not support the assumption that the carriage of the SLC and SLC TC haplotypes means significant susceptibility to Hungarian patients with CD.

6. Analyzing the effect of SLC22A4 C1672T and SLC22A5 G-207C SNPs for the carnitine status of patients with Crohn's disease, no association was observed between the different genotype variations and the altered carnitine ester profile.

7. We investigated the carnitine status of patients with ulcerative colitis by tandem mass spectrometry first in the literature. Significant decreases were observed in fasting plasma levels of C3-, C4- and C5 carnitine esters, which refers to an altered fatty acid metabolism.

## 6. LIST OF PUBLICATIONS

### The thesis is based on the following publications

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