CARNITINE

ANALYTICAL AND PHYSIOLOGICAL ASPECTS

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1. SUMMARY

Carnitine is an endogenous amino acid derivative which plays a key role in energy metabolism. Through acylation of its β-hydroxy group, carnitine is involved in the interorganellar transport of long-chain fatty acids and in the regulation of the coenzyme A pool. Carnitine is present in most mammalian tissues in its free form or as acylcarnitines of several chain lengths.

The present work consists of three projects investigating analytical and physiological aspects of carnitine and its derivatives and emphasizing the essential function of carnitine in detoxifying processes and energy balance.

The interest of the first project is the carnitine homeostasis in patients treated with the antiepileptic drug valproate. Although generally well tolerated, valproate is associated with carnitine deficiency through an unknown mechanism. We studied the effects of valproate on the carnitine balance in long term patients and in one patient starting a treatment with valproate, as well as in an *in vitro* model of the renal transport of carnitine by OCTN2.
We show that a patient starting valproate treatment has decreased carnitine plasma concentrations in the initial phase of the therapy. After long term treatment, plasma concentrations of carnitine and acylcarnitines are similar to control patients, with the exception of valproylcarnitine which is only present in treated patients. Urinary excretions of carnitine are substantially lower in valproate patients, suggesting a renal compensatory mechanism. We pointed out that valproylcarnitine although not reabsorbed, barely contributes to the carnitine loss at the beginning of a therapy.

The focus of the second project is the optimization of a LC-MS/MS method for the simultaneous determination of carnitine and acylcarnitines in plasma. For a rapid sample workup, we integrated an online solid phase extraction step to the chromatographic run after protein precipitation. Quantification was achieved with the addition method to account for matrix effects. We report a reliable method for the quantification of carnitine, acetylcarnitine, octanoylcarnitine, and palmitoylcarnitine requiring minimal sample workup, short analysis time and low sample volumes.

In the last project we investigated the effects of carnitine, acetylcarnitine and propionylcarnitine on the carnitine muscle balance and on the performance of mice completing a running exercise until exhaustion. Carnitine and its two acyl-derivatives did not influence the total muscle carnitine content or the physical performance. Accordingly, we did not observe significant differences of muscular respiration or energy parameters such as lactate, glycogen or phosphocreatine between treated and control groups.

2. ABBREVIATIONS

ACN	Acetyl-L-carnitine
ADAM	9-anthryldiazomethane
ATP	Adenosine-5'-triphosphate
γ-BB	γ-Butyrobetaine
Br-DMEQ	3-bromomethyl-6,7-dimethoxy-1-methyl-2-quinoxalinone
BSA	Bovine serum albumin
CACT	Carnitine-acylcarnitine translocase
CAT	Carnitine acetyltransferase
CE	Capillary electrophoresis
CN	L-Carnitine
CoA	Coenzyme A
CoASH	Free Coenzyme A
COT	Carnitine octanoyltransferase
CPT	Carnitine palmitoyltransferase
DBS	Dried blood spot
DTNB	5,5'-dithiobis-2-nitrobenzoic acid
EDAC	1-ethyl-3-(3-dimethylaminopropyl) carbodiimide
ESI	Electrospray ionization
FAB	Fast atom bombardment
FAD	Flavin adenine dinucleotide
$FADH_2$	Flavin adenine dinucleotide reduced form
FDA	Food and Drug Administration
FMN	Flavin mononucleotide
GC	Gas chromatography
HPLC	High performance liquid chromatography
HTML	3-hydroxy-trimethyl lysine
IMM	Inner mitochondrial membrane
JVS	Juvenile visceral steatosis
MCAD	Medium-chain acylcarnitine dehydrogenase

MCD	Muscular carnitine deficiency
MS	Mass spectrometry
NADH	Nicotinamide adenine dinucleotide
NBD-ED	4-(2-aminoethylamino)-7-nitro-2,1,3-benzoxadiazole
OAT	Organic anion transporter
OCTN	Organic cation/carnitine novel transporter
OMM	Outer mitochondrial membrane
PCN	Propionylcarnitine
PDC	Pyruvate dehydrogenase complex
PPARα	Peroxisome proliferator-activated receptor alpha
REA	Radioenzymatic assay
RIE	Radio isotopic exchange
SCD	Systemic carnitine deficiency
SDH	Succinate dehydrogenase
THP	Trimethyl hydrazinium propionate
TMABA	4-trimethylaminobutyraldehyde
TML	Trimethyllysine
UV	Ultra violet
VDAC	Voltage dependent anion channel
VPA	Valproic acid
$VO_{2\ max}$	Maximal aerobic capacity

3. INTRODUCTION

3.1. General aspects

Carnitine is a small water soluble molecule that owes its name to the Latin word *caro, carnis* meaning flesh. It was discovered in muscle tissue by two independent groups in the same year: Gulewitsch and Krimberg (Gulewitsch *et al.*, 1905) who gave carnitine its actual name and Kutscher who named this new substance “novaine” (Kutscher, 1905), a name that was abandoned three years later (Krimberg, 1908). The structure of carnitine was elucidated in 1927 by Tomita and Sendju (Tomita *et al.*, 1927). Carnitine is a zwitterionic molecule physiologically present as L-enantiomer (**Figure 1**).

Because of its essential character for the survival of the meal worm *Tenebrio molitor*, carnitine was first thought to be a vitamin and was also called vitamin BT (Fraenkel, 1948).

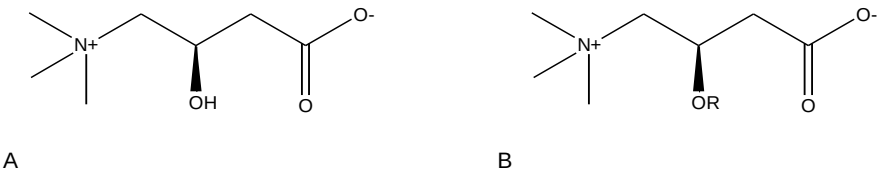


Figure 1*: Structure of L-carnitine (A) and acyl-L-carnitines (B)*

In the mid 1950s, the best known role of carnitine as fatty acyl carrier was brought to light by Fritz, who observed a stimulation of long-chain fatty acids oxidation with addition of carnitine (Fritz, 1955). Growing interest and growing research led to the discovery of other important roles of carnitine such as its acetyl-CoA/CoA buffering capacity or acyl groups detoxifying faculty (Bieber, 1988; Childress *et al.*, 1967).

A 70-kg healthy man contains a pool of about 20 g of carnitine, principally located in the skeletal and heart muscles (Brass, 1995). In the body, carnitine can be found in its free or acylated form, so-called acylcarnitine (**Figure 1**). Depending on the chain length of the acyl group, the acylcarnitine is referred to as short chain (C1-C5), medium chain (C5-C11) or long chain acylcarnitine (> C12) (Roth, 2009). Very-long chain acylcarnitines have also been described (C >22), involved in peroxisomal trafficking (Duranti *et al.*, 2008).

Our main carnitine source is food such as meat or dairy products; about 25% of our body pool comes from biosynthesis. Endogenous carnitine is produced from the two amino acids methionine and lysine in a four-step enzymatic pathway (**Figure 2**).

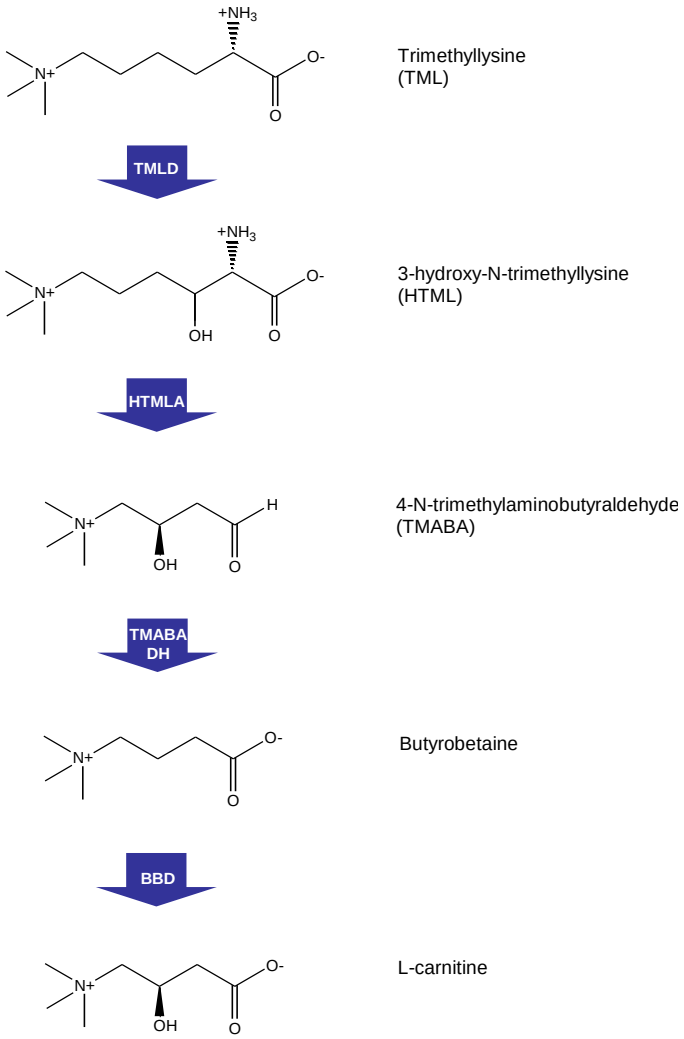


Figure 2: *Biosynthesis of L-carnitine (adapted from Vaz and Wanders, 2002)*

The first building block in the biosynthesis of carnitine is trimethyllysine (TML), the methylation product of protein bound lysine with S-adenosylmethionine. TML is hydroxylated by TML dioxygenase to generate 3-hydroxy-TML (HTML). HTML is cleaved to 4-trimethylaminobutyraldehyde (TMABA) and glycine by the enzyme HTML aldolase. The enzyme TMABA dehydrogenase catalyzes the dehydrogenation step of TMABA to γ-butyrobetaine (γ-BB). This molecule is hydroxylated at its 3-position by γ-BB dioxygenase, yielding carnitine. The enzymes of the carnitine biosynthesis pathway are ubiquitous except for the enzyme of the last step, mostly active in liver and kidney and to a lesser extent in the brain. Cofactors such as vitamin B6, vitamin C, α-ketoglutarate or iron are needed for the biosynthesis of carnitine (Vaz *et al.*, 2002b).

3.2. Functions of carnitine

3.2.1. Acyl-CoA trafficking

3.2.1.1. Across the mitochondrial membranes

The most well-known function of carnitine is its shuttling activity of long chain fatty acids from the cytosol to the mitochondrial matrix for β-oxidation. This function came to light with the work of Fritz who observed enhanced oxygen consumption and labeled CO_2 formation from marked palmitate in liver homogenates incubated with carnitine (Fritz, 1955). Long chain fatty acids, unlike short and medium chain fatty acids entering via diffusion, have to be coupled to carnitine to enter the mitochondrion (Bremer, 1983).

The translocation of long chain fatty acids into the mitochondrion is a four-step process (**Figure 3**). First, fatty acids are activated to fatty acyl-CoA by the action of the enzyme acyl-CoA synthetase, also called acyl-CoA ligase, classified according to their chain length specificity (Groot et al., 1976).

The long chain fatty acyl-CoAs are esterified to the β-hydroxy group of carnitine, a reaction catalyzed by a protein of the outer mitochondrial membrane (OMM), carnitine-palmitoyl transferase 1 (CPT1). Three isoforms of CPT1 have been described: CPT1A, present in the liver and kidney mainly, CPT1B, in the skeletal and cardiac muscles, and CPT1C in the

brain. CPT1 is considered as the rate limiting step of β-oxidation. The liver isoform is regulated by malonyl-CoA, an intermediate of fatty acid biogenesis (Bonnefont *et al.*, 2004).

It has been suggested recently that acyl-CoA synthetase, CPT1 and the voltage-dependent anion channel (VDAC) tightly interact, allowing the transfer of long-chain fatty acids through the OMM (Lee *et al.*, 2011).

The acylcarnitines produced by CPT1 cross the inner mitochondrial membrane via the protein carnitine-acylcarnitine translocase (CACT), embedded in the inner mitochondrial membrane (IMM). CPT2, also a protein of the IMM is responsible for the conversion of acylcarnitines back to carnitine and acyl-CoA, subsequently entering β-oxidation. CPT2 is ubiquitous and present as one isoform (Bonnefont *et al.*, 2004).

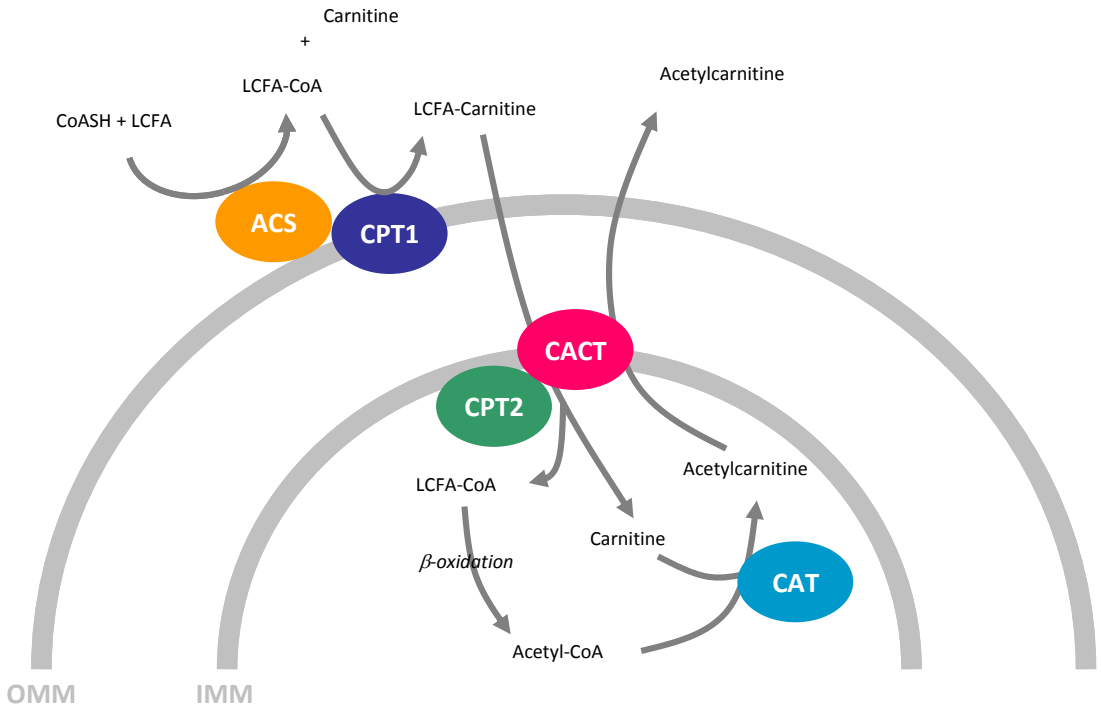


Figure 3: *The carnitine-fatty acid shuttle (adapted from Vaz 2002)*

3.2.1.2. From peroxisomes

Carnitine also plays a role in the metabolic function of peroxisomes. These organelles are involved in the oxidation of several chemical entities such as very-long chain fatty acids or branched fatty acids. To some extent, peroxisomes also contribute to the β-oxidation of long chain fatty acids (Steiber *et al.*, 2004). In the mitochondria, fatty acids are completely oxidized to CO_2; in peroxisomes, their oxidation yields acetyl-CoA, propionyl-CoA or chain shortened fatty acyl-CoA (Wanders *et al.*, 2010).

Fatty acids import into peroxisomes is not a carnitine dependent process as it is in mitochondria. First theories suggested the existence of an unspecific pore-forming protein in the peroxisomal membrane, through which fatty acids could freely diffuse (Van Veldhoven *et al.*, 1987). Nowadays evidence supports the presence of a specific import system for activated fatty acids, involving ABC transporters (Theodoulou *et al.*, 2006; Visser *et al.*, 2007).

While the import of fatty acids into the peroxisome has been shown to be carnitine independent, the export of shortened fatty acids requires the presence of carnitine. Jakobs and Wanders brought evidence that oxidation of the branched fatty acid pristanic acid needs the carnitine-acylcarnitine translocase to run to completion (Jakobs *et al.*, 1995). Metabolomic studies on fibroblasts with known peroxisomal defects further indicated that the complete oxidation of branched fatty acids to CO_2 requires all the enzymes of the carnitine shuttle but CPT1 (Verhoeven *et al.*, 1998).

Together with the fact that peroxisomes contain carnitine acetyl-transferase (CAT) and carnitine octanoyl-transferase (COT), this indicates that shortened fatty acid are converted to carnitine esters in the peroxisomes. Carnitine esters are then exported from the peroxisomes to the cytosol and finally translocated in the mitochondrion via CACT, where they are oxidized to CO_2 (Jakobs *et al.*, 1995). The transporter responsible for the export of acylcarnitines was suggested to be OCTN3 (Lamhonwah *et al.*, 2005).

3.2.1.3. Other compartments

Specific studies of acylcarnitines profiles and localization of carnitine acyltransferases extended the list of carnitine's functions beyond the well-known translocation of fatty acids into the mitochondria.

In erythrocytes, carnitine plays a role in the phospholipids turnover, explaining the presence of CPT1 in these cells devoid of mitochondria (Arduini *et al.*, 1992). Acylcarnitines serve as reserve of acyl-groups for the renewal of phospholipids, ensuring membrane integrity of erythrocytes.

In neurons, carnitine has been suggested to be an intermediate of the synthesis of ceramide and sphingolipids, via the action of the microsomal CPT1c (Sierra *et al.*, 2008).

Evidence supports a role of carnitine in the transfer of acetyl groups from the mitochondria to the nucleus for histone acetylation, justifying CAT activity in the nuclear compartment (Madiraju *et al.*, 2009).

3.2.2. CoA homeostasis

3.2.2.1. Acetyl-CoA buffering

Acetyl-CoA is an intermediate at the crossways of many metabolic pathways with regulatory influence on key enzymes such as the pyruvate dehydrogenase complex (PDC) (Constantin-Teodosiu *et al.*, 1991). To avoid a misbalance of the acetyl-CoA to CoA ratio, the enzyme carnitine acetyltransferase (CAT) conjugates acetyl-CoA to carnitine, generating acetylcarnitine. In conditions enhancing acetyl-CoA formation, such as exhaustive exercise, acetylcarnitine concentrations increase (Friolet *et al.*, 1994).

3.2.2.2. Acyl detoxifier

Carnitine buffering capacity is not limited to acetyl-CoA. Accumulating CoA derivatives, which are impermeable to membranes, are converted to their carnitine derivative for elimination. Several metabolic disorders or drug treatments have been associated with specific acylcarnitines patterns. For example in plasma of patients with medium-chain acyl-CoA dehydrogenase deficiency, elevated octanoylcarnitine concentrations, as an expression of accumulating octanoyl-CoA, are noticeable (Van Hove *et al.*, 1993). During treatment with pivalate prodrugs such as pivampicillin, pivaloylcarnitine is excreted (Melegh *et al.*, 1987). Profiling of acylcarnitines as markers of metabolic disorders is widespread for many clinical applications.

3.2.3. Other functions of carnitine

A multitude of other functions have been attributed to carnitine. Carnitine has been shown to be osmoprotective for certain bacteria or in organs such as the lens (Peluso *et al.*, 2000). Carnitine has also been involved in the immune response in several models (Famularo *et al.*, 2004; Fortin *et al.*, 2009) or in the glucose homeostasis and insulin sensitivity (Ringseis *et al.*, 2012). Hormon-like activity has been associated to carnitine, as allosteric regulator of glucocorticoid receptor (Alesci *et al.*, 2003) or as peripheral antagonist of thyroid hormones (Benvenga *et al.*, 2004) for example. Also antioxidative properties are conferred to carnitine (Cao *et al.*, 2011).

3.3. Carnitine homeostasis

Carnitine balance is a tightly regulated process involving biosynthesis, intestinal absorption, renal reabsorption and tissue distribution. An important concentration gradient between plasma and skeletal muscle where no carnitine synthesis takes place and efficient renal reabsorption of more than 90% of the filtered carnitine implicate an active transporter. The main and most investigated carnitine transporter is the novel organic cation transporter 2 OCTN2 (SLC22A5) cloned in 1998 (Tamai *et al.*, 1998). OCTN2 is a multi-specific cation transporter taking up carnitine with an affinity in the low micromolar range (Berardi *et al.*, 2000; Tamai *et al.*, 1998). Unlike other substrates of OCTN2, carnitine is transported in a sodium dependent way in a 1:1 stoichiometry (Ohashi *et al.*, 1999; Stieger *et al.*, 1995).

OCTN2 is present in various tissues: it is expressed in kidney, heart, skeletal muscle, intestine, placenta, testis, and brain among other organs (Tamai *et al.*, 1998). Compromised uptake of carnitine in fibroblasts, myocytes, liver or renal tubule of OCTN2-deficient mice or patients illustrates the central role of OCTN2 for carnitine homeostasis (Pons *et al.*, 1997; Yokogawa *et al.*, 1999).

Carnitine is also transported by other members of the SLC22 family: OCTN1, OCTN3, and OCT6 (CT2) are polyspecific carnitine/organic cations transporters (Koepsell *et al.*, 2007). These transporters differ in their mode of transport and in their affinity to carnitine. OCTN1 is a proton/cation exchanger. Whereas OCTN2 mediates carnitine transport is a Na+ dependent manner, OCTN3 and OCT6 showed no sodium-dependency. All of these transporters except OCTN1 carry carnitine with high affinity. In mouse, the anion transporter Oat9 has been reported to transport carnitine with high affinity in liver and kidney (Tsuchida *et al.*, 2010). The multi-component transport reported in many organs (Kato *et al.*, 2006; Stieger *et al.*, 1995; Yokogawa *et al.*, 1999) relies probably on the presence of OCTN2, other transporters and passive diffusion.

The absorption of carnitine depends on the dietary intake. In function of the diet, whether strict vegetarian or omnivorous, an individual can ingest between 0.05-15 μmol/kg of carnitine per day (Rebouche *et al.*, 1993). The main site of carnitine absorption is the small intestine (Matsuda *et al.*, 1998). Bioavailability of dietary carnitine exceeds 50% whereas the bioavailability of supplemented carnitine is about 15% (Rebouche, 2004).

The part not taken up is degraded mainly to trimethylamine and butyrobetaine by the intestinal flora. Parenteral carnitine is found almost exclusively unchanged in urine, indicating that no metabolism takes place in humans (Rebouche *et al.*, 1991).

Daily rate of carnitine biosynthesis has been estimated at 1.2 μmol/kg, taking place essentially in the liver (Vaz *et al.*, 2002b).

The renal filtration clearance of the small and polar carnitine is close to the glomerular filtration rate (100-120 mL/min). In a normal situation though, carnitine is almost completely reabsorbed, mainly by the action of OCTN2 in the apical membrane of the proximal tubule (Tamai *et al.*, 2001). In healthy adults, the renal carnitine clearance is in the range 1-3 mL/min with a renal threshold of about 50 μM (Evans *et al.*, 2003).

Kidney adapts to the dietary intake or more precisely to the circulating carnitine to ensure an optimal balance of the carnitine pool (Rebouche *et al.*, 1993; Schurch *et al.*, 2010). Individuals on a strict vegetarian diet typically have lower carnitine clearance to compensate for their low dietary intake (Lombard *et al.*, 1989).

Carnitine homeostasis is tightly regulated. In mice, expression of OCTN2 has been shown to be enhanced by PPARα activation as is the expression of enzymes of carnitine biosynthesis (Ringseis *et al.*, 2009; Wen *et al.*, 2011). Hormonal influence has also been established (Genuth *et al.*, 1981; Stephens *et al.*, 2007b).

3.4. Carnitine deficiency

Deficient carnitine levels can lead to an accumulation of long chain fatty acids or potentially toxic acyl-products, impairing the cellular function. Symptoms such as muscle weakness, fatigue, encephalopathy, cardiomyopathy, and hypoketotic hypoglycaemia are associated with carnitine deficiency. Low carnitine levels can be primary as inherited carnitine transporter defect, or secondary as a result of an excessive loss or a diminished supply of carnitine. While decreased plasma or tissue concentrations of carnitine can be observed in many clinical situations, symptomatic carnitine deficiencies are rather uncommon. Symptoms are believed to appear at very low concentrations of carnitine only (<10% of normal) (Stanley, 2004), in accordance with the fact that individuals with primary carnitine deficiency can remain undetected although their carnitine plasma levels are almost non-existent (El-Hattab *et al.*, 2010; Spiekerkoetter *et al.*, 2003).

3.4.1. Primary deficiency

Two forms of primary carnitine deficiencies have been distinguished, muscular carnitine deficiency (MCD – OMIM 212160) and systemic carnitine deficiency (SCD – OMIM 212140).

First described in 1973 (Engel *et al.*, 1973), MCD is characterized by low carnitine levels in skeletal muscle associated with muscle weakness and lipid storage myopathy, affecting mostly type I fibers. Onset of myopathy occurs generally during early adulthood. Symptoms can range from mild myalgia to fatal outcome. Since carnitine plasma concentrations are usually not altered, a deficiency in a low affinity muscular transporter has been suggested (Martinuzzi *et al.*, 1991). Some patients with MCD benefit from a carnitine supplementation while others do not. Corticosteroid therapy improved symptomatology in some cases (Vielhaber *et al.*, 2004).

The first case of systemic carnitine deficiency was reported in 1975 (Karpati *et al.*, 1975). Symptoms of SCD, usually appearing during early childhood, are multiple. Muscle weakness, progredient cardiomyopathy, lethargy, hypoketotic hypoglycemia, vomiting, seizures, fatigue, and failure to thrive can be clinical signs of systemic carnitine deficiency. Not only carnitine concentrations in muscle are reduced but also in other organs such as the liver. Systemic carnitine deficiency is an autosomal recessive disorder due to a genetic mutation of the SLC22A5 gene encoding OCTN2 (Nezu *et al.*, 1999). Many point mutations have been described with varying phenotypes (Rose *et al.*, 2011).

First described in 1988, the juvenile visceral steatosis (jvs) mouse is a model of systemic carnitine deficiency. Characterized by retarded growth and hepatic microvesicular infiltrations, jvs mice have a mutation of the OCTN2 gene, resulting in an impaired carnitine transport (Koizumi *et al.*, 1988; Kuwajima *et al.*, 1991).

3.4.2. Secondary deficiency

Secondary carnitine deficiencies have several etiologies. They can be due to increased carnitine loss or impaired supply of genetic, physiological or iatrogenic origin.

3.4.2.1. Genetic origin

Several metabolic disorders such as fatty acid oxidation disorders or organic acidurias are associated with an increased urinary loss of carnitine. Accumulating fatty acids or organic acids are converted to their carnitine derivatives for elimination. In the kidney, acylcarnitines are reabsorbed with much lower affinity than carnitine itself, causing a depletion in the carnitine pool (Ohnishi *et al.*, 2008). For example, in plasma and urine samples of patients with medium chain acyl-CoA dehydrogenase deficiency (MCAD), increased medium chain acylcarnitine such as octanoylcarnitine are observed in consequence of the accumulating activated esters. In these patients, plasmatic octanoylcarnitine concentrations can be 100 times higher than normal (Chace *et al.*, 1997). Another example is methylmalonic aciduria, as result of a defective methylmalonyl-CoA mutase, characterized by elevated propionyl- and methylmalonylcarnitine levels, enhancing a renal loss of carnitine (Chalmers *et al.*, 1984).

Mutations of the enzymes of the carnitine shuttle have also been described, leading to more or less severe phenotypes not necessarily accompanied by insufficient carnitine concentrations (Bonnefont *et al.*, 2004; Stanley *et al.*, 1992).

3.4.2.2. Iatrogenic carnitine deficiencies

Hemodialysis

Patients with chronic renal failure have an impaired carnitine homeostasis, characterized by reduced free carnitine concentrations and elevated acylcarnitines to carnitine ratio in plasma (Vernez *et al.*, 2006). During dialysis, small and hydrophilic compounds such as carnitine or its metabolic precursor butyrobetaine are filtered out, contributing to a loss of carnitine via filtration and reduced biosynthesis. Patients with hemodialysis can profit from carnitine supplementation for muscular, cardiac or anemic symptoms but no general consensus exist because of the lack of large-scale clinical studies (Calo 2012).

Drug induced

Several drugs are associated with impaired carnitine homeostasis. While some of them directly interact with the carnitine transporter or the carnitine biosynthesis pathway, others induce a carnitine loss via formation of unabsorbable carnitine derivatives.

Valproate (VPA) is a branched fatty acid used as antiepileptic, mood stabilizer or migraine prophylaxis. VPA is usually well tolerated but has been associated with hepatic failure and/or carnitine deficiency. More than ten metabolites of VPA have been described, one of them being valproylcarnitine (Peterson *et al.*, 2005).

Many studies have reported changes in carnitine and/or acylcarnitines levels in patients taking valproate in monotherapy or polytherapy. Some authors observed a decrease in total and free carnitine in plasma samples of VPA patients (Chung *et al.*, 1997; Rodriguez-Segade *et al.*, 1989) while others did not (Silva *et al.*, 2001). The age of the patients seemed to be linked to decreased plasma carnitine levels, children less than 10 years of age being more sensitive.

In two studies (Silva *et al.*, 2001; Werner *et al.*, 2007), the fate of C8 acylcarnitines (octanoylcarnitine and valproylcarnitine) was investigated. Werner and colleagues observed a slight increase of the C8 species with valproate treatment while Silva and coworkers did not report any differences.

To explain the carnitine deficiency associated with VPA, several modes of action have been suggested (Tein *et al.*, 1993): inhibition of carnitine uptake by valproylcarnitine or another VPA metabolite, interference of metabolites such as 4-ene-valproate with β-oxidation or other energetic pathways were brought into question. The direct inhibitory effect of valproic acid on OCTN2 was studied by Wagner et al. (Wagner *et al.*, 2000), but it showed no significant results, which presumes another mechanism. Okamura and colleagues (Okamura *et al.*, 2006) investigated the effect of valproylcarnitine on OCTN2 and concluded on a non-competitive inhibition with a Ki of 41.6 μM.

Mildronate (trimethyl hydrazinium propionate, THP) is a carnitine analogon that has been used as cardioprotective agent. THP induces a carnitine deficiency through inhibition of OCTN2 and γ-butyrobetaine dioxygenase, the last enzyme of the carnitine biosynthesis pathway. This substance has been used to generate a carnitine deficiency in a rat model (Spaniol *et al.*, 2001).

Pivalic acid has been used to enhance the bioavailability of several drugs such as the antibiotic ampicillin. Rapidly hydrolyzed from the active part, the pivaloyl moiety is converted to pivaloyl-CoA. The non-metabolized CoA derivative is conjugated to carnitine for elimination (Brass, 2002). Pivaloylcarnitine was shown to inhibit the OCTN2-dependent renal reabsorption of carnitine in a competitive manner. The renal loss of carnitine in the form of the weakly reabsorbed pivaloylcarnitine rather than the uptake inhibition underlies the carnitine deficiency observed with pivalate prodrugs treatment (Todesco *et al.*, 2009).

Other direct inhibitors of OCTN2 have been described (Ohashi *et al.*, 1999; Wu *et al.*, 1999). Some inhibitors structurally unrelated to carnitine such as emetine or zidovudine (Wu *et al.*, 1999) have been associated with clinical manifestations of carnitine deficiency (Dalakas *et al.*, 1994; Kuntzer *et al.*, 1990). Modulators of OCTN2 expression such as antineoplastic platinum-derivatives have also been described (Haschke *et al.*, 2010).

3.4.2.3. Physiological carnitine deficiency

The carnitine pool of an omnivorous individual comes mostly from the diet. Decreased intake of carnitine can be observed in patients with parenteral nutrition (Nanni *et al.*, 1983), malabsorption disorder such as celiac disease (Lerner *et al.*, 1993), or vegetarian diet (Lombard *et al.*, 1989). Low intake alone is rarely associated with carnitine deficiency, but concomitant pathological states can favor a carnitine deficiency (Wennberg *et al.*, 1992).

Liver is central for the biosynthesis and turnover of carnitine. Depending on the etiology of the liver disease, increased or decreased carnitine plasma concentrations have been observed (Krähenbühl, 1996).

During pregnancy, plasma carnitine concentrations drop to 50% of the initial concentration (Cederblad *et al.*, 1986). Asymptomatic in most cases, the carnitine decrease can be critical with an underlying disease (Angelini *et al.*, 1978; Cederblad *et al.*, 1986; Donnelly *et al.*, 2007). Other high energy demanding states such as severe burns (Cederblad *et al.*, 1980) or sepsis (Eaton *et al.*, 2005) are also associated with disturbed plasmatic and urinary carnitine concentrations, reflecting increased demand or muscle wasting.

3.5. Analysis of carnitine and acylcarnitines

3.5.1. Early approaches

The first quantitative approach for the determination of carnitine was reported in 1954 (Fraenkel, 1954). The assay was based on the essential character of carnitine for the growth of the larvae of *Tenebrio molitor*. After hatching, the worms can grow and survive for 3-4 weeks in the absence of carnitine; after this period, survival is compromised. Knowing the amount of carnitine needed for an optimal survival and a suboptimal growth rate, the concentration of carnitine in a testing material could be assessed. Although the *Tenebrio* test showed quite reproducible results according to its authors, it was limited in terms of precision, specificity and time efficiency.

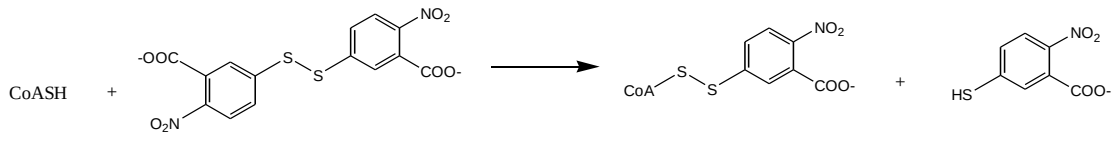
The *Tenebrio molitor* assay was supplanted by colorimetric methods such as the one developed by Friedman (Friedman, 1958). Bromophenol blue was added to purified extracts of meat or urine containing carnitine, leading to the formation of a carnitine ester of bromophenol. The colorimetric determination of the ester allowed a quantification of carnitine in the low micromolar range (0.03-0.19 μmol). Because of the reactivity of quaternary ammonium groups with bromophenol, this method requires pure extracts to ensure selectivity.

3.5.2. Enzymatic assays

A major progress for the analysis of carnitine was achieved in 1964: Marquis & Fritz developed an enzymatic assay (Marquis *et al.*, 1964) relying on the reaction catalyzed by the enzyme carnitine acetyltransferase (CAT) (1). In the first version of this enzymatic assay, the product of the CAT reaction was determined spectrophotometrically. Following reactions are involved in the assay:

(1) Acetyl-CoA + Carnitine $\rightleftharpoons$ Acetylcarnitine + CoASH

(2)



In the presence of a compound with a sulfhydryl group such as CoASH, DTNB is cleaved into two 5-thio-2-nitrobenzoate moieties. One of the benzoates reacts with the sulfhydryl group while the other remains free (2). This free part is yellow and its absorbance can be determined at 412 nm. With constant levels of substrates, the carnitine present in the biological sample will generate an equimolar amount of CoASH and subsequently of the free thionitrobenzoate.

Any sulfhydryl group can cleave DTNB, generating a signal independent of the CoASH formed by CAT. To avoid this interference, SH groups were oxidized while the tissue extracts were heated at 90°C for 5 minutes at pH 8.5. The assay of Marquis and Fritz could detect carnitine amounts as low as 5 nmol in rat tissues such as liver, brain, muscle or serum.

Cederblad and Lindstedt published a method for the measurement of carnitine in the picomole range, relying as well on the carnitine acetyltransferase reaction (Cederblad *et al.*, 1972). This assay is the first so-called REA – radio enzymatic assay – for the determination of carnitine, based on the use of radioactive labeled acetyl-CoA as CAT substrate. Perchloric acid extracts of plasma and muscle samples were incubated with ^{14}C-acetyl-CoA and CAT, and then purified on an anion exchange column to remove unreacted acetyl-CoA. The generated ^{14}C-acetylcarnitine was detected in a scintillation counter. The carnitine content of plasma and muscle extracts ranging from 20 to 2000 picomoles could be assessed.

Since the enzyme CAT catalyzes an equilibrium reaction, endogenous acetylcarnitine can integrate a labeled acetyl group. Unless the reaction (1) is shifted to the acetylcarnitine side, both carnitine and acetylcarnitine will be detected as labeled acetylcarnitine and not all the carnitine present in the testing material will be converted to ^{14}C-acetylcarnitine. If ^{14}C-acetyl-CoA and carnitine are present in similar concentrations, the response between carnitine and ^{14}C-acetylcarnitine will not be in a linear range. This can be circumvented by a large excess of acetyl-CoA or by trapping the CoASH formed. Due to the high cost of ^{14}C-acetyl-CoA, the second alternative has been further developed.

Bohmer and colleagues used DTNB, known to react with sulfhydryl groups, as CoASH scavenger (Bohmer *et al.*, 1974). The problem was partially solved, since relative high amounts of acetyl-CoA were still needed and DTNB was found to be an inhibitor of the enzyme CAT.

In another version of the radioenzymatic assay, DTNB has been replaced by the oxidizing agent sodium tetrathionate (McGarry *et al.*, 1976). The method allows the determination of carnitine and total carnitine with good linearity and no interference with short-chain acylcarnitines. To optimize the time frame of the procedure, plasma without protein precipitation as well as anion exchange resin in solution were used.

Pace and colleagues adapted the method of McGarry to liver and muscle samples (Pace *et al.*, 1978). Unlike plasma, those organs contain notable amounts of acetyl-CoA potentially interfering with the assay. To avoid cross reactivity, higher amounts of labeled acetyl-CoA were used. The samples were treated with perchloric acid, so that differentiation between carnitine, short chain- and long chain-acylcarnitines in function of the extract fraction was achieved.

Another variation of the enzymatic assay based on the CAT reaction was published by Parvin and Pande. The CoASH trapping agent N-ethylmaleimide or oxidized glutathione was substituted to sodium tetrathionate. Instead of the anion exchange resin, charcoal was used to adsorb the unreacted labeled acetyl-CoA (Parvin *et al.*, 1977).

Carnitine determination with REA provides accurate results with a relatively good sensitivity. However, the assay is time consuming and not optimal for routine analysis. Besides, only a rough differentiation of acylcarnitines in chain length categories is possible, with no distinction of the individual acylcarnitines.

Other enzymatic reactions have been exploited for the determination of carnitine. Matsumoto and colleagues used the enzyme carnitine dehydrogenase to convert carnitine into dehydrocarnitine with concomitant formation of NADH. This NADH was then processed by the enzyme diaphorase to transform resazurin into the fluorescent resorufin (Matsumoto *et al.*, 1990). With a flow injection system and immobilized enzymes, this assay is quite rapid but like the CAT reaction, it has the disadvantage of assessing carnitine without differentiation of its esters.

3.5.3. Chromatographic methods

The major advantage of a chromatographic approach is the possibility to separate carnitine and acylcarnitines. Under appropriate conditions, any carnitine ester can theoretically be distinguished from the next related acylcarnitine.

Various chromatographic techniques have been applied to the identification and quantification of carnitine and its derivatives. While thin layer chromatography (Wittels *et al.*, 1965) played a precursor role for the analysis of carnitine, gas chromatography (GC) and especially high performance liquid chromatography (HPLC) made a major contribution to the domain.

Analysis of carnitine and its esters with GC requires a few detours given the non-volatile nature and the thermal instability of the analytes. These non-ideal properties have been exploited in different manners. In a first GC approach, Lewin and colleagues took advantage of the thermal lability of carnitine and monitored its fragments after heat degradation (Lewin *et al.*, 1975). Other authors determined acyl moieties after hydrolysis of the acylcarnitines (Bieber *et al.*, 1977), with the disadvantage of losing the integrity of the acylcarnitines and potentially assessing acyl groups coming from other esters. Another way to get heat stable volatile compounds is by derivatization. Several methods have been reported: Lowes & Rose analyzed acylcarnitines in urine via conversion to acyl-containing lactones and detection with mass spectrometry (Lowes *et al.*, 1990). Huang and colleagues also used GC-MS but with another derivatization procedure: acylcarnitines were first esterified with propyl chloroformate followed by on-column N-dealkylation mediated by potassium iodide (Huang *et al.*, 1991).

Liquid chromatography has been widely used for qualitative and quantitative purposes, with various detection methods such as UV (Bhuiyan *et al.*, 1987; Minkler *et al.*, 1990), fluorescence (Kamimori *et al.*, 1994; Longo *et al.*, 1996), radioactivity measurement (Hoppel *et al.*, 1986; Kerner *et al.*, 1983), or mass spectrometry (Vernez *et al.*, 2004). Mass spectrometric methods, coupled or not to HPLC will be discussed in the next section.

The simultaneous chromatographic separation of carnitine and its derivatives is a challenging task: the carnitine family covers a wide interval of lipophilicities, ranging from the small and polar carnitine up to protein bound long and very-long chain acylcarnitines (Marzo *et al.*, 1991). Normal phase HPLC has been marginally used for the determination of carnitine (Yoshida *et al.*, 1988), while reversed phase is the most popular option when analyzing both carnitine and its derivatives (Minkler *et al.*, 1990; Takeyama *et al.*, 1986; Vernez *et al.*, 2003). To optimize the retention of carnitine and short-chain acylcarnitines, ion pairing reagents such as butanesulfonic acid (Kerner *et al.*, 1983), heptafluorobutyric acid (Vernez *et al.*, 2003) or IPCC-MS3 (Maeda *et al.*, 2007) have been used.

The first objective of chemical derivatization, besides ameliorating chromatographic properties, is to couple a chromophore to carnitine and acylcarnitines for ultraviolet or fluorescence detection. Carnitine and its derivatives are only weakly detected in the UV range with poor sensibility and sensitivity (Hoppel *et al.*, 1986). Several derivatization agents have been used for both UV and fluorescence detection.

One of the first HPLC-UV methods is based on the reaction of the carboxyl group of carnitine with 4'-bromophenacyl trifluoromethylsulfonate, yielding the 4'-bromophenacylester absorbing at 254 nm (Minkler *et al.*, 1990). The reaction can occur under mild conditions and is completed within 10 minutes. Other derivatizing agents such as p-bromophenacyl bromide (Poorthuis *et al.*, 1993; van Kempen *et al.*, 1992), yielding the same bromophenacyl chromophore, require longer reaction time – up to 90 minutes – and higher temperatures.

Derivatization on the hydroxy group of carnitine has been reported for the enantioselective separation and quantification of L-carnitine and its isomer D-carnitine (De Witt *et al.*, 1994; Mardones *et al.*, 1999).

Carnitine and acylcarnitines have been coupled to a variety of fluorescent reagents for detection: 9-anthryldiazomethane (ADAM) (Yoshida *et al.*, 1988), 3-bromomethyl-6,7-dimethoxy-1-methyl-2-quinoxalinone (Br-DMEQ) (Kamimori *et al.*, 1994), 4-(2-aminoethylamino)-7-nitro-2,1,3-benzoxadiazole (NBD-ED) (Matsumoto *et al.*, 1994), 2-(2,3-naphthalimino) ethyl trifluoromethanesulfonate (Minkler *et al.*, 1995), 1-aminoanthracene (Longo *et al.*, 1996) or 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDAC) (Cao *et al.*, 2007). With fluorescence detection, sensitivity is enhanced compared to UV detection with a 500-fold gain and limit of detection in the range 10-100 fmol injected (Minkler *et al.*, 1995).

Radioactivity measurement is another detection method that has been associated with HPLC for the determination of carnitine. Hoppel and colleagues applied the radioenzymatic assay after HPLC-separation of carnitine and acylcarnitines (Hoppel *et al.*, 1986). The acylcarnitine fractions were roughly identified with UV at 210 nm and hydrolyzed under alkaline conditions. Carnitine was then quantified as labeled acetylcarnitine, produced by the enzyme carnitine acetyltransferase with labeled acetyl-CoA.

Tritium labeled carnitine was used for the so-called radioisotopic-exchange method (RIE/HPLC) (Kerner *et al.*, 1983; Schmidt-Sommerfeld *et al.*, 1995). The analytes range measured depends on the substrate specificity of carnitine acetyltransferase. Tissue (Kerner *et al.*, 1983) or plasma samples (Schmidt-Sommerfeld *et al.*, 1995) are in a first phase incubated with CAT and ^{3}H-carnitine. Since the enzymatic reaction is reversible, acylcarnitines incorporate the labeled carnitine. After equilibration, the reaction is shifted to the acylcarnitine side, while trapping CoASH with N-ethylmaleimide. HPLC fractions are then analyzed in a β-counter. Although specific and precise, these methods have the disadvantage of using radioactivity and being time-consuming.

3.5.4. Capillary electrophoresis

Capillary electrophoresis (CE) is a separation technique based on the motion of a molecule in an electric field. A CE apparatus consists of a capillary containing a buffer and connecting two buffer reservoirs subjected to an electric potential. The different charge and size of the analytes are determinant for their mobility in the capillary (Li, 1992). Charged analytes are particularly well suited for capillary electrophoresis. Carnitine and derivatives with their positive charge on the trimethyl ammonium group are good candidates for this separation method.

After electrophoretic separation, the analytes can be detected by several methods such as UV spectroscopy (Vernez *et al.*, 2000), fluorescence spectroscopy (Kiessig *et al.*, 1997), mass spectrometry (Heinig *et al.*, 1999) or contactless conductivity (Pormsila *et al.*, 2011).

When using UV or fluorescence spectroscopy, derivatization remains a necessary step to introduce a chromophore in the analytes. Similar derivatization agents as for HPLC-UV or HPLC-fluorescence have been used (Kiessig *et al.*, 1997; Vernez *et al.*, 2000). The detection limits are comparable to the ones obtained with HPLC separation and are situated in the low micromolar range. Mass spectrometry and contactless conductivity both have the advantage of not requiring any modification of the analyte for its detection.

Capillary electrophoresis has been coupled to contactless conductivity detection for the simultaneous quantification of carnitine and six acylcarnitines in plasma and urine (Pormsila *et al.*, 2011). Based on the conductance between electrodes, detection with capacitively coupled contactless conductivity (C^4D) is particularly adapted for charged molecules such as carnitine. Pormsila and colleagues developed a method with an easy sample workup for plasma and urine with detection limits between 1-3 μmol/L. With the addition of cyclodextrin in the running buffer, carnitine and six acylcarnitines, including the isobaric octanoylcarnitine and valproylcarnitine, could be separated within a 7 minutes run.

Separation with capillary electrophoresis, in comparison to HPLC, has advantages: the sample volume and the solvent requirements are typically lower, separation times are usually shorter and minimal sample workup is necessary (Heinig *et al.*, 1999; Vernez *et al.*, 2000).

3.5.5. Mass spectrometry

Mass spectrometry has been applied for the qualitative and quantitative determination of carnitine and its esters since the 1980s. A first utilization of this technique was reported in 1984 (Millington *et al.*, 1984). In this study, acylcarnitines from urine samples of children with organic acidurias or Reye's syndrome were ionized with a FAB source. The method has been extended to other matrices such as blood, tissue (Millington *et al.*, 1989) or dried blood spots (DBS) (Millington *et al.*, 1990).

With the availability of mass spectrometry, considerable advances have been realized in the field of carnitine determination. Mass spectrometry allows the identification and elucidation of acylcarnitines as well as their quantification. Thanks to mass spectrometry, profiling of acylcarnitines, as marker of metabolic disorders, is widespread for many clinical applications, such as new born screening (Chace *et al.*, 2003; Zytkovicz *et al.*, 2001), post-mortem examinations (Rashed *et al.*, 1995; Roe *et al.*, 1987), prenatal diagnosis (Braida *et al.*, 2001; Van Hove *et al.*, 1993) or clinical monitoring (Vernez *et al.*, 2006).

Screenings for metabolic disorders were often done in urine (Lowes *et al.*, 1990; Millington *et al.*, 1984), with the limitation to renally excreted acylcarnitines. With plasma or blood dried on filter paper, protein bound acylcarnitines could be included in the profile. In the past years urine analysis has been re-implanted as meaningful tool for the identification of some disorders (Duranti *et al.*, 2008; Tortorelli *et al.*, 2005).

Several ionization sources have been implemented when analyzing acylcarnitines (Briand *et al.*, 1995; Millington *et al.*, 1989), the most common being electrospray ionization (ESI) (Fingerhut *et al.*, 2001; Vernez *et al.*, 2003).

Although not essential for the detection with mass spectrometry, derivatization is a common step in the sample workup process to increase sensitivity. Typically, carnitine and acylcarnitines are derivatized within 15 minutes at 65°C with methanol or butanol, yielding methyl or butyl esters respectively (Chace *et al.*, 1997; Inoue *et al.*, 1999). This derivatization step can be critical and be source of imprecision. Hydrolysis of the acylcarnitines can occur, leading to erroneous results (Johnson, 1999).

A fragment typically used for carnitine and derivatives in tandem mass spectrometry is 85 m/z (**Figure 4**). This fragment is obtained from carnitine and any acylcarnitine after loss of the trimethyl amine group and the acyl moiety (Möder *et al.*, 2005).

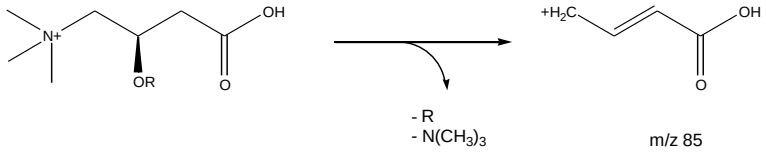


Figure 4: *Common fragment of carnitine and derivatives*

This common feature has been exploited for screening purposes in the precursor ion mode, allowing the identification of a wide range of acylcarnitines (Clayton *et al.*, 1998; Mueller *et al.*, 2003). Neutral loss mode with the m/z 59, corresponding to the trimethyl amine, is also a fragment common to all acylcarnitines that can be used for the screening of acylcarnitines (Zuniga *et al.*, 2011).

Mass spectrometry has often been combined to various separation methods to allow the differentiation of isobaric compounds with similar fragmentation pattern. The most common combination is with HPLC (Minkler *et al.*, 2008; Vernez *et al.*, 2003), while methods using capillary electrophoresis (Chalcraft *et al.*, 2009; Heinig *et al.*, 1999) or gas chromatography (Lowes *et al.*, 1990) have also been described. Separation prior to detection allowed the identification of new metabolites such as valproylcarnitine, isomer of octanoylcarnitine (Millington *et al.*, 1985).

Given the endogenous nature of carnitine and acylcarnitines, classical quantification as recommended for a bioanalytical method (FDA, 2001) is not a straight forward process. Since most of the biological fluids or tissues contain carnitine, surrogate matrices or analytes have often been used. Blank plasma has been mimicked with dialyzed plasma (Longo *et al.*, 1996) or bovine serum albumin (BSA) 4% (Minkler *et al.*, 2008; Vernez *et al.*, 2004), corresponding to a plasma albumin concentration of 40 g/L. Water is a common urine ersatz (Maeda *et al.*, 2007; Vernez *et al.*, 2003). Standard curves with deuterated standards have been built to circumvent the presence of the analytes in the matrix (Liu *et al.*, 2008). In dried blood samples, quantification is often based on the intensity of labeled internal standards.

Semi-quantitative approaches using the ratios of specific acylcarnitines and carnitine (Fingerhut *et al.*, 2001; Rashed *et al.*, 1995) are common with defined cut-off values to identify pathological conditions.

3.6. Carnitine in muscle

3.6.1. Muscular energy supply

Any muscular effort requires energy from the hydrolysis of ATP. This ATP can come from several sources, such as phosphocreatine, glycogen, and fatty acids (**Figure 5**).

Phosphocreatine is a high energy molecule serving as ATP reserve. At the onset of exercise, phosphocreatine is rapidly used to generate ATP, a reaction catalyzed by the enzyme creatine kinase (Hultman *et al.*, 1991).

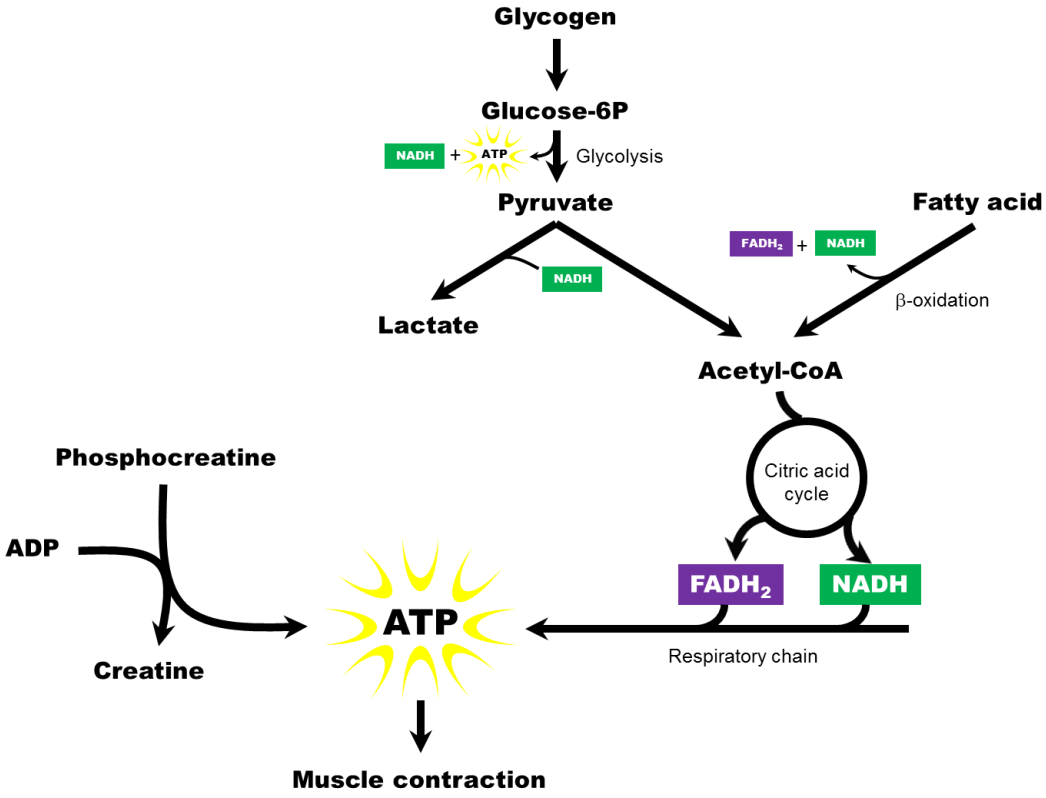


Figure 5*: Overview of the ATP producing pathways*

Glycogen, a branched glucose polymer, is the storage form of glucose. When needed, glycogen is broken down to single glucose-6-phosphate units that can enter glycolysis. During glycolysis, one glucose molecule is degraded to two pyruvates with net formation of two ATP and two NADH + H^+. Pyruvate, the end product of glycolysis, is either reduced to lactate with concomitant oxidation of NADH to NAD^+ or decarboxylated to acetyl-CoA entering subsequently the mitochondrial citric acid cycle. While the conversion of pyruvate to lactate is anaerobic, the entire processing of acetyl-CoA in the citric acid cycle and in the electron transport chain requires the presence of oxygen.

The main site of fatty acid degradation is the mitochondrion. After translocation in the mitochondrial matrix via the carnitine shuttle, long chain fatty acyl-CoAs enter β-oxidation. In a four-step enzymatic process, they are shortened by C2 acetyl-CoA units. Per cycle of β-oxidation, one acetyl-CoA is formed, while one $FADH_2$ and one NADH + H^+ are generated, both delivering electrons to the electron transport system.

In the mitochondrion, the citric acid enzymes allow the complete oxidation of the high energy acetyl-CoA to CO_2 with the production of three NADH, one GTP and one $FADH_2$. NADH and $FADH_2$ are two electron transferring substrates involved in the respiratory chain.

The respiratory chain is the main cellular ATP producing machinery. It is composed of five complexes located in the mitochondrial inner membrane (**Figure 6**).

Complex I, also known as NADH:coenzymeQ oxidoreductase, catalyzes the electron transfer from NADH to ubiquinone, the oxidized form of coenzyme Q, by the intermediary of flavin mononucleotide (FMN) and iron-sulfur clusters.

Complex II, the succinate:coenzyme Q oxidoreductase, transfers electrons from succinate to ubiquinone via flavin adenine dinucleotide (FAD) and iron-sulfur clusters. FAD is a prosthetic group reduced during the oxidation of succinate to fumarate by the succinate dehydrogenase, part of the citric acid cycle and complex II.

The electrons of ubiquinol, the reduced form of coenzyme Q, are processed in complex III, the coenzyme Q:cytochrome C reductase. Via hemes and an iron-sulfur cluster, electrons are transferred from ubiquinol to cytochrome c, a peripheral membrane protein of the inner mitochondrial membrane.

The last complex of the electron transport chain is the cytochrome c oxidase, complex IV. By the intermediary of hemes and copper containing centers, electrons from cytochrome c are transferred to molecular oxygen, yielding water.

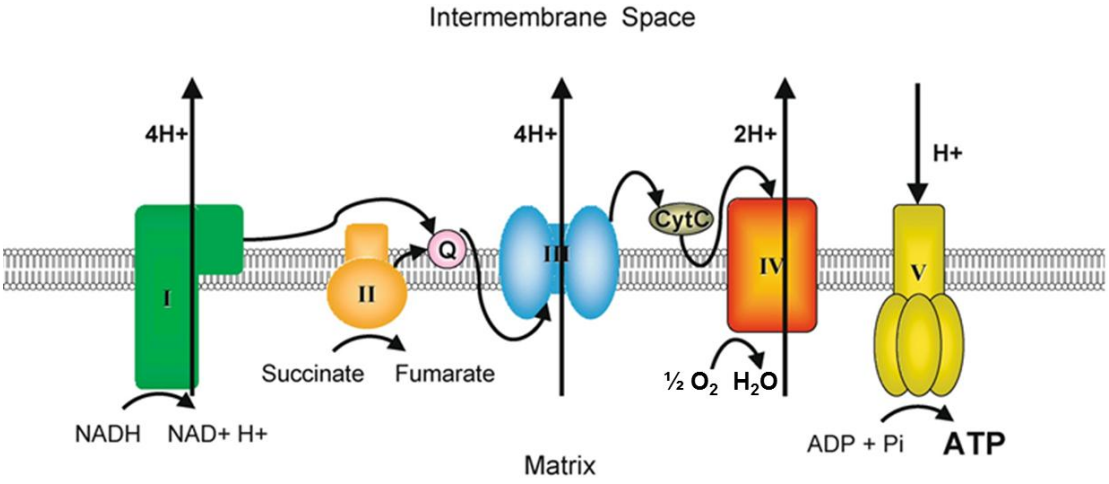


Figure 6:*The respiratory chain (adapted from Cuperus et al., 2010)*

During the electron transport process, from NADH and $FADH_2$ to oxygen, protons are pumped from the mitochondrial matrix into the intermembrane space, creating an electrochemical gradient. The proton gradient drives complex V, also called F_1F_0-ATP synthase, generating ATP. Complex V functions as a rotor, converting the chemical energy of the proton gradient into mechanical energy (Voet *et al.*, 2004).

3.6.2. Muscle types

The energy source of a contracting muscle depends on the exercise intensity. A long and moderate effort tends to be mainly supplied by lipid oxidation whereas a short and intense effort is powered by glucose degradation (van Loon *et al.*, 2001).

In the regulation between the aerobic and anaerobic energetic pathways, the pyruvate dehydrogenase complex (PDC) plays a pivotal role. It catalyzes the decarboxylation of pyruvate to acetyl-CoA with concomitant reduction of NAD^+. The phosphorylation state of PDC and the inhibitory effect of its products acetyl-CoA and NADH regulate the activity of PDC and the acetyl-CoA flux towards the citric acid cycle (Greenhaff *et al.*, 2002).

The energy supply depends also on the type of the muscle. According to their morphological and physiological characteristics –ATPase activity, myosin heavy chain type or succinate dehydrogenase content– muscle fibers have been divided into three main categories: type I, type IIA and IIB fibers. The proportion of fiber types in a given muscle shows interspecies and interindividual variability.

Type I fibers are slow oxidative fibers, relying on lipid oxidation as their main source of ATP. These fibers are fatigue resistant and are typically found in postural muscles. They contain a high mitochondrial volume and a rich pool of oxidative enzymes, supplied in oxygen by a dense capillary network. Because of their high amount of myoglobin for the transport of oxygen, type I fibers are typically red.

Type II fibers rely mostly on glucose for their energy supply. These fibers are fast at producing energy but are more fatigue-prone than type I fibers. Type II fibers contain high levels of glycolytic enzymes and glycogen. They require lower oxygen concentrations, explaining their white color. Type II fibers are subdivided into type IIA and IIB. Type IIA fibers are relatively fatigue resistant and besides the glycolytic machinery contain also oxidative enzymes, as revealed by a strong succinate dehydrogenase (SDH) staining. They are referred to as fast-twitch oxidative glycolytic fibers. Type IIB fibers, the fast-twitch glycolytic fibers, are practically devoid of oxidative enzymes and are quickly fatigable. Other type II fibers subtypes have been described with characteristics between type IIA and type IIB (Schiaffino *et al.*, 2011).

3.6.3. Carnitine and muscle function

Due to its position at strategic points in energy metabolism, carnitine has been the focus of many studies investigating muscle function and performance. On the one hand, as fatty acid importer, carnitine could enhance fatty acid oxidation, thereby potentially sparing glycogen reserves. CPT1, the rate limiting step of β-oxidation, with a Km value of about 0.5 mM for carnitine (McGarry *et al.*, 1983) is under resting conditions saturated (muscle concentrations 2-4 mmol/kg wet weight). While the total muscular carnitine content is barely affected during exercise, the free carnitine to acylcarnitines ratio can dramatically change. At rest or during low intensity exercise, acylcarnitines, especially acetylcarnitine, represent 10-20% of the total carnitine pool, with about 80% of free carnitine. After exhaustive exercise, the acetylcarnitine concentration increases, accounting for more than 60% of the total carnitine content (Constantin-Teodosiu *et al.*, 1996). Under these conditions and considering possible compartment differences, carnitine availability might become a limiting factor for the activity of CPT1 (Jeppesen *et al.*, 2012; Stephens *et al.*, 2007a).

On the other hand, as acetyl-CoA buffering agent, carnitine could enhance the PDC flux while lowering the acetyl-CoA to CoA ratio. An increased turnover of pyruvate by PDC would in turn reduce lactate formation (Brass, 2000).

Many studies assessing potential effects of carnitine on muscle function exist. While the benefits of carnitine on muscle function in pathological states are positively documented, the situation in healthy volunteers is controversial (Brass, 2004).

Most studies conducted in humans failed to show a clear impact of carnitine supplementation on muscle carnitine concentrations, respectively on muscle performance. Whether oral or intravenous, doses up to 4 g generally did not affect the muscular carnitine pool, even though increasing the plasma concentrations (Stephens *et al.*, 2007a). Markers of muscle metabolism (lactate, glycogen content) or markers of performance ($VO_{2\ max}$, respiratory quotient) are often unaffected by a supplementation. Reported positive results concern mostly one metabolic marker and are not consistent between the studies (Brass, 2004). Divergent results can partly be attributed to broad differences in study designs in terms of exercise, type, dose and duration of supplementation, training status of the subjects and control of dietary carnitine (Galloway *et al.*, 2005).

Recently, Stephens and coworkers highlighted the enhancing effect of hyperinsulinemia on the carnitine muscle concentration. With direct insulin infusion or carbohydrate-induced hyperinsulinemia, the carnitine levels could be increased by 10 to 15% in muscle (Stephens *et al.*, 2006; Stephens *et al.*, 2007c). The effects of such an elevation were investigated in 14 healthy volunteers supplemented with 2 g of carnitine twice daily with 80 g carbohydrates or placebo during 12 or 24 weeks. Wall and colleagues reported a 20% increase of carnitine muscle concentration, associated with a diminished lactate, increased glycogen and enhanced PDC activity in muscle at 80% $VO_{2\ max}$. These effects were observed only after the longer period of supplementation (Wall *et al.*, 2011). This study supports the dual effect of carnitine in muscle function, as lipid oxidation enhancer at low intensity exercise and as PDC indirect activator during high intensity workout.

Acetylcarnitine and propionylcarnitine are two short chain carnitine derivatives. Both substances have been studied for several indications; acetylcarnitine shows beneficial effects in cognition while propionylcarnitine has a protective effect in heart failure and ischemia-reperfusion studies. Their potential advantages over carnitine as exercise enhancers are unknown (Galloway *et al.*, 2005).

4. AIMS OF THE THESIS

The general aim of this thesis is to highlight the importance of carnitine from a physiological and from an analytical point of view. This thesis had three specific aims developed in three projects.

The aim of the first project was to gain a better insight into the carnitine homeostasis under valproate therapy with focus on the metabolite valproylcarnitine. We aimed to understand the molecular mechanism leading to or counteracting a renal loss of carnitine in patients treated with valproate. We performed uptake experiments in cells overexpressing OCTN2, the main transporter responsible for the renal reabsorption of carnitine and investigated the transport of radiolabeled carnitine in presence of valproylcarnitine. In parallel, we conducted a study with one patient starting a therapy with valproate and with ten patients under long term therapy with this drug. In plasma and urine samples we determined the concentrations and clearances of carnitine and several acylcarnitines including valproylcarnitine as well as the carnitine precursor butyrobetaine.

The goal of the second project was to optimize a LC-MS/MS method for the simultaneous determination of carnitine and acylcarnitines in plasma. For a rapid determination, we performed an online solid phase extraction on a cation exchange column before elution on the analytical column. For quantification, we applied the addition method to account for matrix effects and work in native matrix, containing endogenous analytes.

The third project aimed to investigate the benefits of acylcarnitines supplementation on the muscular capacity of mice *in vivo*. After oral supplementation with carnitine, acetylcarnitine or propionylcarnitine, mice completed a treadmill exercise until exhaustion. We investigated the mitochondrial function in skeletal muscle fibers with high resolution respirometry. Energy parameters such as lactate, glycogen, creatine, and phosphocreatine were determined. Carnitine concentrations in plasma, urine and muscles were analyzed and total carnitine balance was assessed.

5. EFFECT OF SHORT- AND LONG-TERM TREATMENT WITH VALPROATE ON CARNITINE HOMEOSTASIS IN HUMANS

Short title: Valproate and carnitine homeostasis

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(Ther Drug Monit, accepted manuscript)

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5.1. Abstract

Aims: To identify mechanisms of hypocarnitinemia in patients treated with valproate.

Methods: Plasma concentrations and urinary excretion of carnitine, acetylcarnitine, propionylcarnitine, valproylcarnitine, and butyrobetaine were determined in a patient starting valproate treatment and in 10 patients on long-term valproate treatment. Transport of carnitine and valproylcarnitine by the proximal tubular carnitine transporter OCTN2 was assessed *in vitro*.

Results: In the patient starting valproate, the plasma carnitine and acetylcarnitine levels dropped for 1 to 3 weeks and had recovered after 3 to 5 weeks, whereas the plasma levels of propionyl- and valproylcarnitine increased steadily over 5 weeks. The renal excretion and excretion fractions (EF) of carnitine, acetylcarnitine, propionylcarnitine, and butyrobetaine decreased substantially after starting valproate. Compared to controls, patients on long-term valproate treatment had similar plasma levels of carnitine, acetylcarnitine and propionylcarnitine, whereas valproylcarnitine was found only in patients. Urinary excretion and renal clearance of carnitine, acetylcarnitine, propionylcarnitine and butyrobetaine were decreased in valproate-treated compared to control patients, reaching statistical significance for carnitine. The EFs of carnitine, acetylcarnitine and propionylcarnitine were <5% of the filtered load in controls and were lower in valproate-treated patients. In contrast, the EF for valproylcarnitine approached 100%, resulting from a low affinity of valproylcarnitine for the carnitine transporter OCTN2 and competition with concomitantly filtered carnitine.

Conclusions: The initial drop in plasma carnitine levels of valproate-treated patients is most likely due to impaired carnitine biosynthesis, whereas the recovery of the plasma carnitine levels is explainable by increased renal expression of OCTN2. Renally excreted valproylcarnitine does not affect renal handling of carnitine in vivo.

Key words: valproate, carnitine, valproylcarnitine, OCTN2, carnitine biosynthesis

5.2. Introduction

Valproic acid (valproate) is a branched, medium-chain fatty acid frequently used as an antiepileptic drug (Beydoun *et al.*, 1997; Marson *et al.*, 2007) and, more recently, also for the prophylaxis of migraine and in patients with bipolar disorder (Bond *et al.*, 2010; D'Amico, 2010). Valproate is primarily metabolized by glucuronidation (DeVane, 2003) A smaller part is transformed to the coenzyme A derivative, which can either be converted to the respective carnitine ester or β-oxidized to propionyl-CoA and acetyl-CoA. Accordingly, valproylcarnitine has been detected in the plasma and in urine of patients treated with valproate (Millington *et al.*, 1985; Murakami *et al.*, 1996; Muro *et al.*, 1995).

Already more than 20 years ago, first reports have been published about children treated with valproate who have decreased free carnitine plasma concentrations (Bohles *et al.*, 1982; Ohtani *et al.*, 1982; Stumpf *et al.*, 1983). Since hypocarnitinemia can be associated with hyperammonemia, this is a potentially severe complication of this treatment (Eubanks *et al.*, 2008; Hamed *et al.*, 2009; Thom *et al.*, 1991). Interestingly, not all patients treated with valproate develop hypocarnitinemia (Coppola *et al.*, 2006; Hirose *et al.*, 1998). In children with long-term valproate ingestion, the frequency of hypocarnitinemia has been reported to be in the range of 40% (Coppola *et al.*, 2006), whereas hypocarnitinemia is less frequent in adolescents or adults treated with this drug (Silva *et al.*, 2001). Accordingly, reported risk factors for hypocarnitinemia in valproate-treated patients include valproate dose, young age and antiepileptic polytherapy (Coppola *et al.*, 2006; Hamed *et al.*, 2009; Thom *et al.*, 1991).

Carnitine is a small molecule which is essential for the transport of long-chain fatty acids in the mitochondrial matrix, where they can be β-oxidized (Fritz, 1955; Fritz *et al.*, 1965; Kerner *et al.*, 2000). Beside this, carnitine is important to maintain a sufficiently high intracellular concentration of coenzyme A, which is used in many metabolic pathways (Brass *et al.*, 1980; Friolet *et al.*, 1994). The acyl-groups of acyl-CoAs can be transferred to carnitine by the action of acylcarnitine transferases, leading to the formation of the respective acylcarnitine (Bremer, 1983; Kerner *et al.*, 2000). In contrast to acyl-CoAs, acylcarnitines can be transported out of cells and can be excreted by the urine. By this mechanism, potentially toxic acyl-groups can be eliminated (Vernez *et al.*, 2006). Carnitine is eliminated by glomerular filtration (Heuberger *et al.*, 1998), but most of the carnitine filtered is reabsorbed by the action of the carnitine carrier OCTN2 (Bremer, 1983; Krahenbuhl *et al.*, 2000; Krahenbuhl *et al.*, 1997), which is located in the proximal tubule (Haschke *et al.*, 2010; Nezu *et al.*, 1999; Todesco *et al.*, 2008). In mammals, most of the carnitine body needs are provided by the diet, and a smaller part is biosynthesized in skeletal muscle, liver and kidneys (Krahenbuhl *et al.*, 2000; Vaz *et al.*, 2002a).

The precise mechanisms leading to hypocarnitinemia in patients treated with valproate are so far not known. Stadler et al. assessed urinary carnitine excretion during the first month of treatment with valproate (Stadler *et al.*, 1999). Interestingly, they found an initial drop in the free carnitine plasma concentration, followed by a complete recovery after one month of treatment. The renal clearance of carnitine first increased and then decreased compared to the pre-treatment values, offering a possible explanation for changes in the plasma carnitine concentrations. In addition, Farkas et al. reported a decrease in carnitine biosynthesis in rats treated with valproate (Farkas *et al.*, 1996), offering an alternative explanation for hypocarnitinemia in patients treated with valproate.

The current study was performed to define better the molecular mechanisms leading to hypocarnitinemia in patients treated with valproate as well as to explore potential mechanisms counteracting the renal loss of carnitine in such patients. We therefore investigated the renal handling of valproylcarnitine and free carnitine in patients treated with valproate and performed *in vitro* investigations regarding transport of valproylcarnitine by OCTN2 and expression of OCTN2 in the presence of valproate.

5.3. Materials and Methods

Reagents

L-Carnitine, acetylcarnitine, and sodium valproate were purchased from Sigma (St. Louis, MO, USA). Propionylcarnitine and valproylcarnitine were obtained from Dr. H. J. ten Brink (VU Medical Center, Amsterdam, The Netherlands). Carnitine-d3 and octanoylcarnitine-d3 were supplied by Cambridge Isotope Laboratories (Andover, MA, USA) and ^{3}H-L-carnitine by GE Healthcare (Amersham, UK). All other chemicals and solvents were from Merck (Darmstadt, Germany) or Sigma.

Collection of plasma and urine samples

Blood was collected by venopuncture into heparinized tubes from one patient immediately before and at several occasions after starting treatment with valproate, from 10 patients on long-term treatment (>1 month) with valproate and from 10 age- and sex-matched control patients not treated with valproate. Plasma was obtained by centrifugation and stored at -70°C until analysis.

From the patient studied during start of therapy with valproate, spot urine samples were obtained when blood samples were drawn. Valproate plasma levels were determined by an enzyme immunoassay routinely used in the University Hospital. From the patients on long-term valproate treatment and from the corresponding control patients, 24-hour urine samples were obtained when the venous blood samples were drawn. The volume of the urine was determined, and a urine sample was stored at -70°C until analysis. The Ethics Committee of the State of Basel had approved the study protocol.

Synthesis of ^{3}H-valproylcarnitine

The synthesis and purification of ^{3}H-valproylcarnitine was performed according to the procedure of Ziegler et al. (Ziegler *et al.*, 1967) and the patent 7916590 of Sigma-Tau with the modifications described previously for pivaloylcarntine (Todesco *et al.*, 2009). The product was analyzed with NMR in deuterated methanol as described (Todesco *et al.*, 2009). ^{3}H-valproylcarnitine was 96.5% pure; the 3.5% impurity was not carnitine.

LC/MS-MS Equipment

The HPLC system consisted of a HTS PAL autosampler (CTC Analytics AG, Zwingen, Switzerland), an oven (CTO-20A), pumps (LC-20AD) and a controller (CBM-20A) from Shimazu (Reinach, Switzerland). The HPLC system was coupled to a triple quadrupole mass spectrometer API 4000 (AB/MDS Sciex, Concord, Canada) with an electron spray ionization source. Separation was performed on a Luna C8 5 μm column (150mm x 2mm) equipped with a C8 (4 x 2.0 mm) precolumn (Phenomenex, Torrance, CA, USA).

Preparation of samples and calibrators, HPLC conditions

Samples and calibrators were prepared as described previously by Vernez et al.(Vernez *et al.*, 2003; Vernez *et al.*, 2004). The chromatographic separation was done with a binary flow (0.2 mL/min) at 30°C. Starting at 20% phase B, the gradient increased to 90% phase B within 8 minutes. After a 5 minutes plateau at 90% phase B, the analytical column was re-equilibrated for 3 minutes with 20% B, for a total run time of 16 minutes.

MS conditions and validation

Carnitine and the acylcarnitines of interest were analyzed in the multiple reaction monitoring mode. A first transition was used for quantification, a second one for qualification. Following transitions (m/z) were used: butyrobetaine 146→87, 146→60 carnitine 162→105, 162→85; carnitine-d3 165→103, 165→85; acetylcarnitine 204→85, 204→145; propionylcarnitine 218→85, 218→159; valproylcarnitine 288→85, 288→229; octanoylcarnitine-d3 291→85, 291→229.

Imprecision and accuracy were assessed with three quality controls (QC) measured on several days (n=5). The concentrations used for carnitine were 3.75, 37.5, and 150 μmol/L, for acetylcarnitine 2, 10, and 40 μmol/L, and for the other analytes 0.2, 1, and 4 μmol/L. In plasma, inter-assay precisions for the QC concentrations were ≤6.0% for all measured analytes and the inter-day accuracies were between 92.9 and 105.5%. For urine, the precision was ≤7.8% for carnitine and valproylcarnitine and ≤14.5% for the other analytes. The accuracy was within 87.9-113.6% for all compounds. The standard curves showed an excellent linearity for all analytes tested both in plasma and urine (r^2 ≥0.993).

Transport of carnitine and valproylcarnitine by OCTN2

Uptake experiments were conducted in L6 cells (rat skeletal muscle myoblasts) overexpressing human OCTN2 (hOCTN2) exactly as described before (Todesco *et al.*, 2008). The protein content was determined with the Pierce Chemical BCA kit (Rockford, IL, USA) as specified by the supplier.

Expression of OCTN2 mRNA in HEK293-FT cells

HEK293-FT cells (human embryonic kidney cells) were supplied by Invitrogen (Paisley, UK). Growth medium (DMEM high glucose) contained 10% FBS, 1% Glutamax, 1% HEPES, 100 U/mL penicillin, and 100 μg/mL streptomycin. Cells were grown in 6-well plates and treated when they reached 60-70% confluency. Valproate 100 or 1000 μmol/L, valproylcarnitine 5 μmol/L, or DMSO as control was added to the cell culture medium.

After 6h, 24h or 48h of treatment, RNA was collected and purified with the RNeasy Mini Kit (Qiagen, Hombrechtikon, Switzerland) according to the supplier's procedure. RNA was then converted to cDNA with the Omniscript RT Kit (Qiagen Hombrechtikon, Switzerland). Amplification and detection were made on an ABI Prism 7900HT RT-PCR machine (Applied Biosystems, Rotkreuz, Switzerland) with the FastStart SYBR green Master (Roche, Basel, Switzerland). The expression of OCTN2 and GAPDH was investigated. Primers were purchased from Microsynth (Balgach, Switzerland) and designed as follows: hOCTN2 forward primer 5'-GCAGCATCCTGTCTCCCTAC-3', reverse primer 5'-GCTGTCAGGATGGTCAGACTT-3', and GAPDH forward primer 5'-AGCCACATCGCTCAGACAC-3', reverse primer 5'-GCCCAATACGACCAAATCC-3'.

Quantification of OCTN2 mRNA levels was performed with GAPDH as an endogenous control with the comparative threshold cycle method. The results are expressed relative to the values obtained in control cells at 6h, 24h and 48h respectively.

Protein expression of OCTN2 in HEK293-FT cells

After incubation in 6-well plates, the cells were trypsinized and washed twice with ice cold PBS. The cell pellet was lysed with 100 μL NET lysis buffer (50 mmol/L Tris-HCl, 50 mmol/L NaCl, 5 mmol/L EDTA, 1% NP-40, and Complete Mini anti-protease cocktail (Roche, Switzerland)) for 15 minutes on ice. The lysate was centrifuged at 15'700 g for 10 minutes at 4°C.

After protein determination, the supernatant was combined with NuPage LDS sample buffer (Invitrogen, Paisley, UK) and heated during 5 minutes at 95°C. The proteins were separated on a NuPage 4–12% Bis-Tris gel (Invitrogen) and then blotted on a PVDF membrane (Millipore, Zug, Switzerland) in a semi-dry transfer cell (BioRad, Reinach, Switzerland). The membrane was blocked with a 5% milk protein solution in PBS for 1 hour, washed and incubated with the primary antibody OCTN2/K33 (1:5000) (Todesco *et al.*, 2008) or β-actin (1:2000) (Santa Cruz Biotechnology, CA, USA) overnight at 4°C. After washing, the membrane was incubated with the HRP-coupled secondary antibody, goat anti-rabbit for OCTN2 and rabbit anti-goat for β-actin (BioRad, Reinach, Switzerland). Detection was made with the ECL Western blotting reagents (GE Healthcare, Amersham, UK). Quantification of OCTN2 was made with the Quantity One software (BioRad) relative to β-actin.

Statistics and calculations

The renal clearance (Cl_{ren}) and the renal excretion fractions of carnitine and acylcarnitines were determined as described previously (Haschke *et al.*, 2010; Heuberger *et al.*, 1998).
Curve fitting of the uptake experiments and determination of the kinetic parameters were done with SigmaPlot® version 11 (Scientific Solutions, Lausanne, Switzerland) as described previously (Todesco *et al.*, 2009).
Median values of the 2 groups were compared with the rank sum test using SigmaStat version 3.5 (Scientific Solutions, Lausanne, Switzerland).

5.4. Results

Carnitine metabolism and urinary excretion in a patient starting treatment with valproate

We first determined the renal handling of carnitine in a 28-years old omnivore female during the initial five weeks after starting a treatment with valproate. Similar to a report in the literature (Stadler *et al.*, 1999), the plasma carnitine concentration showed an initial drop and then gradually recovered, reaching pre-treatment values after one month of treatment (**Figure 1A and 1B**). The plasma acetylcarnitine and butyrobetaine concentrations showed a similar behavior as carnitine with a more accentuated initial drop for acetylcarnitine. In

contrast, the plasma concentrations of valproylcarnitine and propionylcarnitine increased with time and correlated with the plasma valproate concentration.

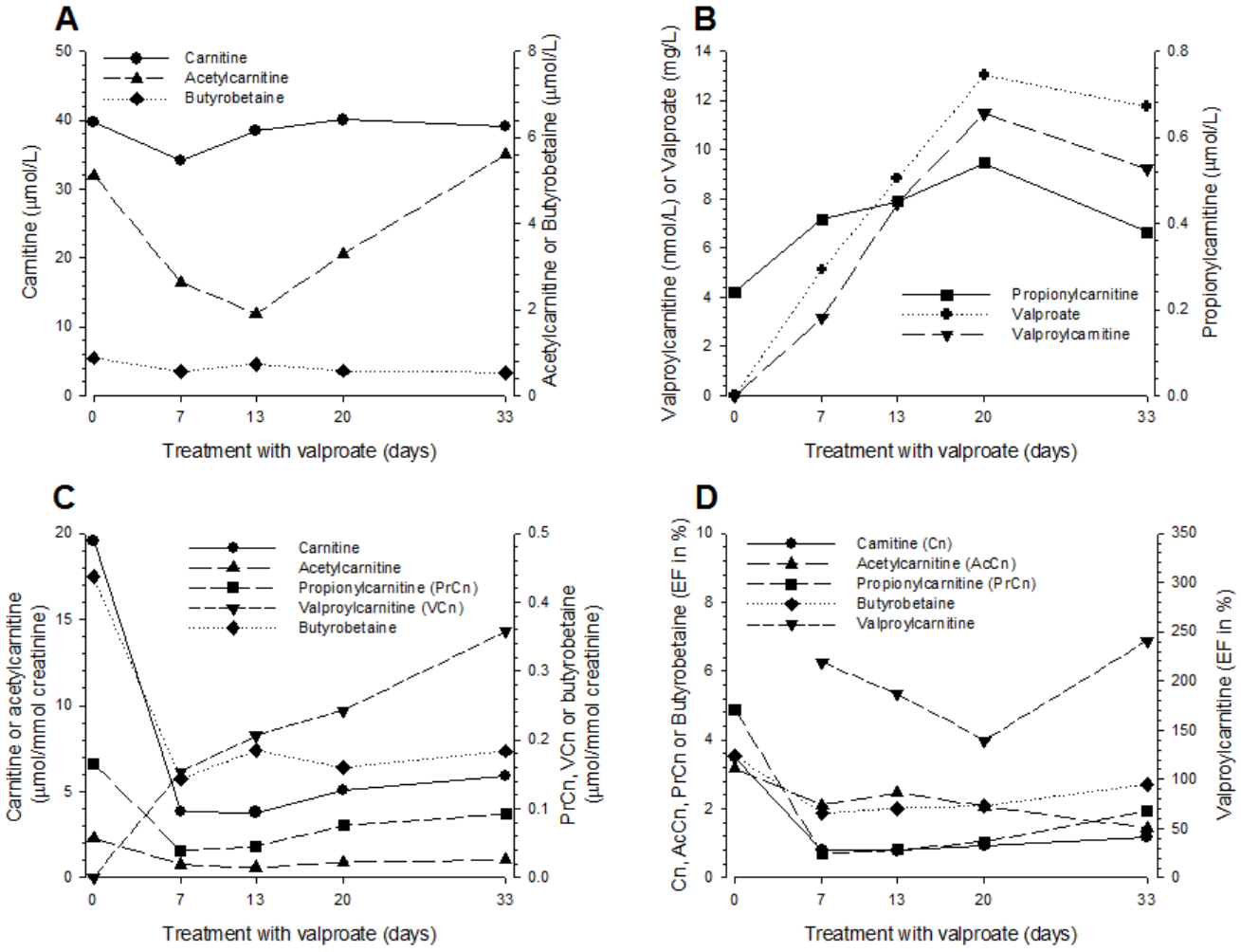

Figure 1: *Plasma concentrations and renal handling of carnitine, acylcarnitines and butyrobetaine in a patient starting treatment with valproate*. (A) After an initial drop, the plasma concentrations of carnitine and acetylcarnitine recover during treatment with valproate. (B) The plasma concentrations of valproylcarnitine and propionylcarnitine (both formed from valproyl-CoA) increase with rising valproate concentrations. (C) The renal excretions of carnitine, acetylcarnitine, propionylcarnitine, and butyrobetaine show a sharp drop after starting valproate treatment, whereas the excretion of valproylcarnitine increases. (D) The excretion fractions of carnitine, acetylcarnitine, propionylcarnitine, and butyrobetaine decrease after starting valproate treatment, whereas the excretion fraction of valproylcarnitine is in the range of 150 to 250%.

In order to detect possible reasons for the initial drop in the plasma carnitine and acetylcarnitine concentrations, we determined the renal excretion of carnitine, acylcarnitines, and butyrobetaine in this patient. As shown in **Figure 1C**, the urinary excretions

of carnitine, acetylcarnitine, propionylcarnitine, and butyrobetaine dropped after starting the treatment with valproate, whereas the excretion of valproylcarnitine steadily increased with the duration of exposure. The excretion fractions of carnitine, acetylcarnitine, propionylcarnitine, and butyrobetaine were <5% of the filtered load before and, in agreement with the reduced renal excretion of these compounds, decreased by 50% or more during treatment with valproate. While the excretion of valproylcarnitine was approximately 50 times lower compared to carnitine, the excretion fraction of valproylcarnitine remained constant between 150 and 250% of the filtered load during treatment with valproate (**Figure 1D**).

Transport of carnitine and valproylcarnitine by OCTN2

The results obtained in the patient described above clearly indicated that the initial drop in the carnitine plasma concentration could not be explained by increased renal losses of carnitine, acylcarnitines or butyrobetaine. In order to understand better the high excretion fraction of valproylcarnitine and to investigate a possible interaction between valproylcarnitine and carnitine at the level of OCTN2, we studied the transport of valproylcarnitine and its interaction with carnitine in L6 cells overexpressing hOCTN2. As reported previously (Todesco *et al.*, 2009; Todesco *et al.*, 2008), the transport of L-carnitine displayed a saturable Michael-Menten kinetics with a K_m of 11.9 ± 1.5 µmol/L and a V_{max} of 263 ± 14 pmol x mg protein^{-1} x min^{-1} (mean ± SD, **Figure 2A**). Valproylcarnitine inhibited the transport of carnitine competitively with a K_i of 78.5 ± 8.6 µmol/L (**Figure 2A and B**). Valproylcarnitine was also transported by OCTN2; the maximal transport activity was similar to L-carnitine (V_{max} 287 ± 9 pmol x protein^{-1} x min^{-1}), but the affinity for hOCTN2 was much lower (K_m 218 ± 20 µmol/L) (**Figure 2C**). L-carnitine inhibited the transport of valproylcarnitine competitively with a K_i of 12.5 ± 0.9 µmol/L (**Figure 2C and D**).

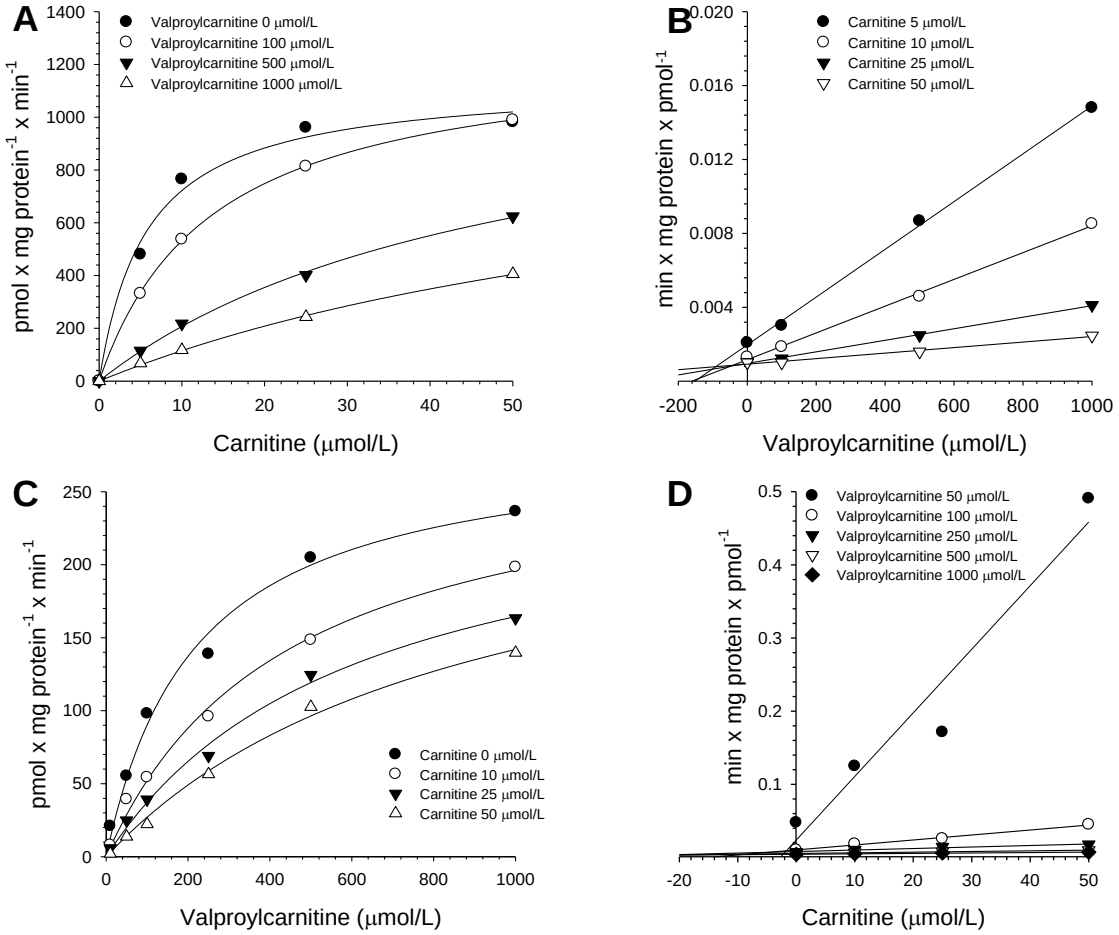

Figure 2: *Transport of carnitine and valproylcarnitine into L6 cells overexpressing hOCTN2*. (A) The transport of L-carnitine (K_m 11.9 ± 1.5 μmol/L) into L6 cells is inhibited by valproylcarnitine. (B) A Dixon plot of this transport is compatible with competitive inhibition by valproylcarnitine (K_i 78.5 ± 8.6 μmol/L). (C) Valproylcarnitine is also transported by OCTN2, but with a low affinity (K_m 218 ± 20 μmol/L). Carnitine inhibits the transport of valproylcarnitine. (D) The inhibition by L-carnitine is competitive (K_i 12.5 ± 0.9 μmol/L). Values are indicated as mean ± SD.

Plasma carnitine and butyrobetaine concentrations in patients on long-term therapy with valproate

To get a better understanding of the effects of long-term treatment with valproate on carnitine metabolism, we determined carnitine, acylcarnitines and butyrobetaine in one plasma sample and in 24h-urine of ten patients treated with valproate for at least one month and ten age- and sex-matched control patients not treated with valproate. Fourteen females (7 in each group) and six males (3 per group) were studied. In the valproate group, four

patients had valproate for the indication epilepsy and six for mood disorders. The median age of the patients in the valproate group was 40 years (range 23-53) and in the control group 39 years (range 26-52). The median BMI and body weight were 24.9 kg/m^2 (range 18.0-35.4) and 73 kg (range 49-102), respectively, in the valproate group and 22.1 kg/m^2 (range 17.6-32.5) and 65 kg (range 49-94), respectively, in the control group.

	Control patients (n=10)	Valproate-treated patients (n=10)	p
Carnitine	35.5 (19.0 – 58.0)	34.3 (13.7 – 45.7)	0.734
Acetylcarnitine	5.17 (3.49 – 11.2)	4.99 (3.70 – 6.70)	0.910
Propionylcarnitine	0.37 (0.19 – 0.60)	0.52 (0.24 – 1.04)	0.088
Valproylcarnitine	<0.01	0.02 (0.01 – 0.09)	<0.001
Butyrobetaine	0.59 (0.45 – 1.05)	0.54 (0.42 – 0.90)	0.521

Table 1: *Carnitine, acylcarnitines and butyrobetaine plasma concentrations in patients on long-term treatment with valproate*. Carnitine, acylcarnitines and butyrobetaine were determined using LC/MS-MS as described in Methods. Data are presented as median (range), units are µmol/L.

As shown in **Table 1** and **Figure 3A**, the inter-individual carnitine and acylcarnitines concentrations showed a large variation, not only between, but also within the groups. We found no significant differences in the concentrations of carnitine and acetylcarnitine between valproate-treated and control patients. Propionylcarnitine concentrations showed a trend to be higher in valproate-treated patients, but this increase did not reach statistical significance. Valproylcarnitine (median plasma concentration 20 nmol/L) was detectable in every patient treated with valproate, but in none of the controls. There were no significant correlations between carnitine and propionylcarnitine (**Figure 3A**), carnitine and valproylcarnitine (**Figure 3B**), total valproate and valproylcarnitine (**Figure 3C**), and valproylcarnitine and propionylcarnitine (**Figure 3D**).

The plasma concentration of the carnitine precursor butyrobetaine was approximately 10% lower in valproate-treated compared to control patients, but this difference did not reach statistical significance (**Table 1**).

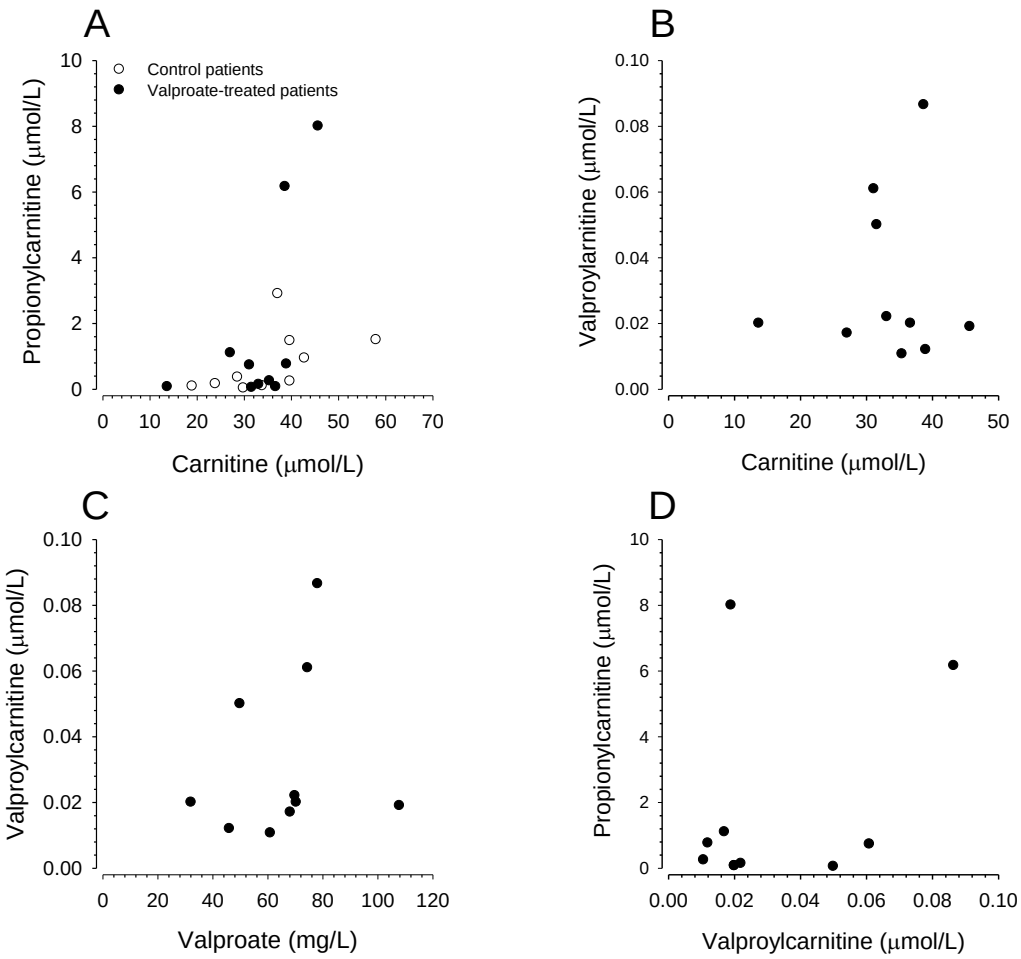

Figure 3: *Plasma concentrations and correlations of carnitine, propionylcarnitine and valproylcarnitine in patients with long-term valproate treatment.* (A) There is no significant correlation between carnitine and propionylcarnitine in patients treated with valproate (n = 10) and in age and sex matched control patients (n = 10). (B) There is no significant correlation between carnitine and valproylcarnitine in valproate-treated patients. (C) There is no significant correlation between valproate and valproylcarnitine in valproate-treated patients. (D) There is no significant correlation between valproylcarnitine and propionylcarnitine in valproate-treated patients.

Urinary excretion and clearance of carnitine

As shown in **Table 2**, the renal excretion of carnitine and acetylcarnitine were 4 to 5 times lower in valproate-treated patients, reaching statistical significance for carnitine. Similar to plasma, valproylcarnitine could not be detected in the urine of control patients, but in all the samples of patients treated with valproate. Similar to carnitine, the excretion of butyrobetaine was also lower in valproate-treated than control patients, but without reaching statistical significance.

The renal clearances of carnitine, acetylcarnitine, propionylcarnitine, and butyrobetaine averaged all <15 mL/min and the medians were all numerically lower in valproate-treated compared to control patients, reaching statistical significance for carnitine (**Table 2**). In comparison, the clearance of valproylcarnitine, which could be calculated only in patients treated with valproate, was almost as high as the creatinine clearance.

	Control patients (n=10)	Valproate-treated patients (n=10)	P
Carnitine and butyrobetaine excretion (µmol/day)			
Carnitine	70.8 (5.1 – 454)	14.8 (5.7 – 119)	0.038
Acetylcarnitine	26.0 (1.2 – 84.8)	6.1 (1.2 – 44.0)	0.162
Propionylcarnitine	1.19 (0.09 – 6.15)	0.83 (0.12 – 9.55)	0.734
Valproylcarnitine	<0.01	3.01 (0.90 – 8.12)	<0.001
Butyrobetaine	2.71 (1.15 – 13.5)	1.67 (0.57 – 6.73)	0.186
Renal clearances (mL/min)			
Carnitine	1.53 (0.12 – 8.48)	0.39 (0.11 – 2.14)	0.014
Acetylcarnitine	3.05 (0.21 – 13.7)	0.82 (0.20 – 6.70)	0.212
Propionylcarnitine	2.22 (0.24 – 11.5)	0.97 (0.23 – 6.36)	0.521
Valproylcarnitine	n.d.	72.5 (49.1 – 213)	n.d.
Butyrobetaine	3.27 (1.06 – 8.93)	2.22 (0.53 – 5.49)	0.241
Renal excretion fraction (% of creatinine clearance)			
Carnitine	1.52 (0.10 – 4.54)	0.43 (0.12 – 1.68)	0.031
Acetylcarnitine	2.77 (0.18 – 7.35)	1.04 (0.27 – 7.64)	0.385
Propionylcarnitine	1.87 (0.20 – 6.18)	1.63 (0.24 – 5.88)	0.791
Valproylcarnitine	n.d.	88.7 (39.9 – 233)	n.d.
Butyrobetaine	3.11 (1.39 – 5.90)	2.44 (1.36 – 5.79)	0.473

n.d.: not determinable

Table 2: *Renal handling of carnitine, acylcarnitines and butyrobetaine in patients on valproate long-term treatment*. Carnitine, acylcarnitines and butyrobetaine were determined using LC/MS-MS as described in Methods. The plasma creatinine concentrations and the creatinine clearance were 69 (45 – 91) µmol/L and 106 (76 – 187) mL/min, respectively, in control and 65 (47 – 79) µmol/L, and 90 (34 – 163) mL/min, respectively, in valproate-treated patients. Data are presented as median (range).

The excretion fractions of carnitine, acetylcarnitine, propionylcarnitine, and butyrobetaine were all <10% and the medians of the excretion fractions were all numerically lower in valproate-treated compared to control patients, again reaching statistical significance only for carnitine (**Table 2**). For valproylcarnitine, the excretion fraction was in the range of 100%, suggesting that filtered valproylcarnitine is not reabsorbed.

Effect of valproate on hOCTN2 mRNA and protein expression in HEK293 cells

To investigate the possibility that valproate or valproylcarnitine influence the expression of OCTN2, we determined mRNA levels of OCTN2 in the human embryonic kidney cell line HEK293 treated with valproate or valproylcarnitine. After 24h of treatment, 100 µmol/L and 1000 µmol/L valproate significantly enhanced the mRNA expression of OCTN2 by 15 and 21%, respectively, compared to control values (**Figure 4A**). At 48h, the corresponding increases were 20% and 11% in the presence of 100 and 1000 µmol/L valproate, respectively, and did not reach statistical significance. Treatment with 5 µmol/L valproylcarnitine was ineffective.

As shown in **Figure 4B**, both valproate (100 and 1000 µmol/L) and valproylcarnitine (5 µmol/L) did not influence the hOCTN2 protein levels after 24h and 48h of treatment.

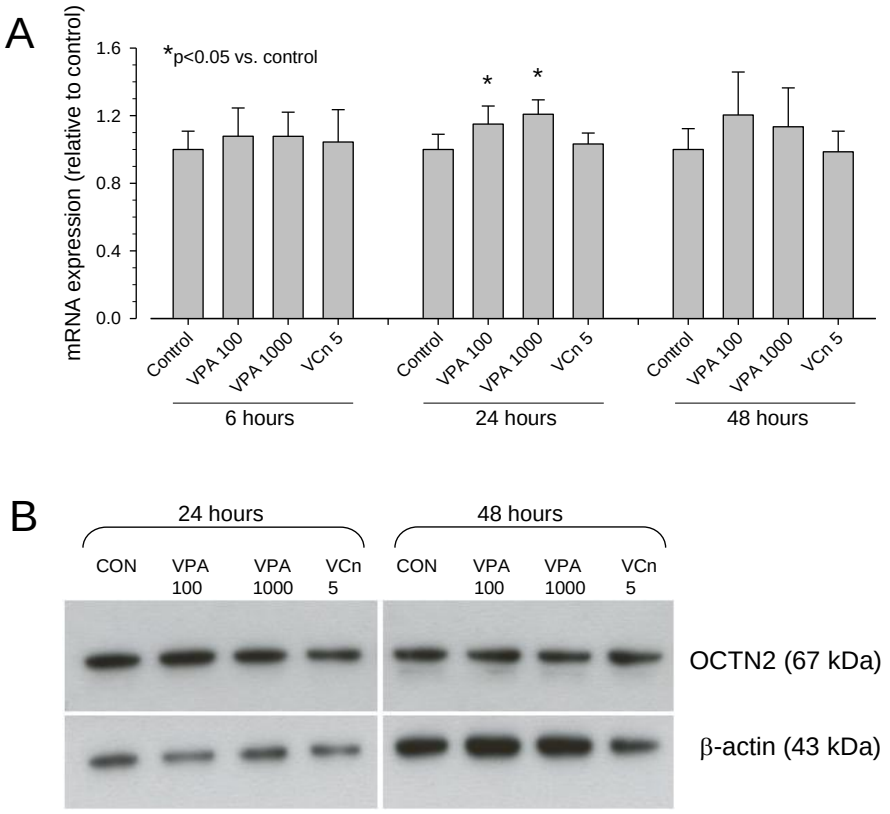

Figure 4: *Effect of valproate and valproylcarnitine on the expression of OCTN2 in HEK293-FT cells.* Cells were treated with valproate (VPA) 100 or 1000 μmol/L or valproylcarnitine (VCn) 5 μmol/L for 6h, 24h and 48h. (A) mRNA expression was measured by quantitative RT-PCR with GAPDH as a control. Expression in treated cells is shown relative to the expression in DMSO control cells. The results are the mean of 5 experiments done in triplicates. In cells treated for 24 h with 100 to 1000 μmol/L valproate, the OCTN2 mRNA expression is significantly increased by 15 to 21% compared to control values. (B) Representative Western blot showing OCTN2 (67 kDa) and β-actin (43 kDa) in treated cells. Treatment with VPA or VCn is not associated with a change in the OCTN2 protein content.

5.5. Discussion

The investigations in the patient starting treatment with valproate showed two effects on the plasma carnitine and acetylcarnitine concentrations: an initial drop reaching its maximum at week 1 or 2 and a slow recovery which was achieved between week 3 and 5. Similar results have been reported by Stadler et al. (Stadler *et al.*, 1999), who determined carnitine plasma concentrations and renal excretion in healthy adults treated with valproate for 28 days. Similar to our findings, Stadler et al. also observed a reduction in the clearance of total and free carnitine, which they claimed to be responsible for the recovery of the plasma carnitine concentration after the initial drop (Stadler *et al.*, 1999).

Since we were able to determine the relevant acylcarnitines and the carnitine precursor butyrobetaine, we could not only confirm the results of Stadler et al. (Stadler *et al.*, 1999), but we can discuss possible mechanisms underlying the changes in carnitine metabolism associated with valproate. The initial drop in the carnitine and acetylcarnitine plasma concentrations can theoretically be explained by a reduced intake and/or biosynthesis, an increased uptake by tissues (mainly skeletal muscle, the main carnitine store in mammals (Bremer, 1983; Friolet *et al.*, 1994; Krahenbuhl *et al.*, 2000)) and/or an increased renal excretion. Decreased dietary intake of carnitine can be excluded, since both in the current study as well as in the study of Stadler et al. (Stadler *et al.*, 1999) the subjects studied did not change their eating habits. Increased renal excretion of carnitine and/or acetylcarnitine could be observed in none of the two studies, excluding also this possibility. In the current study, the renal excretion of carnitine, acetylcarnitine and propionylcarnitine decreased dramatically within the first week after starting treatment with valproate and was low also during long-term valproate treatment. The only carnitine species with a higher renal excretion compared to pretreatment conditions or patients not treated with valproate was valproylcarnitine, which could be detected only in subjects treated with valproate. The renal excretion of valproylcarnitine is low compared to the excretion of carnitine, however, and can therefore not explain the initial drop in the plasma carnitine concentrations of valproate-treated subjects.

The most likely explanations for this initial drop are therefore an increased uptake of carnitine by tissues (skeletal muscle) and/or decreased carnitine biosynthesis. Increased uptake by tissues can be expected to be self-limiting, since export from tissues (either by

diffusion or transport proteins) must also increase when a new steady state is reached. Our observation that urinary excretion of carnitine and acylcarnitines remains low also during long-term treatment with valproate argues therefore against this possibility. The most likely reason for the initial drop in the plasma carnitine concentration is therefore a decrease in carnitine biosynthesis. This hypothesis is supported by our observation that the urinary excretion of butyrobetaine, the direct biosynthetic precursor of carnitine, also decreased sharply after starting valproate and remained low during long-term treatment with this drug. Farkas et al. have reported a decrease in carnitine biosynthesis in rats treated with valproate (Farkas et al., 1996) and have suggested an impaired conversion of butyrobetaine to carnitine as a possible mechanism. The data obtained in our patients argue against this mechanism, which should be associated with an increase in the butyrobetaine plasma concentration and also in its urinary excretion. Our data, which showed a decrease in renal excretion of butyrobetaine, favor an impact on carnitine biosynthesis proximal to the conversion of butyrobetaine to carnitine. In this context it is interesting to note that children have been reported to have a lower carnitine biosynthesis than adults (Rebouche *et al.*, 1986), possibly explaining why children treated with valproate are more susceptible to hypocarnitinemia than adults.

As shown in the current study and in the study of Stadler et al. (Stadler et al., 1999), subjects treated with valproate develop mechanisms to maintain their plasma carnitine concentrations after an initial drop. The most likely mechanism is an increased renal reabsorption of carnitine and short-chain acylcarnitines, which is supported by the current study and the study of Stadler et al. (Stadler et al., 1999). In patients with long-term valproate treatment, we observed a decrease in the excretion fractions of carnitine, acetylcarnitine, propionylcarnitine, and butyrobetaine, which are all transported by OCTN2 (Todesco et al., 2008), the high affinity carnitine transporter responsible for the renal reabsorption of carnitine (Nezu *et al.*, 1999; Stieger *et al.*, 1995; Tamai *et al.*, 1998). These observations suggest an increase in renal expression of OCTN2 in subjects treated with valproate, which could possibly be triggered by the initial drop in the plasma carnitine concentration. This hypothesis is supported by observations in vegetarians. Vegetarians have not only lower carnitine plasma concentrations compared to omnivores, but also a lower renal carnitine clearance (Lombard et al., 1989). Further support comes from a recent study in which we could show that rats with pharmacologically induced hypocarnitinemia

have an increased renal OCTN2 expression and transport of carnitine into proximal tubular brush border membrane vesicles (Schurch et al., 2010).

Another mechanism by which OCTN2 could be upregulated is by the activation of PPARα, which is known to be involved in the regulation of OCTN2 expression in liver and intestine (Maeda *et al.*, 2008; Ringseis *et al.*, 2007). Since fatty acids and fatty acid derivatives are known activators of PPARα (Hawkins *et al.*, 1987) valproate and/or valproylcarnitine could have similar properties taking into account their fatty acid structure. Moreover, older studies have demonstrated hepatic peroxisomal proliferation in rodents treated with valproate (Horie *et al.*, 1985), indicating that valproate stimulates the PPARα signaling pathway. In human embryonic kidney cells, we observed an approximately 20% increase in the mRNA expression of OCTN2 after 24 to 48 h of incubation with 100 µmol/L valproate, but not in the presence of 5 µmol/L valproylcarnitine. Since we observed no effect of valproate or valproylcarnitine on the OCTN2 protein content, it appears unlikely, however, that this increase in mRNA expression is functionally relevant. The initial drop in the carnitine plasma concentration is therefore a more probable trigger of the suspected increase of the renal expression and function of OCTN2 in valproate-treated patients.

In contrast to carnitine, acetylcarnitine and propionylcarnitine, the excreted fraction of the filtered valproylcarnitine approximated 100% in patients with long-term valproate treatment, indicating that valproylcarnitine is hardly reabsorbed by OCTN2. This finding could be confirmed by our *in vitro* studies with L6 cells overexpressing hOCTN2. Valproylcarnitine was transported by OCTN2, but only with a low affinity (K_m 218 µmol/L). This affinity is comparable to the affinity of pivaloylcarnitine to OCTN2, another drug metabolite studied in the same transport system (Todesco *et al.*, 2009). The transport of valproylcarnitine was inhibited by carnitine (K_i 12.5 µmol/L), suggesting that this inhibition is important under *in vivo* conditions. Taking into account the low plasma concentration (median 20 nmol/L) in comparison to the low affinity of valproylcarnitine to OCTN2 (K_m 218 µmol/L) and the expectable competition with carnitine for OCTN2, the renal reabsorption of valproylcarnitine could be predicted to be negligible. Similar conclusions have been reached by Ohnishi et al. who studied the transport of carnitine and valproylcarnitine in perfused rat kidneys and in cells expressing hOCTN2 (Ohnishi *et al.*, 2008). Interestingly, in perfused rat kidneys, the reabsorption of valproylcarnitine was almost as efficient as for carnitine (Ohnishi *et al.*, 2008), suggesting species differences in the interaction of valproylcarnitine with OCTN2.

The plasma concentrations of valproylcarnitine (median 20 nmol/L) are far from its inhibitory constant for the transport of carnitine (K_i 80 μmol/L), suggesting that any inhibitory effect of valproylcarnitine on the reabsorption of carnitine would be marginal. Accordingly, patients treated with valproate did not have significantly higher clearances or excretion fractions of carnitine and short-chain acylcarnitines compared to controls in our study. The data obtained *in vitro* are therefore in full agreement with the renal handling of carnitine and acylcarnitines in patients on long-term valproate treatment.

5.6. Conclusions

The current study shows that treatment with valproate does affect carnitine homeostasis. At the beginning of a therapy with valproate, patients develop hypocarnitinemia most probably due to an impairment of proximal steps of carnitine biosynthesis. During long-term treatment, the plasma carnitine levels normalize in most patients due to a higher renal reabsorption of carnitine and short-chain acylcarnitines. The plasma concentration of valproylcarnitine is too low to affect the reabsorption of carnitine and acylcarnitines at the level of OCTN2.

6. RAPID QUANTIFICATION OF PLASMA CARNITINE AND ACYLCARNITINES BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY-TANDEM MASS SPECTROMETRY USING ONLINE SOLID PHASE EXTRACTION

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6.1. Summary

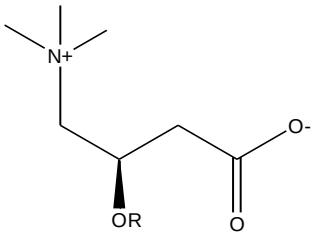
Carnitine is an amino acid derivative that plays a key role in energy metabolism. Endogenous carnitine is found in its free form or bound to acyl groups of several chain lengths. Quantification of carnitine and acylcarnitines is of interest for the newborn screening of metabolic disorders and for the monitoring of patients at risk for hypocarnitinemia.

We developed a method with online solid phase extraction coupled to high performance liquid chromatography and tandem mass spectrometry to quantify carnitine, acetylcarnitine, octanoylcarnitine, and palmitoylcarnitine, three acylcarnitines with different polarity. Plasma samples were deproteinized with methanol and loaded on a cation exchange trapping column. The analytes were separated on a reversed-phase C8 column using heptafluorobutyric acid as ion pairing reagent. Because of the endogenous nature of the analytes, we applied the addition method for their quantification. By adding known concentrations of carnitine, acetylcarnitine, octanoylcarnitine, and palmitoylcarnitine to plasma aliquots, we generated a standard curve with an intercept corresponding to the endogenous concentration. We determined the accuracy and precision of the method as well as stability and recovery of the analytes. Results were compared with an established radioenzymatic assay.

We report a method for simultaneous quantification of carnitine and three acylcarnitines with an easy sample workup. This approach requires small sample volumes and short analysis time, providing a useful tool for clinical routine analysis.

6.2. Introduction

Carnitine is an endogenous compound that plays a crucial role in energy metabolism. Carnitine is essential for the transport of long-chain fatty acids into the mitochondrial matrix for subsequent β-oxidation (Fritz, 1955) and for the regulation of coenzyme A (CoA) and acyl-CoA pools (Brass *et al.*, 1980; Friolet *et al.*, 1994). These functions of carnitine involve the acylation of its β-hydroxy group, leading to the formation of various acylcarnitines with different chain lengths (Steiber et al., 2004)(**Figure 1**).



Compound	*-R*
Carnitine	-H
Acetylcarnitine	$-COCH_3$
Octanoylcarnitine	$-CO(CH_2)_6CH_3$
Palmitoylcarnitine	$-CO(CH_2)_{14}CH_3$

Figure 1: *Structure of L-carnitine and three acyl-L-carnitines of different chain lengths*

Analytical profiling of carnitine and its acyl-derivatives is used to screen newborns and children for metabolic disorders or to monitor patients at risk or with established hypocarnitinemia (Millington et al., 1990). The traditional methods for the quantification of carnitine and acylcarnitines in plasma use HPLC separation coupled to MS/MS detection. The sample preparation can consist of several steps such as solid phase extraction (Vernez et al., 2003; Vernez et al., 2004) and esterification of carnitine and acylcarnitines at their carboxyl groups with different alcohols to enhance sensitivity (Chace et al., 1997; Minkler et al., 2008). Sample processing is time-consuming and imprecisions can arise from incomplete derivatization and/or hydrolysis of acylcarnitines during the derivatization process (Johnson, 1999).

Because of the endogenous nature of carnitine and acylcarnitines, their quantification is mainly achieved with surrogate matrices such as dialyzed plasma or an albumin solution (Vernez et al., 2004), or with surrogate analytes (e.g. deuterated compounds) (Liu *et al.*, 2008; Longo *et al.*, 1996; Minkler *et al.*, 2008). Both approaches use substitute parameters for the original matrix or analyte so that comparative studies are required to ensure the validity of the model (Chau et al., 2008).

For the analysis of clinical samples, both time and precision are important factors. Our aim was therefore to improve our existing method for plasma carnitine and acylcarnitine analysis (Vernez et al., 2004) by minimizing the time used for sample preparation and by optimizing the quantification procedure. We report a rapid and precise method for the simultaneous quantification of carnitine, acetylcarnitine, octanoylcarnitine, and palmitoylcarnitine in plasma samples using online solid phase extraction. To minimize matrix effects the addition method was used to quantify these endogenous analytes.

6.3. Materials and Methods

Reagents

L-Carnitine, L-acetylcarnitine, and D,L-palmitoylcarnitine were purchased from Sigma (St. Louis, MO, USA). L-Octanoylcarnitine was obtained from Dr. H. J. ten Brink (VU Medical Center, Amsterdam, The Netherlands). L-Carnitine-d3, L-acetylcarnitine-d3, L-octanoylcarnitine-d3, and L-palmitoylcarnitine-d3 were supplied by Cambridge Isotope Laboratories (Andover, MA, USA).

All other chemicals and solvents were from Merck (Darmstadt, Germany) or Sigma and were of analytical grade.

LC/MS-MS Equipment

The HPLC system consisted of a HTS PAL autosampler (CTC Analytics AG, Zwingen, Switzerland), a column-oven (CTO-20A), four pumps (LC-20AD) and a controller (CBM-20A) from Shimadzu (Reinach, Switzerland). The HPLC system was coupled to an API4000 triple quadrupole mass spectrometer from AB/MDS Sciex (Concord, Canada) with an electron spray ionization source.

Samples were loaded on an Oasis MCX column (30 μm, 2.1 x 20 mm; Waters Corporation, Milford, MA, USA) and separated on a Luna C8 5 μm column (150mm x 2mm) equipped with a C8 precolumn (4 x 2.0 mm, Phenomenex, Torrance, CA, USA).

Sample preparation and carnitine determination by LC/MS-MS

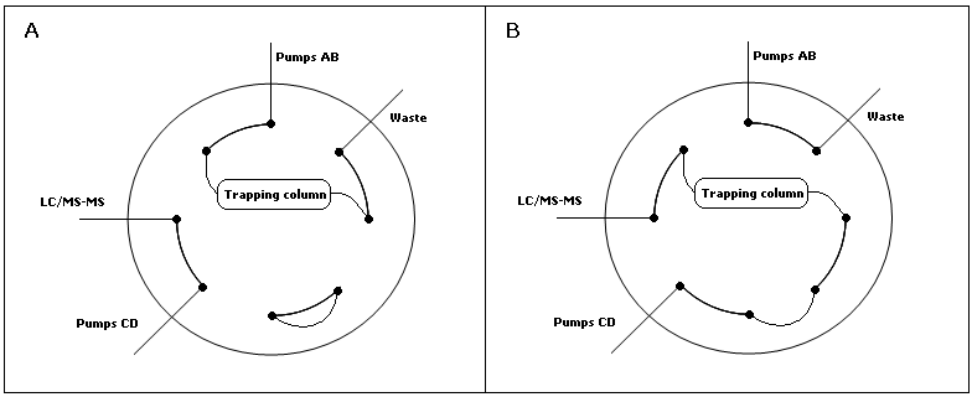
Blood samples from seven healthy volunteers were collected in tubes containing lithium heparin. Plasma was obtained after centrifugation at 1'811 g for 10 minutes and stored at -20°C until analysis. For the determination of total carnitine, acylcarnitines were hydrolyzed under alkaline conditions. 25 μL of sample were mixed with 25 μL KOH 0.5 M (pH 13) and incubated at 40°C for 30 minutes. The mixture was then neutralized with 50 μL 1% formic acid in water (V:V) and centrifuged for 10 minutes at 1'811 g.

The plasma or hydrolyzed plasma sample to be analyzed was divided in five aliquots of 10 μL. To each of the 10 μL aliquots, 0 to 40 μL of aqueous standard solution was added in 10 μL steps. The missing standard solution volume in lower concentrations was compensated with the corresponding volume of water. The final concentrations in the standard solutions were as follows: carnitine 10-40 μmol/L, acetylcarnitine 2.5-10 μmol/L, octanoylcarnitine 0.02-0.08 μmol/L, and palmitoylcarnitine 0.1-0.4 μmol/L. The plasma samples were then deproteinized with the addition of 200 μL of methanol containing the internal standards. The end concentrations of the internal standards were the following: carnitine-d3 20 μmol/L, acetylcarnitine-d3 5 μmol/L, octanoylcarnitine 0.04 μmol/L, and palmitoylcarnitine 0.2 μmol/L. The samples were mixed for 15 minutes at full speed on a Multi-tube Vortexer (VX-2500, VWR International) and centrifuged at 3'220 g for 30 minutes.

Quantification was made using the standard addition method. For this the plasma sample to be analyzed was divided into 5 aliquots of 10 μL. One aliquot served as "blank" (no addition of exogenous carnitine and acylcarnitines) and four as standards (addition of carnitine and acylcarnitine standards at increasing concentrations). For total carnitine, aliquots of the hydrolyzed plasma after neutralization and centrifugation were used.

HPLC Conditions

The chromatographic separation was done with a binary flow at 50°C. Phase A was an aqueous solution of 10 mmol/L of heptafluorobutyric acid and 10 mmol/L of ammonium acetate; phase B was methanol with the same additives. During 1.5 minutes, the analytes were loaded on the trapping column using 0.1% formic acid in water (V:V) as mobile phase (flow 0.5-1.0 mL/min), while the analytical column was conditioned with 10% phase B (flow 0.35 mL/min). After valve switching, the analytes were transferred to the analytical column starting at 10% phase B with a linear increase of the gradient to 95% phase B within 4 minutes. After a plateau of 2 minutes at 95% phase B, the analytical column was re-equilibrated for 2 minutes with 10% B, resulting in a total run time of 8 minutes (**Figure 2**).



Time [min]	Flow [mL/min]	Running buffers	B concentration [%]	Valve position
0	0.5	Water + 0.1% FA		Loading (A)
1.5	1.0			
2	0.35	Water/MeOH + 10 mmol/L HFBA + 10 mmol/L AmAc	10	Elution (B)
2.1			50	
4			95	
6			95	
8			10	

Figure 2: *Valve switching configuration and chromatographic conditions*. A: Valve is in the loading position. B: Valve is in the elution position. FA: formic acid; MeOH: methanol; HFBA: heptafluorobutyric acid; AmAc: ammonium acetate.

MS conditions

Carnitine and the acylcarnitines of interest were analyzed in the multiple reaction monitoring mode. A first transition was used for quantification, a second one for qualification. Following positive mode transitions (m/z) were used: carnitine 162/103 and 162/60, carnitine-d3 165/103 and 165/63, acetylcarnitine 204/85 and 204/145, acetylcarnitine-d3 207/85 and 207/145, octanoylcarnitine 288/85 and 288/229, octanoylcarnitine-d3 291/85 and 291/229, palmitoylcarnitine 400/85 and 400/341, and palmitoylcarnitine-d3 403/85 and 403/341, respectively.

The ion spray voltage was 5'500 eV, the probe temperature 450°C, and the dwell time 50 ms for each analyte.

Quantification with the standard addition method

The quantification of carnitine, acetylcarnitine, octanoylcarnitine, and palmitoylcarnitine was achieved with the standard addition method. As described above, known amounts of the analytes were added to the plasma samples and a standard curve with an equation of the type $y = ax + b$ was generated. The endogenous concentration was calculated as the intercept of the standard curve on the y-axis, defined as $x = \frac{-b}{a}$.

Qualification of the method

We determined the accuracy and precision of the method as well as the stability and recovery of the analytes. Because of the endogenous nature of carnitine and its derivatives and the lack of blank plasma, we used spiked plasma samples for the determination of the accuracy. Pooled plasma was spiked with three concentrations of carnitine, acetylcarnitine, octanoylcarnitine, and palmitoylcarnitine (low, medium and high quality controls, QC), containing the unknown endogenous concentration of the pooled plasma plus 12.5, 25, 37.5 μmol/L carnitine, 3, 6, 9 μmol/L acetylcarnitine, 0.03, 0.06, 0.09 μmol/L octanoylcarnitine, and 0.125, 0.25, 0.375 μmol/L palmitoylcarnitine, respectively.

For free carnitine and total carnitine, the results obtained with our method were compared with the results obtained with a radioenzymatic assay originally described by Cederblad & Lindstedt (Cederblad *et al.*, 1972) and modified by Brass and Hoppel (Brass *et al.*, 1978).

Recovery was assessed with three concentrations of the four analytes spiked before or after the protein precipitation step. For the assessment of short-term stability, samples were spiked, stored at room temperature for 8h, analyzed and compared to freshly spiked samples. For stability under storage conditions, samples were spiked and kept frozen at -20°C for one week. The samples were then analyzed and compared to freshly spiked plasma samples. For freeze/thaw stability, freshly prepared samples were frozen at -20°C for at least 12h and then thawed at room temperature for 30 minutes. After three freeze/thaw cycles, the samples were analyzed and compared to freshly prepared samples. To determine the stability of the analytes in solution, freshly made stock solutions were analyzed and compared to stock solutions that had been stored at -20°C for four months.

6.4. Results

Method qualification

We first tested the feasibility of a classical quantification with standard curves prepared in carnitine-free matrices such as water or 4% bovine serum albumin (BSA). Since the slopes of the standard curves in water containing BSA were different from the slopes in pooled plasma for carnitine and acetylcarnitine (**Figure 3A-D**), we applied the addition method to account for the observed matrix effects.

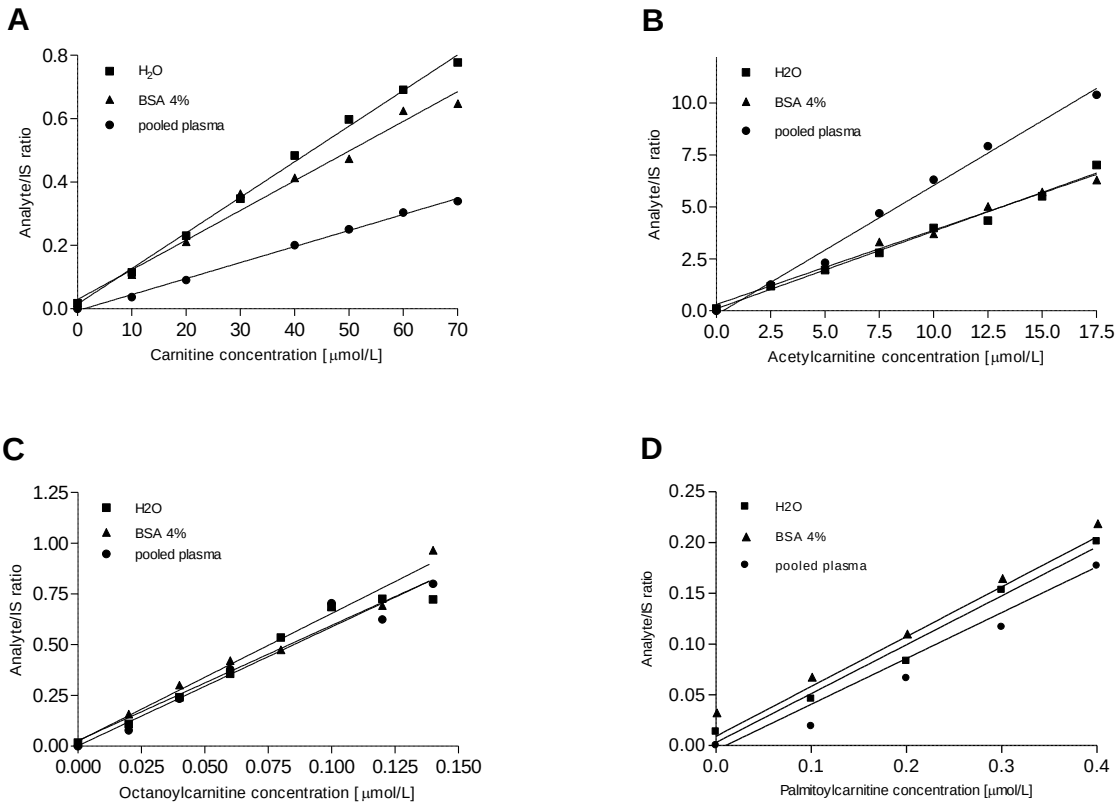


Figure 3: Standard curves of carnitine (A), acetylcarnitine (B), octanoylcarnitine (C) and palmitoylcarnitine (D) generated in water (■), BSA 4% (▲) or pooled plasma (●). Different slopes for carnitine and acetylcarnitine in plasma prompted us to use the addition method for quantification to account for matrix effects. For pooled plasma, the endogenous concentration was subtracted for clarity. Concentrations are given in µmol/L.

The mean recoveries of carnitine, acetylcarnitine, octanoylcarnitine, and palmitoylcarnitine for three different concentrations are reported in **Table 1**. The analytes, added to pooled plasma, were stable after three freeze-thaw cycles. After one week at -20°C, acetylcarnitine and octanoylcarnitine showed a drop by 7.4% and 11.4%, respectively. Similarly, after 8h at room temperature, acetylcarnitine and octanoylcarnitine had decreased by 5.9 and 6.4%, respectively, while carnitine and palmitoylcarnitine remained stable (**Table 1**).

	Recovery	**Freeze-thaw stability**	**1 week stability**	**8h stability**	**Stability of stock solution**
Carnitine	100.6±0.7%	103.2±1.5%	95.2±4.1%	103.1±5.5%	99.0±16.3%
Acetylcarnitine	98.4±2.7%	105.3±2.7%	92.6±3.7%	94.1±2.3%	102.8±16.5%
Octanoylcarnitine	102.3±3.8%	103.7±3.7%	89.6±7.1%	93.6±2.9%	110.0±24.9%
Palmitoylcarnitine	105.6±2.2%	103.6±4.1%	95.8±1.2%	98.7±6.3%	99.2±27.8%

Table 1: *Recovery and stability*. Stability studies of the four analytes were performed on seven replicates of the three quality controls.

Accuracy was assessed with three quality controls measured on three different days. The QCs were generated from pooled plasma spiked with three known concentrations of the four analytes. The target values were calculated by adding the determined concentration of the pooled plasma to the spiked concentrations. For total carnitine, carnitine, and acetylcarnitine, the measured concentrations were within ± 7.5% of the expected concentrations. For octanoylcarnitine and palmitoylcarnitine, the variability was higher, exceeding 20% for the low octanoylcarnitine QC (**Table 2**).

Precision was determined from plasma samples of seven healthy volunteers, one pooled plasma sample and three quality controls at different concentrations (low, medium and high). The samples were analyzed with the addition method on three different days. For free carnitine, acetylcarnitine, and total carnitine, the interday coefficients of variation (CV) did not exceed 10%. For low concentrations of octanoylcarnitine and palmitoylcarnitine we observed a higher variability with interday CVs up to 28% (**Table 3**).

		Low QC	Medium QC	High QC
Free carnitine	Expected	62.1	74.6	87.1
	Measured	59.6	74.2	88.3
	Accuracy	96.0%	99.5%	101.4%
Acetylcarnitine	Expected	8.5	11.5	14.5
	Measured	8.6	11.1	13.9
	Accuracy	101.5%	97.0%	96.0%
Octanoylcarnitine	Expected	0.10	0.13	0.16
	Measured	0.13	0.12	0.13
	Accuracy	126.8%	93.0%	81.1%
Palmitoylcarnitine	Expected	0.29	0.41	0.54
	Measured	0.34	0.39	0.64
	Accuracy	119.5%	93.3%	118.2%
Total carnitine	Expected	81.9	97.6	113.2
	Measured	80.3	90.3	111.4
	Accuracy	98.0%	92.5%	98.4%

Table 2: *Accuracy.* Accuracy was assessed with three quality controls (low, medium, high) generated from pooled plasma spiked with three different concentrations of carnitine and three acylcarnitines. The expected concentration was calculated from the concentration measured in pooled plasma plus the spiked concentrations. Concentrations are given in μmol/L.

	Free carnitine		Acetyl-carnitine		Octanoyl-carnitine		Palmitoyl-carnitine		Total carnitine	
	mean	CV%	mean	CV%	mean	CV%	Mean	CV%	mean	CV%
Subject 1	54.4	4.8	6.8	7.3	0.07	16.3	0.15	16.0	71.7	13.8
Subject 2	52.3	9.6	5.9	7.7	0.06	14.7	0.13	11.9	64.2	5.8
Subject 3	44.5	2.1	4.5	2.7	0.10	13.9	0.12	12.0	57.5	1.7
Subject 4	58.1	6.2	5.0	5.6	0.06	12.9	0.18	13.6	72.5	9.1
Subject 5	46.5	9.6	4.6	8.6	0.05	27.4	0.11	9.9	61.2	10.4
Subject 6	60.0	9.5	6.5	7.0	0.07	12.1	0.14	15.2	71.5	14.5
Subject 7	45.9	8.1	8.0	7.0	0.11	8.7	0.17	26.7	57.4	3.4
Pooled	49.6	8.8	5.5	7.8	0.07	6.9	0.16	10.0	66.3	13.5
QC L	59.6	8.1	8.6	4.5	0.13	9.37	0.34	16.9	80.3	8.4
QC M	74.2	10.0	11.1	6.8	0.12	16.11	0.39	21.4	90.3	16.9
QC H	88.3	1.3	13.9	7.1	0.13	5.70	0.64	7.80	111.4	7.9

Table 3: *Precision*. The precision of the method was evaluated with repetitive measurements (n=3) of seven plasma samples, one pooled plasma sample and three quality controls. Concentrations are given in μmol/L.

Cross-comparison of plasma samples from seven healthy volunteers was performed with an established radioenzymatic method. The two methods provided comparable values with a correlation of 0.93 for free carnitine and a correlation of 0.92 for total carnitine (**Figure 4**). For free carnitine, we observed a mean systematic shift of 7 μmol/L between our method and the radioenzymatic assay. The difference exceeded 15% for only one sample.

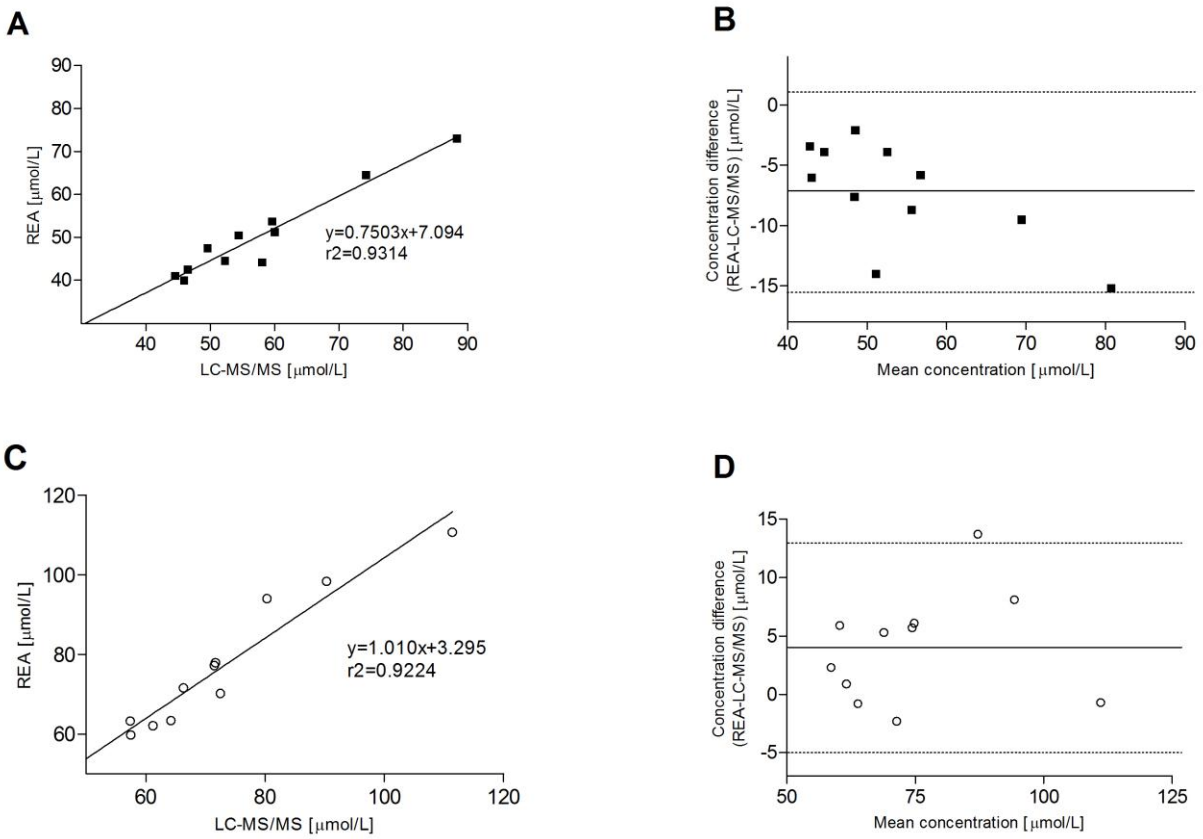


Figure 4: Carnitine (■) and total carnitine (○) concentrations were measured with the developed method (LC-MS/MS) and with an established radioenzymatic assay (REA). The two methods were compared using linear regression (A, C) and Bland-Altman plots (B, D). Concentrations are given in μmol/L.

Application of the method

The method was applied for the quantification of carnitine and three acylcarnitines in plasma samples of one healthy volunteer completing a cycling exercise until exhaustion. As expected, we observed increasing acetylcarnitine concentrations with increasing effort (**Figure 5A**). Carnitine concentrations slightly decreased (**Figure 5A**) while octanoylcarnitine and palmitoylcarnitine concentrations were not affected by exercise (**Figure 5B**).

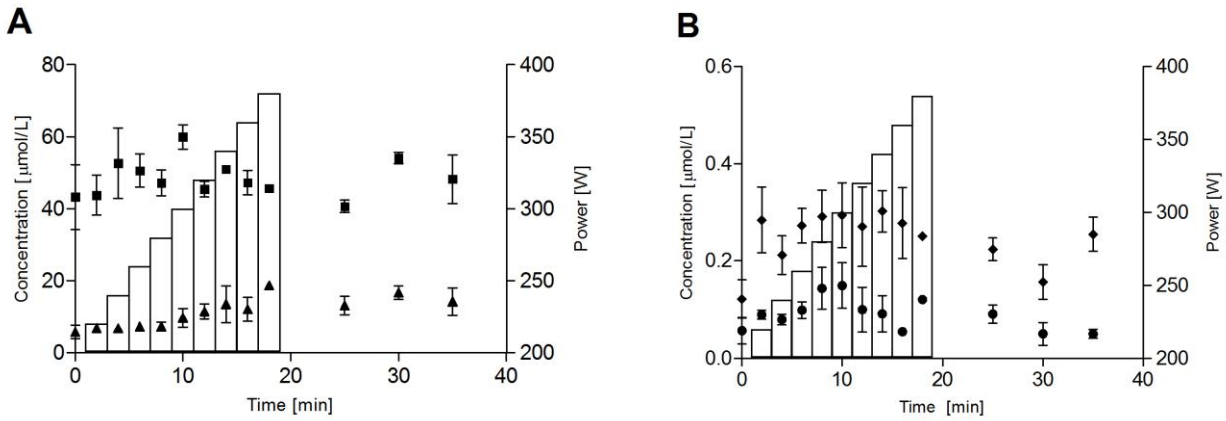


Figure 5: The new method was applied for the quantification of carnitine and acylcarnitines in plasma samples of one healthy volunteer completing a cycle exercise with increasing work load until exhaustion. The workload (bars) started at 220 W and was increased by 20 W every 2 min. Exhaustion was reached after 18 min at 380 W. A: Carnitine (■) and acetylcarnitine (▲), B: octanoylcarnitine (●) and palmitoylcarnitine (♦). Mean concentration in µmol/L ±SD of 3 determinations are shown.

6.5. Discussion

We have developed a fast and simple method for the determination of carnitine and three of its acyl derivatives in human plasma. The method is based on protein precipitation and online solid phase extraction followed by direct determination of the analytes by LC-MS/MS. The analytes in the reported method cover a wide range of lipophilicity – from the small and polar carnitine to the lipophilic palmitoylcarnitine – and would be applicable to other acylcarnitines in this solubility range. The small sample volume needed – 75 µL for the determination of acylcarnitine profile and total carnitine – is advantageous for situations where only small blood volumes are available, as for instance in pediatric patients.

Solid phase extraction (SPE) is widely used during sample preparation for the determination of carnitine and acylcarnitines (Minkler *et al.*, 2008; Vernez *et al.*, 2004). Since carnitine is a cation under acidic conditions, cation exchangers are the preferred matrices to optimize retention (Vernez et al., 2004). Solid phase extraction can enhance the sensitivity of the chromatographic assays, but the process can be time-consuming when operated off-line. In comparison, online solid phase extraction coupled to chromatographic

separation in one run conciliates the advantages of SPE with time efficiency. This is demonstrated with our method, enabling solid phase extraction and sample analysis within 8 minutes.

Derivatization is common for the analysis of acylcarnitines, with the advantages of increasing the sensitivity and optimizing the chromatographic properties of the analytes while prolonging sample preparation. On the other hand, disadvantages of derivatization include incomplete reaction, possible degradation of the analytes and prolongation of the sample work-up. We therefore prefer methods without derivatization for the reasons previously described (Vernez et al., 2003; Vernez et al., 2004).

Quantification of carnitine and acylcarnitines is complicated by the endogenous nature of the analytes. Classical quantification with standard curves in a blank matrix as used for exogenous compounds can only be achieved with denatured matrices such as dialyzed plasma (Liu *et al.*, 2008; Vernez *et al.*, 2004). An alternative are albumin-containing solutions, which have been used for the radioenzymatic method (Brass *et al.*, 1978) or also for LC/MS methods (Vernez et al., 2004). In the current study, standards in water or in albumin-containing solutions had a substantially different slope compared to standards in plasma, precluding their use as a matrix for standards. One possible solution of this problem is the above-mentioned pre-treatment of plasma, but procedures such as dialysis are time consuming. The addition method, which allows the quantification of the analytes in native matrix, avoids artifacts of surrogate or denatured matrices by directly accounting for matrix effects. A possible disadvantage of this method is the fact that a single sample has to be analyzed several times in order to obtain a reliable curve which can be used for quantification. Taking into account the short analysis time, this disadvantage is acceptable. Besides, the same standard solution in different volumes is added to the plasma aliquots, enabling a semi-automated sample processing within 5 minutes. Together with the 30 minutes hydrolysis time for the determination of total carnitine, mixing and centrifugation steps, reliable quantification of total carnitine, free carnitine and acylcarnitines can be achieved within half a day. Our method consists of a rapid and simple procedure in comparison to published methods using derivatization times of typically 15 minutes, hydrolysis times of one hour for total carnitine, LC runs of 30 minutes or more (Maeda *et al.*, 2007; Minkler *et al.*, 2008; Sun *et al.*, 2006), or potential evaporation and reconstitution steps.

In terms of precision, our method shows comparable results as described in the literature (Ghoshal et al., 2005; Minkler et al., 2008). Coefficients of variation were below 10% for carnitine and acetylcarnitine and below 15% for total carnitine. We observed higher variability for octanoylcarnitine and palmitoylcarnitine, remaining nevertheless within acceptable limits, considering the mean variation obtained for all plasma samples and considering the low concentrations measured. For palmitoylcarnitine, similar interday precisions have been reported by Ghoshal and colleagues (13.7%) and Minkler and colleagues (0.11±0.02, corresponding to a CV of 18%) (Ghoshal et al., 2005; Minkler et al., 2008).

Regarding the sensitivity of our method, we defined the lower limits of quantification (LLOQ) for octanoylcarnitine and palmitoylcarnitine with the lowest QC measured with reliable precision and accuracy, in order to avoid a surrogate matrix or matrix dilution. For octanoylcarnitine, the LLOQ was 0.03 μmol/L and for palmitoylcarnitine 0.125 μmol/L.

We describe a fast and reliable method for the quantification of carnitine and three acylcarnitines requiring small sample volumes and short total analysis time. This simple approach with minimal sample workup represents a useful method for clinical analysis.

7. EFFECTS OF CARNITINE, ACETYLCARNITINE AND PROPIONYLCARNITINE SUPPLEMENTATION ON SKELETAL MUSCLE PARAMETERS AND PHYSICAL PERFORMANCE OF MICE

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7.1. Abstract

Carnitine is a zwitterionic molecule with key functions in energy metabolism. Whereas most studies failed to show an accumulation of carnitine in skeletal muscle while supplementing carnitine, the effects of high oral doses of acetylcarnitine or propionylcarnitine on skeletal muscle composition and function are unknown. We therefore investigated the effects of carnitine, acetylcarnitine and propionylcarnitine supplementation on body carnitine homeostasis and skeletal muscle capacity of mice. Animals were supplemented with 2 mmol/kg/d carnitine, acetylcarnitine or propionylcarnitine via drinking water for four weeks. After this period, half of the mice were submitted to an exhaustive treadmill exercise while the other half served as control group. We determined endurance parameters, carnitine and acylcarnitines concentrations in muscle, plasma and urine of supplemented animals compared to a control group not treated with carnitine. In soleus and in gastrocnemius muscle biopsies, we investigated the muscular oxidative capacity in presence of specific mitochondrial substrates. Energy parameters such as lactate in plasma, phosphocreatine, creatine and glycogen in white and red portions of the quadriceps femoris muscle were determined.

In the supplemented groups, the total carnitine concentrations in plasma and in urine were significantly higher than in controls groups, whereas we observed no difference in muscle carnitine content. The bioavailability of supplemented carnitine or acylcarnitines, estimated as the difference between carnitine supplementation and urinary excretion, was in the range of 20 to 23%. The supplemented acylcarnitines were hydrolyzed before reaching the systemic circulation. The *ex vivo* oxygen consumption by isolated muscle fibers from soleus or gastrocnemius was not different between supplemented and control mice. The supplementation of carnitine or acylcarnitines had no influence on the running capacity of the animals or key substrates of energy metabolism.

In conclusion, oral supplementation of carnitine, acetylcarnitine or propionylcarnitine was associated with increased plasma concentrations of total carnitine and increased urinary excretion of carnitine, but did not affect the skeletal muscle carnitine content. Accordingly, physical performance and skeletal muscle energy metabolism were not significantly affected by carnitine supplementation.

7.2. Introduction

Carnitine is an amino acid derivative which plays an essential role in cellular energy metabolism due to acylation of its β-hydroxy group. The main body carnitine pool is in skeletal muscle (Krahenbuhl et al., 2000; Spaniol et al., 2001), where carnitine functions as carrier of long chain-fatty acids (Fritz, 1955) and as a regulator of the CoASH/acetyl-CoA ratio (Brass et al., 1980; Friolet et al., 1994).

Carnitine is an obligatory intermediate for the transport of long chain fatty acids into the mitochondria. In order to reach the mitochondrial matrix for subsequent β-oxidation, long chain fatty acids have first to be converted into the corresponding acyl-CoA and then into the acylcarnitine derivative. The second step is achieved by carnitine palmitoyltransferase 1 (CPT 1), the rate limiting enzyme of β-oxidation (Roepstorff et al., 2005). As acylcarnitines, long-chain fatty acids are transported across the inner mitochondrial membrane through the carnitine/acylcarnitine carrier (Indiveri et al., 2011) into the mitochondrial matrix, where they enter the β-oxidation cycle after reconversion into the acyl-CoA derivative by CPT2 (Bremer, 1983).

During high intensity exercise, acetyl-CoA generated by pyruvate oxidation or β-oxidation overwhelms the capacity of the Krebs cycle. The limited CoA pool would rapidly be depleted by the accumulating acetyl-CoA, if carnitine could not function as a buffer for the acetyl groups. Carnitine acetyltransferase converts acetyl-CoA to acetylcarnitine, thereby ensuring the availability of free coenzyme A (CoASH) (Brass et al., 1980; Friolet et al., 1994).

Taking into account these important functions of carnitine, it is not surprising that carnitine supplementation has been studied intensively as a potential performance enhancer (Brass, 2004). In *ex vivo* models it has been reported that an elevation of the carnitine muscle content increases skeletal muscle force and delays fatigue (Brass et al., 1993; Dubelaar et al., 1991). In humans, most studies failed to show a positive effect of carnitine supplementation on physical performance, correlating with the fact that the muscle carnitine content could not be increased by oral or parenteral means despite elevated plasma concentrations (Brass, 2000). High oral doses of carnitine during several days or intravenous infusion for several hours before exercise failed to show an impact on the

physical performance of healthy volunteers or athletes (Barnett et al., 1994; Oyono-Enguelle et al., 1988; Wachter et al., 2002). Recently, Wall et al. reported an approximately 20% increase of the carnitine muscle content in human subjects ingesting 80 g carbohydrate per day and oral carnitine intake (Wall et al., 2011). This increase in the skeletal muscle carnitine content was associated with an increase in work output, maintained skeletal muscle glycogen stores and a reduced production of lactate.

In rodents, studies investigating the accumulation of carnitine in skeletal muscle associated with carnitine supplementation are controversial. An increase of the skeletal muscle carnitine content of supplemented animals was reported by several groups (Bacurau et al., 2003; Negrao et al., 1987), while in other investigations, no such increase could be detected (Lambert et al., 2009; Primassin et al., 2008).

Whereas the dose und duration of carnitine supplementation seems to influence skeletal muscle carnitine accumulation in rodents, little is known about the effect of supplementation with carnitine derivatives, e.g. acetylcarnitine or propionylcarnitine, on the skeletal muscle carnitine content and physical performance. Elevated carnitine plasma concentrations are not necessarily associated with an increase in the skeletal muscle carnitine content due to saturation of the carnitine transport system into skeletal muscle at normal carnitine plasma concentrations (Berardi et al., 2000) and due to a large concentration gradient between plasma and skeletal muscle (Brass, 2000; Friolet et al., 1994). For acetylcarnitine and propionylcarnitine, concentrations differences between plasma and skeletal muscle are not as extreme, possibly also allowing passive diffusion as a transport mechanism.

In this study we therefore aimed to investigate the potential benefits of acetylcarnitine or propionylcarnitine supplementation in comparison to carnitine on the muscular carnitine content and working capacity of mice. After oral supplementation during four weeks, mice were subjected to exhaustive exercise and the plasma and skeletal muscle carnitine pools as well as metabolic markers in muscle and plasma were analyzed.

7.3. Material and methods

Animals

The experiments were performed in agreement with the guidelines for the care and use of laboratory animals and were approved by the cantonal veterinary authority (License 2509). The animals were adult male C57BL/6 mice (Janvier, Le Genest-Saint-Isle, France), housed in a standard facility with a 12h:12h light-dark cycle. The mice were fed with a standard pellet chow and water ad libitum. Before starting supplementation, the animals were acclimatized to the animal facility for at least 7 days.

Supplementation

L-carnitine and acetyl-L-carnitine HCl were purchased from Sigma (St. Louis, MO, USA). Propionyl-L-carnitine HCl was a kind gift of Sigma Tau (Pomezia, Rome, Italy).

The solutions of carnitine, acetylcarnitine and propionylcarnitine were made in tap water once a week and were kept at 4°C during that time. After one week of acclimatization, the mice were supplemented with carnitine (CN), acetylcarnitine (ACN) or propionylcarnitine (PCN) via drinking water during four weeks (4 groups, each group n=6). The concentration of the salts in tap water was 10 mmol/L so that mice of about 25 g drinking 5 mL/d would be exposed to approximately 2 mmol/kg/d of each compound. Water consumption was monitored daily. The carnitine content in the standard chow and in the drinking water was evaluated with a LC-MS/MS method previously described (Morand, 2012).

Protocol

Between week 1 and 2 of supplementation, the mice spent 24h in individual metabolic cages with supplemented water and food ad libitum. 24h urine was collected for the analysis of the excretion of carnitine and acylcarnitines.

After four weeks of supplementation, the mice were submitted to an exhaustive exercise on a treadmill with previous acclimatization to the apparatus (Exer-3/6, Columbus Instruments, Columbus, OH, USA). The starting speed was 5 m/min; after 5 minutes, the speed was increased by 1 m/min every minute until the maximal speed of 20 m/min was reached. Exhaustion was defined as immobility for more than 5 seconds on the end lane grid despite electric stimulation (0.4 mA, 2 Hz).

Performance of the mice was calculated from the distance and the duration of the run according to the following equation:

$$\text{Performance (W)} = \frac{m\,(\text{kg}) \times a\,(\text{m/s}^2) \times d\,(\text{m})}{t\,(\text{s})} \quad (1)$$

Biological sample collection

After exhaustion the mice were anaesthetized with an intraperitoneal application of ketamine (100 mg/kg) and xylazine (10 mg/kg). The soleus and gastrocnemius muscle were excised and conserved in ice cold BIOPS buffer (10 mmol/L Ca-EGTA buffer, 0.1 μmol/L free calcium, 5.77 mmol/L ATP, 6.56 mmol/L $MgCl_2$, 20 mmol/L taurine, 15 mmol/L phosphocreatine, 0.5 mmol/L dithiothreitol, and 50 mmol/L K-MES, pH 7.1) until analysis. Blood was collected into heparin coated tubes by an intracardiac puncture or a tail incision. Plasma was separated by centrifugation at 3000 g for 15 minutes.

For chemical analysis muscle samples from the red and white quadriceps femoris were frozen in liquid nitrogen immediately after excision. Plasma, urine and muscle samples were kept at -80°C until analysis.

High resolution respirometry

All oxygen measurements were performed at 30°C with an Oxygraph 2k apparatus equipped with the Datlab software (OROBOROS, Innsbruck, Austria). Muscle biopsies preserved in BIOPS buffer were prepared according to an established protocol (Pesta et al., 2012). Muscle fibers were permeabilized during 30 min in BIOPS buffer containing saponin (50 μg/mL). Per respiration chamber, 1-2 mg of permeabilized fibers was added to 2.0 mL of MiR05 buffer (EGTA 0.5 mmol/L, $MgCl_2$ 3 mmol/L, taurine 20 mmol/L, KH_2PO_4 10 mmol/L, HEPES 20 mmol/L, D-sucrose 110 mmol/L, BSA essentially fatty acid free 1 g/L, and lactobionic acid 60 mmol/L).

The following protocol was used to evaluate the activity of the different mitochondrial complexes: glutamate (10 mmol/L) and malate (2 mmol/L), ADP (2.5 mmol/L), rotenone (0.5 μmol/L), succinate (10 mmol/L), malonate (20 mmol/L), antimycin A (2.5 μmol/L), and TMPD/ascorbate (0.5/2 mmol/L). To verify the integrity of the outer mitochondrial membrane, cytochrome c (10 μmol/L) was added at the end of the respiration protocol. Respiration rates are expressed in picomoles O_2 per minute per gram wet weight.

Urinary carnitine concentrations

A LC-MS/MS method previously described was adapted for the determination of carnitine and acylcarnitines in urine (Morand, 2012). The method was extended to the analysis of propionylcarnitine (transitions 218/85 and 218/159) and creatinine (114/44 and 114/86). Hydrolysis of total carnitine was achieved with 0.5 mol/L potassium hydroxide, neutralization and dilution with 0.1% formic acid.

Plasma parameters

Plasma concentrations of carnitine were determined as previously described with adaptation to propionylcarnitine (Morand, 2012). Venous lactate concentrations were analyzed with an enzymatic assay (Olsen, 1971). Creatine kinase activity in venous plasma was determined before and after exhaustive exercise with a kit according to the supplier's instructions (BioAssay Systems, Hayward, CA, USA).

Muscle parameters

Muscle tissue was homogenized with a Mikro-dismembrator during 1 minute at 2000 rpm (Sartorius Stedim Biotech, Göttingen, Germany). 50 mg muscle was extracted with 1 mL of extracting solvent depending on the metabolites determined as described below.

Phosphocreatine, creatine, and ATP were photometrically determined in an acidic extract (perchloric acid 0.5 mol/L, EDTA 1 mmol/L) of muscle powder as previously described (Harris et al., 1974). Muscle glycogen content was analyzed in alkaline (NaOH 0.1 mmol/L) muscle extracts according to Harris (Harris et al., 1974). Carnitine, acylcarnitines and total carnitine were determined in aqueous muscle extracts with an established LC-MS/MS (Morand, 2012).

Statistical analysis

Data is expressed as mean ± SEM. Statistical analyses were performed using Student's t-test or one-way ANOVA with the software Prism version 5 (Graph Pad Software, San Diego, CA). Statistical significance was set at *p < 0.05 and **p < 0.01.

7.4. Results

Characterization of the animals

During the study, we observed no weight differences between the groups (**Figure 1A**). Food intake was similar in all the groups. The daily water intake was increased in all treated groups compared to control group (**Figure 1B**). The control animals (n=12) drank 4.1 ± 0.6 mL/d, whereas the animals of the carnitine (CN), acetylcarnitine (ACN), and propionylcarnitine (PCN) group ingested 5.2 ± 0.3 mL/d, 5.7 ± 0.9 mL/d, and 5.6 ± 0.4 mL/d, respectively (mean ± SD; no difference between treated animals). The mean daily exposure to exogenous carnitine, acetylcarnitine or propionylcarnitine was 2.2 mmol/d/kg (1.9-2.7 mmol/d/kg) in the treated groups and below 10 μmol/kg/d in the control group, taking into account carnitine ingested by the drinking water and by food (**Figure 1C**). The carnitine content of the food was 60 nmol/g. The concentrations of carnitine, acetylcarnitine, and propionylcarnitine in the drinking solutions remained stable during their storage in the water bottles at room temperature (data not shown).

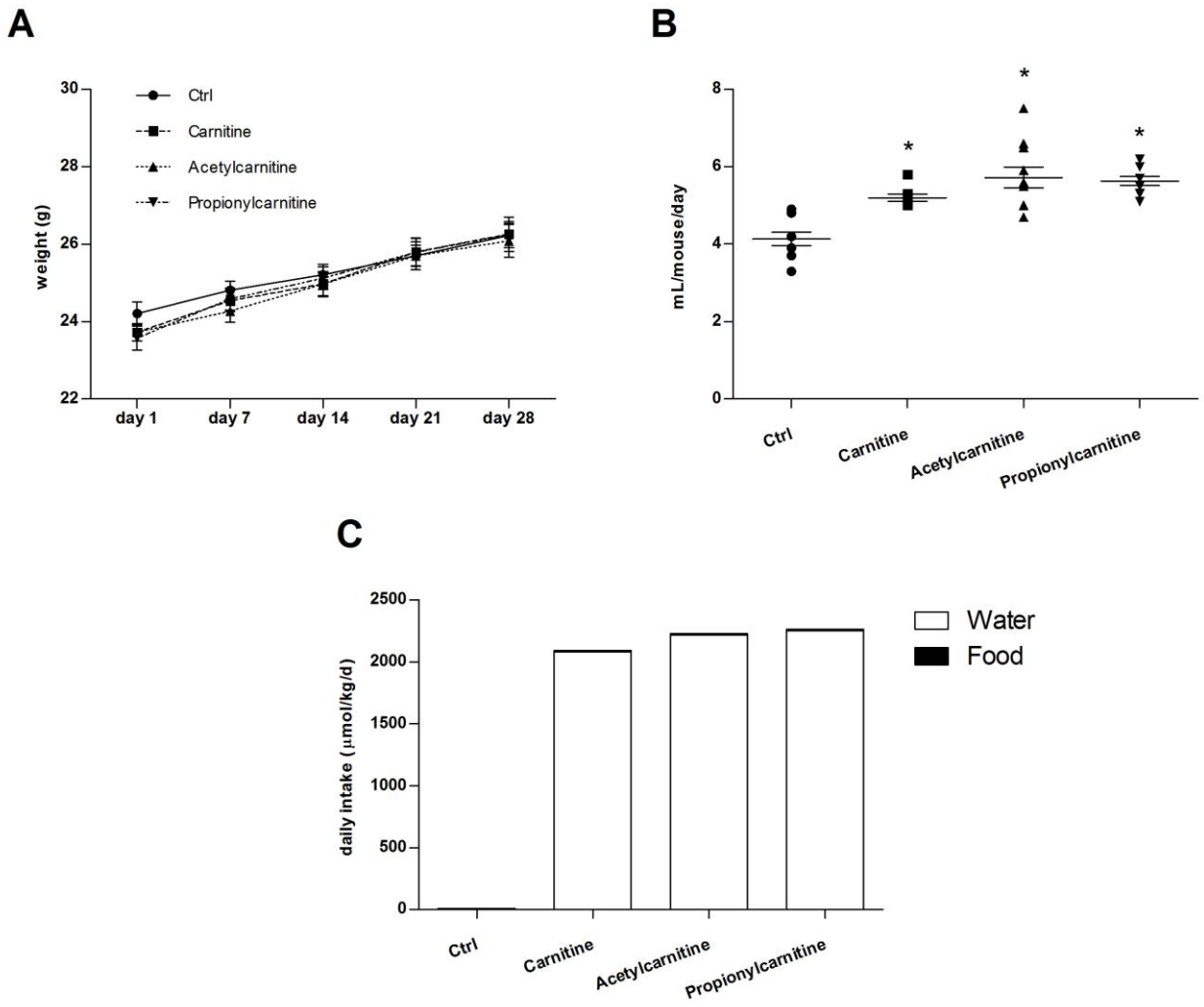

Figure 1: *Characterization of the animals*. The weight gain (in grams) was similar in the treatment groups compared to control (A). Water consumption (in mL/mouse/d) was significantly higher in the treated groups (B). Daily consumption of carnitine or acylcarnitines was below 10 µmol/kg/d in the control group and in the range of 2000 µmol/kg/d in the supplemented groups (C). Data is represented as mean ± SEM of 12 animals per group.

Carnitine and acylcarnitine pools in plasma and skeletal muscle and renal carnitine excretion

In plasma, total carnitine concentrations were significantly higher in the treated groups (**Table 1**), associated with higher free carnitine concentrations. Acetylcarnitine, propionylcarnitine, and palmitoylcarnitine plasma concentrations were similar between the groups.

In red or white quadriceps muscle, we observed no difference between the groups treated with carnitine or acylcarnitines and the control group. The acetylcarnitine concentrations accounted for 20% of the total pool in red fibers and for 15% in the white fibers. Total carnitine, free carnitine, acetylcarnitine, and palmitoylcarnitine concentrations were by trend lower in the white compared to red quadriceps. Propionylcarnitine concentrations in skeletal muscle were below detection limits, also in mice treated with propionylcarnitine (**Table 1**).

	Control	**Carnitine**	**ACN**	**PCN**
Plasma concentrations (μmol/L)				
Carnitine	31.0±0.5	37.2±2.2	36.1±1.4	35.6±3.4
Acetylcarnitine	7.8±0.8	8.5±1.3	9.3±0.6	7.6±0.7
Propionylcarnitine	0.30±0.04	0.24±0.07	0.27±0.04	0.76±0.38
Palmitoylcarnitine	0.25±0.01	0.29±0.04	0.25±0.02	0.30±0.03
Total carnitine	36.4±1.4	47.7±1.8**	47.6±1.6**	42.7±1.6*
Skeletal muscle concentrations (μmol/kg wet weight)				
Red quadriceps				
Free carnitine	205±29	272±32	216±25	189±14
Acetylcarnitine	66±7	69±11	70±3	62±9
Propionylcarnitine	<0.5	<0.5	<0.5	<0.5
Palmitoylcarnitine	44±8	55±6	32±12	28±5
Total carnitine	291±31	309±59	359±56	249±43
White quadriceps				
Free carnitine	110±7	115±10	127±11	130±11
Acetylcarnitine	39±5	37±10	26±3	28±5
Propionylcarnitine	<0.5	<0.5	<0.5	<0.5
Palmitoylcarnitine	15±5	20±3	14±3	19±5
Total carnitine	195±17	207±14	169±20	152±14

Table 1: *Carnitine and acylcarnitines concentrations in plasma and skeletal muscle under resting conditions*. Mean values ± SEM of six animals are represented. **: $p<0.01$ compared to control, * $p<0.05$ compared to control. ACN: Acetylcarnitine, PCN: Propionylcarnitine.

Treated animals excreted about 17 times more total carnitine than controls animals (24 ± 4 μmol/kg/d in the control group vs. 417 ± 143, 390 ± 76, and 448 ± 36 μmol/kg/d in the carnitine, ACN, and PCN treated groups respectively) (**Table 2**). The excreted carnitine was found principally in the form of free carnitine and acetylcarnitine (**Figure 2**). In all treated groups, independently of the supplemented form of carnitine, the excretion of acetylcarnitine was significantly higher (41 ± 10 μmol/kg/d in the carnitine group, 54 ± 13 in the ACN group, and 46 ± 6 for the PCN group) compared to control animals (2 ± 0 μmol/kg/d). Urinary excretion of propionylcarnitine could only be detected in the three treated groups, whereas the propionylcarnitine concentration was below the limit of detection in the urine of control mice. The highest urinary propionylcarnitine concentrations were found in the PCN group (3.3 ± 1.1 μmol/kg/d vs 1.0 ± 0.3 μmol/kg/d, and 1.1 ± 0.3 μmol/kg/d in the CN and ACN groups respectively). Due to the high variability, this difference did not reach statistical significance (**Table 2**).

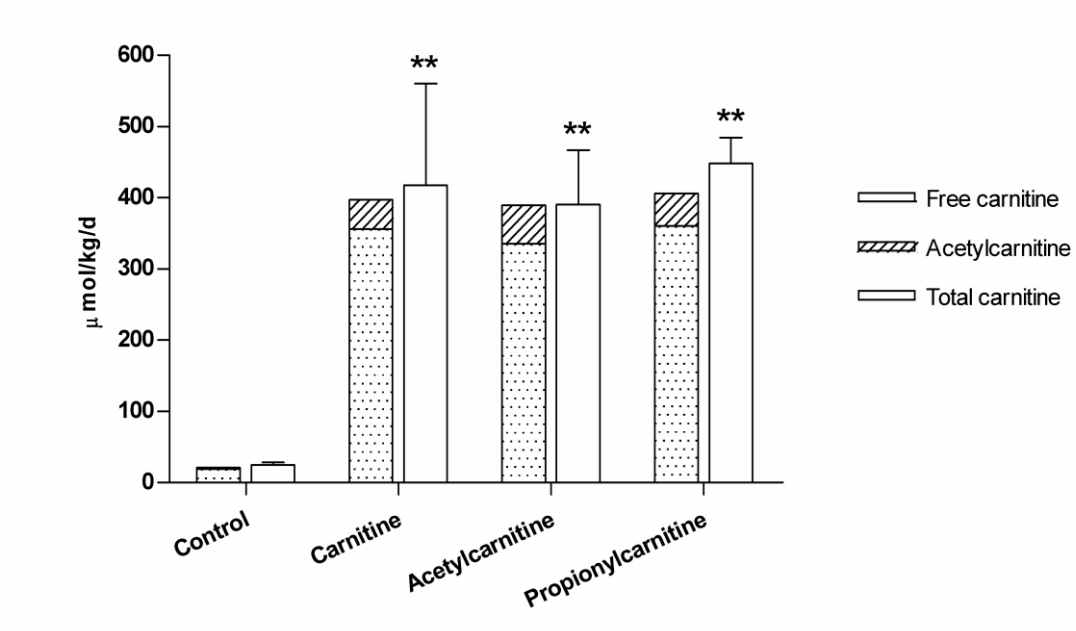

Figure 2: *Mean urinary excretion of carnitine, acetylcarnitine, and total carnitine*. Mice supplemented with carnitine, acetylcarnitine or propionylcarnitine excreted significantly higher amounts of carnitine and acetylcarnitine than the control group. Data is expressed as μmol/kg/d (mean ± SEM of 6 animals per group). **: $p<0.01$ compared to control

	CTRL	CARNITINE	ACN	PCN
Free carnitine	19±2	356±71**	335±68**	360±34**
Acetylcarnitine	2±0	41±10**	54±13**	46±6**
Propionylcarnitine	n.d.	1.0±0.3**	1.1±0.3**	3.3±1.1**
Total carnitine	24±4	417±143**	390±76**	448±36**

Table 2: *Excretion of carnitine, acetylcarnitine, propionylcarnitine, and total carnitine*. Excreted amounts are mean values ± SEM of 6 animals, given in μmol/kg/d. **: $p<0.01$ compared to control group.

Carnitine balance under resting conditions

Total carnitine input and output in control and treated animals are presented in **Table 3**. The total intake of carnitine was 200 times higher in animals supplemented with carnitine, acetylcarnitine or propionylcarnitine. Excretion, as discussed above, was 10 to 20 times higher in the treated groups as compared to the control group. The tissue accumulation was calculated from the mean total carnitine concentration in red and white muscles and from the estimated muscle weight gain during the observation period of one month. The difference was calculated by subtracting the excretion plus tissue accumulation from total intake of carnitine. In control mice, the negative difference represents mainly carnitine biosynthesis. This value is in agreement with reported values in rats obtained by a similar method (Krahenbuhl et al., 2000). In treated animals, the positive value is mainly explained by incomplete bioavailability. Bioavailability, as estimated from carnitine ingestion and urinary excretion, was between 20% and 23% without a difference between the groups supplemented with carnitine or acylcarnitines.

Treatment group (n = 6)	Intake		Excretion	Tissue accumulation	Difference
	Water	Food			
Control	0±0	9.5±0.1	31±4	0.32±0.06	-22±4
Carnitine	2072±27	9.6±0.2	472±152	0.40±0.10	1609±155
Acetylcarnitine	2254±114	9.6±0.2	451±80	0.30±0.06	1812±140
Propionylcarnitine	2259±48	9.6±0.2	501±40	0.44±0.08	1767±63

Table 3: *Total carnitine balance under resting conditions*. The difference was calculated by subtracting the excreted and accumulated amounts from the intake. Values are given in μmol/kg/d ± SEM.

High resolution respirometry

For the soleus and the gastrocnemius muscle biopsies, we observed no significant difference in oxygen uptake for the different conditions tested between the groups investigated. These findings suggest that mitochondrial content and capacity remained unchanged in the supplemented compared to the control group (**Figure 3**). As expected, the maximal respiration rate in the red soleus muscle was higher than in the white gastrocnemius.

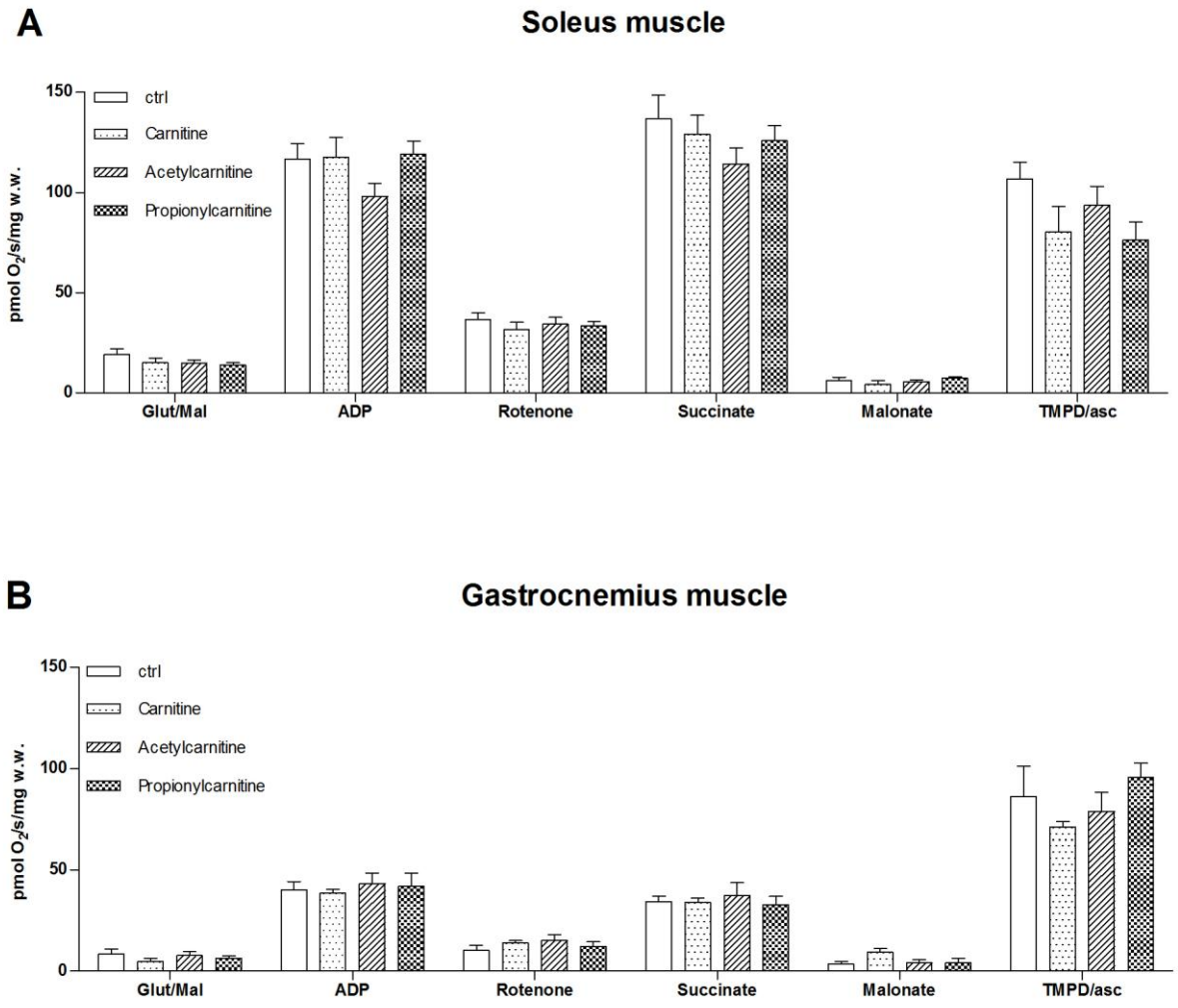

Figure 3: *Oxygen consumption by isolated muscle fibers*. We observed no differences between supplemented mice and control mice. Data is represented as mean in pmol O_2/s/mg wet weight ± SEM of six animals.

Exercise

As shown in **Figure 4**, the mean running distance of untreated mice was 561 ± 150 m (n=6). The distance covered by treated mice was by trend increased in all groups but no statistical significance was observed because of high interindividual variability (carnitine 699 ± 265 m, acetylcarnitine 772 ± 320 m, propionylcarnitine 820 m ± 525 m). The performance of the animals was in the range of 67 – 69 mW and did not differ between the groups.

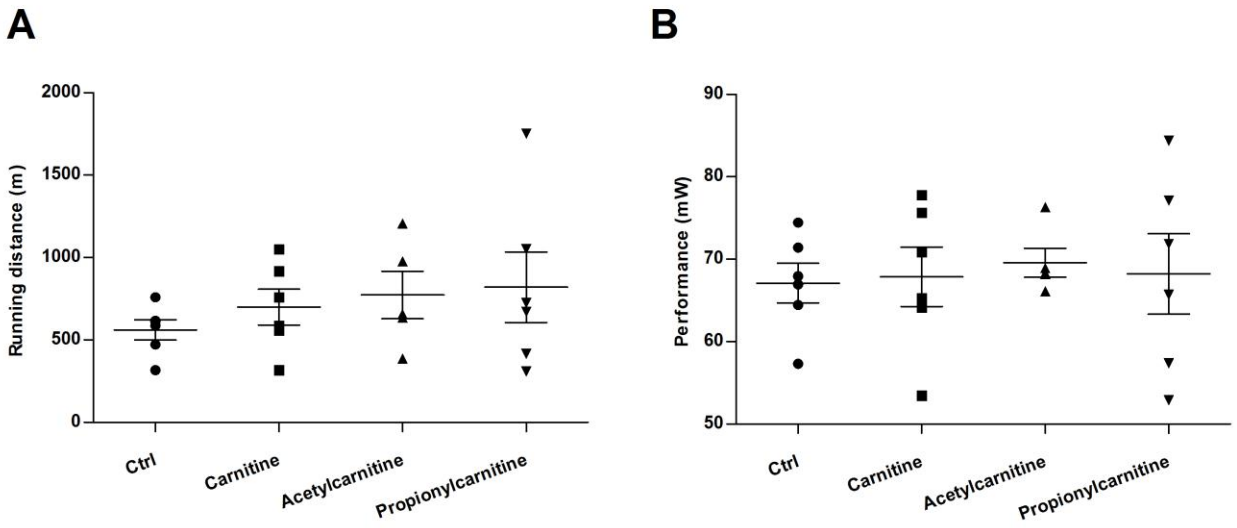

Figure 4: *Running capacity of mice completing a treadmill exercise until exhaustion*. We observed a slight upward trend for the running distance (A) in treated groups. Performance expressed in mW (B) did not differ between the groups. Mean values ± SEM of six animals are represented.

Energy parameters in plasma and skeletal muscle in resting mice and mice after exhaustive exercise

As shown in **Table 4**, plasma lactate levels increased significantly with exercise in all the groups. By trend, lactate concentrations after exercise were lower in the three supplemented groups as compared to the control group, though not reaching statistical significance. Creatine kinase activity was not different between supplemented and control animals and showed a slight elevation with exercise compared to resting conditions (results not shown).

Energy parameters were determined in separate samples of white and red quadriceps femoris (**Table 4**). The creatine and phosphocreatine concentrations were not different between supplemented and control groups and were not significantly influenced by exercise. Similarly, the skeletal muscle ATP content was not affected by exercise. There were no significant differences between control and supplemented groups, although there was a trend for a higher ATP content in red fibers from supplemented mice. The glycogen content was higher in white skeletal muscle fibers compared to red fibers. After exercise, a significant decrease of the glycogen content was observed in white fibers, while no

difference was noticed in red fibers. The glycogen content after exercise was by trend higher in treated groups, but again without reaching statistical difference.

	CTRL	CN	ACN	PCN	CTRL	CN	ACN	PCN
Red quadriceps								
	Resting conditions				Exhaustive exercise			
Creatine (μmol/kg w.w.)	25.4±1.5	23.6±1.3	28.2±0.8	25.2±2.1	25.8±1.2	26.6±1.3	24.8±1.8	28.3±1.4
Phosphocreatine (μmol/kg w.w.)	3.1±1.2	7.5±1.8	7.1±1.0	6.1±1.8	4.4±1.1	7.7±1.8	5.5±1.4	7.0±1.2
ATP (μmol/kg w.w.)	4.3±0.6	8.6±1.7	9.9±0.9	8.5±1.4	6.6±1.2	11.0±1.6	8.8±1.0	9.3±1.2
Glycogen (mmol/kg w.w.)	9.5±1.2	11.8±1.0	12.8±1.0	11.5±2.9	7.3±1.7	14.7±2.3	11.1±0.8	8.5±1.9
White quadriceps								
	Resting conditions				Exhaustive exercise			
Creatine (μmol/kg w.w.)	23.7±2.5	21.8±2.5	21.8±2.3	21.6±1.5	26.1±2.0	26.1±1.4	23.0±2.1	24.5±1.2
Phosphocreatine (μmol/kg w.w.)	11.8±2.0	12.1±0.4	10.9±2.5	10.3±2.0	8.1±1.3	7.7±2.2	12.1±0.9	9.3±1.3
ATP (μmol/kg w.w.)	8.2±1.0	9.7±1.0	9.6±0.6	9.2±0.9	9.4±1.0	9.1±2.0	10.9±1.1	9.3±0.7
Glycogen (mmol/kg w.w.)	17.0±2.9	13.6±1.5	20.9±1.7	15.1±1.4	5.6±2.0*	9.7±1.4	11.3±1.1*	12.6±2.5
Plasma								
	Resting conditions				Exhaustive exercise			
Lactate (mmol/L)	2.2±0.2	2.2±0.2	2.1±0.2	1.9±0.1	6.2±0.5**	4.8±0.3**	5.0±0.2**	5.2±0.5**

Table 4: *Effect of exercise on skeletal muscle and plasma parameters.* Mean values ± SEM of six animals are represented. **: $p<0.01$ compared to resting values, * $p<0.05$ compared to resting values.

7.5. Discussion

In this study we investigated the effects of carnitine, acetylcarnitine, and propionylcarnitine on the muscle carnitine balance and on exercise capacity of mice.

We could show that carnitine, acetylcarnitine or propionylcarnitine supplementation does not influence the skeletal muscle carnitine pool when administered orally for 4 weeks at a dose of 2 mmol/kg/d. Other animal studies also reported no effect of carnitine supplementation on the skeletal muscle carnitine content. In rats, low oral doses of carnitine or acetylcarnitine (200 μmol/kg/d) for 14 days did not increase the muscle carnitine pool (Lambert et al., 2009). In mice, a higher carnitine dose (200 mg/kg/d) for five weeks also failed to show an effect on the skeletal muscle carnitine pool (Primassin et al., 2008). The few animal studies performed in rats or mice reporting a positive effect of carnitine supplementation on the skeletal muscle carnitine pool, either used high parenteral doses during a relatively long period of time (4 mmol/kg/d during 8 weeks) (Negrao et al., 1987) or determined the carnitine content only in soleus muscle (Bacurau et al., 2003). We could not confirm this finding in deep quadriceps biopsies which mainly contained red fibers similar to the soleus muscle.

In humans, most studies failed to show an impact of carnitine supplementation on total muscle carnitine content and on performance (Barnett et al., 1994; Wachter et al., 2002). Recently, increased carnitine muscle concentrations were reported in muscle of humans treated with high amounts of carbohydrates to induce hyperinsulinemia (Wall et al., 2011). It was hypothesized that insulin enhances carnitine transport via stimulation of the Na^+/K^+ ATPase, leading to the formation of a higher Na^+ gradient to drive the sodium-dependent carnitine transporter OCTN2. In a similar study, a higher expression of OCTN2 5 hours after an infusion of carnitine and insulin was demonstrated, rendering a second possible explanation for the observed increase in the skeletal muscle carnitine content under hyperinsulinemic conditions (Stephens et al., 2006). In the study of Wall et al. the increase of the skeletal muscle carnitine content was observed only in the group treated for 24 weeks and not in the group treated for 12 weeks, suggesting that long supplementation periods are required to increase the muscle carnitine pool (Wall et al., 2011).

It has to be pointed out that the transport mechanisms of carnitine into the muscle are not fully characterized. It is established that the sodium-dependent carnitine carrier OCTN2 is expressed in skeletal muscle (Nezu et al., 1999; Spaniol et al., 2001; Stephens et al., 2006; Tamai et al., 1998; Tamai et al., 2000). On the other hand, the study of Berardi et al. (Berardi et al., 2000) using rat skeletal muscle membrane vesicles revealed carnitine transport characteristics which are not completely compatible with the function of OCTN2. It is therefore currently unclear whether the transport of carnitine into skeletal muscle can fully be explained by OCTN2 or whether additional carnitine carriers exist.

In mice, the bioavailability of low doses of carnitine (250 ng/kg) was reported to be 67% (Yokogawa et al., 1999), which is clearly higher than the values found in the current study. For acetylcarnitine and propionylcarnitine, the absorption profile in mice is not known. In humans, although it was suggested that acetylcarnitine is partly hydrolyzed in enterocytes, oral doses of acetylcarnitine increased the corresponding plasma concentrations by 40% (Rebouche, 2004). The bioavailability of propionylcarnitine has not been determined in mice, but has been estimated to be less than 20% (Mancinelli et al., 2005). Our study was not designed as a pharmacokinetic study, but nevertheless allowed to estimate some kinetic parameters of the compounds administered. As shown in **Table 3**, the bioavailability of carnitine, acetylcarnitine and propionylcarnitine is in the range of 20 – 23% irrespectively of the compound administered and based on urinary excretion of carnitine and oral ingestion. The comparison with the study of Yokogawa et al. suggests that carnitine absorption is concentration-dependent (Yokogawa et al., 1999). Furthermore, the current study indicates that orally ingested acylcarnitines undergo hydrolysis either in the intestinal tract or in the liver, since we could not detect increased concentrations of acylcarnitines in the systemic circulation. Further studies assessing the effect of acylcarnitines on the skeletal muscle carnitine content and physical performance would have to be carried out with parenteral administration.

As mentioned above, the supplemented carnitine, acetylcarnitine or propionylcarnitine were excreted mainly as carnitine and to a lesser extent as acetylcarnitine, independently of the supplemented carnitine derivative. Whereas propionylcarnitine was below detection limits in urine of control mice, it was present in the urine of all treated animals, also of the mice supplemented with carnitine and acetylcarnitine. Similarly, in humans treated with oral carnitine, elevated acetylcarnitine and propionylcarnitine were observed in plasma and urine (Cao et al., 2009). Furthermore, in patients with end-stage renal failure undergoing

hemodialysis, intravenous administration of carnitine was associated with increased plasma concentrations of acylcarnitines and increased elimination of acylcarnitines by dialysis (Vernez et al., 2006). These findings suggest that the circulating plasma carnitine is exchanged with the tissue pool, where it undergoes intensive transacetylation processes.

After exercise, we did not observe significant differences in the skeletal muscle phosphocreatine content compared with resting conditions, suggesting that exercise was not intensive enough, even though the mice were exhausted. Although the interval between end of exercise and sacrifice of the mice was in no case longer than 15 minutes, this period may have been long enough to allow at least partial recovery of the phosphocreatine pool. Exhaustive exercise is supported by the observed increase in the plasma lactate concentrations in exercising mice and in the decrease of the glycogen content in skeletal muscle.

In conclusion, oral supplementation of carnitine, acetylcarnitine or propionylcarnitine was associated with increased plasma concentrations of total carnitine and increased urinary excretion of carnitine, but did not affect the skeletal muscle carnitine content. Accordingly, physical performance and skeletal muscle energy metabolism were not affected by carnitine supplementation.

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8. CONCLUSION AND OUTLOOK

Carnitine has been the center of intensive research since its discovery in 1905. After these years, the interest about carnitine remains intact. The present work was focused on methodological aspects for the determination of carnitine and its derivatives and on functional aspects of this substance.

In the first project, we investigated the effects of valproate on carnitine homeostasis. We observed that the carnitine balance can be disturbed by exogenous factors such as valproate. In healthy individuals, this disturbance has no implication since the initial carnitine loss is compensated by a more effective renal reabsorption. The mechanisms leading to this compensation are not fully understood yet. The carnitine body pool is an elaborate equilibrium between biosynthesis, enteral absorption and renal reabsorption, as illustrated in vegetarians who compensate their low carnitine intake with efficient renal reabsorption (Lombard *et al.*, 1989). However, the fact that patients under valproate therapy do develop hypocarnitinemia in some cases as reported in the literature (Chung *et al.*, 1997; Rodriguez-Segade *et al.*, 1989), points out the complexity and subtlety of this balance. Many parameters are at stake, like genetic predispositions or concomitant pathologies, and the prevention of hypocarnitinemia and its potentially severe consequences depends on the comprehension of the involved factors.

Analytical methods to determine carnitine and its acyl-derivatives were developed parallel to the studies about the functions of carnitine. Any research depends on the robustness of the methods applied to the production of the data: no method means no data, fragile methods mean questionable data. The determination of carnitine and acylcarnitines remains a challenging task because of the endogenous nature of the analytes, their broad range of polarity and their variable physiological concentrations. We developed a method for the simultaneous analysis of carnitine and three of its derivatives, representative of the endogenous polarity range. Confronted to matrix effects, we applied the addition method for the quantification of the analytes, overcoming at the same time the problem of the ubiquity of carnitine and acylcarnitines in biological matrices.

The importance of reliable analytical methods is highlighted by their application. Carnitine profiling is well established for the new born screening of metabolic diseases such as organic acidurias or fatty acid oxidation disorders (Chace *et al.*, 2003). Certain pathologies or treatments are as well associated with specific acylcarnitines patterns. For example, a biotin deficiency can be detected by the presence of 3-hydroxyvalerylcarnitine in plasma or urine of affected subjects (Stratton *et al.*, 2010; Stratton *et al.*, 2011). Another example illustrated in the first project of this thesis is the detection of valproylcarnitine in plasma and urine samples of patients treated with this substance (Millington *et al.*, 1985). When considering these examples, the potential of carnitine profiling could go even beyond its actual utilizations. If substances like valproate or pivalate, as mentioned in the introduction, are eliminated as carnitine derivatives, it is probable that other substances with similar structures or structures convertible to CoA derivatives can be conjugated to carnitine and eliminated as such. These specific acylcarnitines are the proof of an exposure to the mother substance and their presence could be a valuable tool in toxicological screenings. Specific acylcarnitines patterns might as well be revealing of a specific disease, like organic acidurias are or another metabolic disease where the carnitine profile has not been investigated yet. Sensitive and reliable analytical methods are indispensable to exploit this potential.

The main roles of carnitine in bioenergetics have been recognized last century (Brass *et al.*, 1980; Childress *et al.*, 1967; Fritz, 1955). Carnitine ensures normal physiological functions as carrier of long-chain fatty acids and as regulator of the acetyl-CoA/CoASH ratio. The utility of carnitine supplementation to enhance physical activity is controversial. Surely carnitine does not harm. With a lethal dose of ~ 8 g/kg for a mouse (Wolff *et al.*, 1971), higher than kitchen salt (Boyd *et al.*, 1963), no cases of carnitine intoxication have been reported so far. But does it positively influence muscle metabolism, particularly in situations where energy metabolism is impaired such as in diabetic or obese patients? Considering the fact that *ex vivo* experiments support a beneficial effect of carnitine (Brass *et al.*, 1993), the question seems rather to be how the muscle could incorporate more than it already contains. As shown in our *in vivo* animal project, oral supplementation of short-chain acylcarnitines seems not to be the answer to that question. Only low concentrations of acetylcarnitine and propionylcarnitine reach the plasma compartment when supplemented in drinking water, with no influence on muscle content. By avoiding incomplete reabsorption, a parenteral application of these acylcarnitines would enhance the plasma

concentrations and the probability of entering the muscle. Other carnitine “prodrugs” or modified pharmaceutical applications might also be considered to increase carnitine muscle content.

Another key for the penetration of carnitine into the muscle relies on a better understanding of the transport mechanisms of carnitine into the muscle and the regulation of the transporters involved. The presence of OCTN2 on the sarcolemmal membrane is undeniable but this transporter alone cannot account for all carnitine movements in and out of the muscle. This is illustrated by the fact that patients with systemic carnitine deficiency can benefit from a carnitine supplementation even though they do not possess a functional OCTN2. Multi-component transport systems have been described in cultured muscle cells and in muscle vesicles (Berardi *et al.*, 2000; Martinuzzi *et al.*, 1991). Whereas OCTN2 expression seems to be influenced by insulin circulating concentrations (Stephens *et al.*, 2006; Wall *et al.*, 2011), the situation regarding other transporters and their regulation is vague, leaving the way open for further research.

In conclusion, research about carnitine and its derivatives still has a bright future lying ahead. This tiny molecule will keep fascinating through its multiple facets and its invaluable involvement in our proper functioning. This thesis wanted to bring a modest contribution to the carnitine research, however raising just as many questions, if not more, as it answered.

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10. REFERENCES

Alesci, S., De Martino, M.U., Mirani, M., Benvenga, S., Trimarchi, F., Kino, T. & Chrousos, G.P. (2003). L-carnitine: A nutritional modulator of glucocorticoid receptor functions. *Faseb J*, **17,** 1553-5.

Angelini, C., Govoni, E., Bragaglia, M.M. & Vergani, L. (1978). Carnitine deficiency: acute postpartum crisis. *Ann Neurol*, **4,** 558-61.

Arduini, A., Mancinelli, G., Radatti, G.L., Dottori, S., Molajoni, F. & Ramsay, R.R. (1992). Role of carnitine and carnitine palmitoyltransferase as integral components of the pathway for membrane phospholipid fatty acid turnover in intact human erythrocytes. *J Biol Chem*, **267,** 12673-81.

Bacurau, R.F., Navarro, F., Bassit, R.A., Meneguello, M.O., Santos, R.V., Almeida, A.L. & Costa Rosa, L.F. (2003). Does exercise training interfere with the effects of L-carnitine supplementation? *Nutrition*, **19,** 337-41.

Barnett, C., Costill, D.L., Vukovich, M.D., Cole, K.J., Goodpaster, B.H., Trappe, S.W. & Fink, W.J. (1994). Effect of L-carnitine supplementation on muscle and blood carnitine content and lactate accumulation during high-intensity sprint cycling. *Int J Sport Nutr*, **4,** 280-8.

Benvenga, S., Amato, A., Calvani, M. & Trimarchi, F. (2004). Effects of carnitine on thyroid hormone action. *Ann N Y Acad Sci*, **1033,** 158-67.

Berardi, S., Stieger, B., Hagenbuch, B., Carafoli, E. & Krahenbuhl, S. (2000). Characterization of L-carnitine transport into rat skeletal muscle plasma membrane vesicles. *Eur J Biochem*, **267,** 1985-94.

Beydoun, A., Sackellares, J.C. & Shu, V. (1997). Safety and efficacy of divalproex sodium monotherapy in partial epilepsy: a double-blind, concentration-response design clinical trial. Depakote Monotherapy for Partial Seizures Study Group. *Neurology*, **48,** 182-8.

Bhuiyan, A.K., Watmough, N.J., Turnbull, D.M., Aynsley-Green, A., Leonard, J.V. & Bartlett, K. (1987). A new simple screening method for the diagnosis of medium chain acyl-CoA dehydrogenase deficiency. *Clin Chim Acta*, **165,** 39-44.

Bieber, L.L. (1988). Carnitine. *Annu Rev Biochem*, **57,** 261-83.

Bieber, L.L. & Choi, Y.R. (1977). Isolation and identification of aliphatic short-chain acylcarnitines from beef heart: possible role for carnitine in branched-chain amino acid metabolism. *Proc Natl Acad Sci U S A*, **74,** 2795-8.

Bohles, H., Richter, K., Wagner-Thiessen, E. & Schafer, H. (1982). Decreased serum carnitine in valproate induced Reye syndrome. *Eur J Pediatr*, **139,** 185-6.

Bohmer, T., Rydning, A. & Solberg, H.E. (1974). Carnitine levels in human serum in health and disease. *Clin Chim Acta*, **57,** 55-61.

Bond, D.J., Lam, R.W. & Yatham, L.N. (2010). Divalproex sodium versus placebo in the treatment of acute bipolar depression: a systematic review and meta-analysis. *J Affect Disord*, **124,** 228-34.

Bonnefont, J.P., Djouadi, F., Prip-Buus, C., Gobin, S., Munnich, A. & Bastin, J. (2004). Carnitine palmitoyltransferases 1 and 2: biochemical, molecular and medical aspects. *Mol Aspects Med*, **25,** 495-520.

Boyd, E.M. & Shanas, M.N. (1963). The Acute Oral Toxicity of Sodium Chloride. *Arch Int Pharmacodyn Ther*, **144,** 86-96.

Braida, L., Crovella, S., Boniotto, M., Luchesi, A., de Vonderweid, U., Casetta, B. & Amoroso, A. (2001). A rapid and quantitative mass spectrometry method for determining the concentration of acylcarnitines and aminoacids in amniotic fluid. *Prenat Diagn*, **21,** 543-6.

Brass, E.P. (2004). Carnitine and sports medicine: use or abuse? *Ann N Y Acad Sci*, **1033,** 67-78.

Brass, E.P. (1995). Pharmacokinetic considerations for the therapeutic use of carnitine in hemodialysis patients. *Clin Ther*, **17,** 176-85; discussion 175.

Brass, E.P. (2002). Pivalate-generating prodrugs and carnitine homeostasis in man. *Pharmacol Rev*, **54,** 589-98.

Brass, E.P. (2000). Supplemental carnitine and exercise. *Am J Clin Nutr*, **72,** 618S-23S.

Brass, E.P. & Hoppel, C.L. (1978). Carnitine metabolism in the fasting rat. *J Biol Chem*, **253,** 2688-93.

Brass, E.P. & Hoppel, C.L. (1980). Relationship between acid-soluble carnitine and coenzyme A pools in vivo. *Biochem J*, **190,** 495-504.

Brass, E.P., Scarrow, A.M., Ruff, L.J., Masterson, K.A. & Van Lunteren, E. (1993). Carnitine delays rat skeletal muscle fatigue in vitro. *J Appl Physiol*, **75,** 1595-600.

Bremer, J. (1983). Carnitine--metabolism and functions. *Physiol Rev*, **63,** 1420-80.

Briand, G., Fontaine, M., Schubert, R., Ricart, G., Degand, P. & Vamecq6, J. (1995). Direct analysis by electrospray ionization and matrix-assisted laser desorption ionization mass spectrometry of standard and urinary acylcarnitines. Comparison with fast atom bombardment and gas chromatography chemical ionization mass spectrometry. *Journal of Mass Spectrometry*, **30,** 1731-1741.

Cao, Q.R., Ren, S., Park, M.J., Choi, Y.J. & Lee, B.J. (2007). Determination of highly soluble L-carnitine in biological samples by reverse phase high performance liquid chromatography with fluorescent derivatization. *Arch Pharm Res*, **30,** 1041-6.

Cao, Y., Qu, H.J., Li, P., Wang, C.B., Wang, L.X. & Han, Z.W. (2011). Single dose administration of L-carnitine improves antioxidant activities in healthy subjects. *Tohoku J Exp Med*, **224,** 209-13.

Cao, Y., Wang, Y.X., Liu, C.J., Wang, L.X., Han, Z.W. & Wang, C.B. (2009). Comparison of pharmacokinetics of L-carnitine, acetyl-L-carnitine and propionyl-L-carnitine after single oral administration of L-carnitine in healthy volunteers. *Clin Invest Med*, **32,** E13-9.

Cederblad, G., Fahraeus, L. & Lindgren, K. (1986). Plasma carnitine and renal-carnitine clearance during pregnancy. *Am J Clin Nutr*, **44,** 379-83.

Cederblad, G., Larsson, J., H, N. & Schildt, B. (1980). Urinary excretion of carnitine in burned patients. *Burns*, **8,** 102-109.

Cederblad, G. & Lindstedt, S. (1972). A method for the determination of carnitine in the picomole range. *Clin Chim Acta*, **37,** 235-43.

Chace, D.H., Hillman, S.L., Van Hove, J.L. & Naylor, E.W. (1997). Rapid diagnosis of MCAD deficiency: quantitative analysis of octanoylcarnitine and other acylcarnitines in newborn blood spots by tandem mass spectrometry. *Clin Chem*, **43,** 2106-13.

Chace, D.H., Kalas, T.A. & Naylor, E.W. (2003). Use of tandem mass spectrometry for multianalyte screening of dried blood specimens from newborns. *Clin Chem*, **49,** 1797-817.

Chalcraft, K.R. & Britz-McKibbin, P. (2009). Newborn screening of inborn errors of metabolism by capillary electrophoresis-electrospray ionization-mass spectrometry: a second-tier method with improved specificity and sensitivity. *Anal Chem*, **81,** 307-14.

Chalmers, R.A., Stacey, T.E., Tracey, B.M., de Sousa, C., Roe, C.R., Millington, D.S. & Hoppel, C.L. (1984). L-Carnitine insufficiency in disorders of organic acid metabolism: response to L-carnitine by patients with methylmalonic aciduria and 3-hydroxy-3-methylglutaric aciduria. *J Inherit Metab Dis*, **7 Suppl 2,** 109-10.

Chau, C.H., Rixe, O., McLeod, H. & Figg, W.D. (2008). Validation of analytic methods for biomarkers used in drug development. *Clin Cancer Res*, **14,** 5967-76.

Childress, C.C., Sacktor, B. & Traynor, D.R. (1967). Function of carnitine in the fatty acid oxidase-deficient insect flight muscle. *J Biol Chem*, **242,** 754-60.

Chung, S., Choi, J., Hyun, T., Rha, Y. & Bae, C. (1997). Alterations in the carnitine metabolism in epileptic children treated with valproic acid. *J Korean Med Sci*, **12,** 553-8.

Clayton, P.T., Doig, M., Ghafari, S., Meaney, C., Taylor, C., Leonard, J.V., Morris, M. & Johnson, A.W. (1998). Screening for medium chain acyl-CoA dehydrogenase deficiency using electrospray ionisation tandem mass spectrometry. *Arch Dis Child*, **79,** 109-15.

Constantin-Teodosiu, D., Carlin, J.I., Cederblad, G., Harris, R.C. & Hultman, E. (1991). Acetyl group accumulation and pyruvate dehydrogenase activity in human muscle during incremental exercise. *Acta Physiol Scand*, **143,** 367-72.

Constantin-Teodosiu, D., Howell, S. & Greenhaff, P.L. (1996). Carnitine metabolism in human muscle fiber types during submaximal dynamic exercise. *J Appl Physiol*, **80,** 1061-4.

Coppola, G., Epifanio, G., Auricchio, G., Federico, R.R., Resicato, G. & Pascotto, A. (2006). Plasma free carnitine in epilepsy children, adolescents and young adults treated with old and new antiepileptic drugs with or without ketogenic diet. *Brain Dev*, **28,** 358-65.

Cuperus, R., Leen, R., Tytgat, G.A., Caron, H.N. & van Kuilenburg, A.B. (2010). Fenretinide induces mitochondrial ROS and inhibits the mitochondrial respiratory chain in neuroblastoma. *Cell Mol Life Sci*, **67,** 807-16.

D'Amico, D. (2010). Pharmacological prophylaxis of chronic migraine: a review of double-blind placebo-controlled trials. *Neurol Sci*, **31 Suppl 1,** S23-8.

Dalakas, M.C., Leon-Monzon, M.E., Bernardini, I., Gahl, W.A. & Jay, C.A. (1994). Zidovudine-induced mitochondrial myopathy is associated with muscle carnitine deficiency and lipid storage. *Ann Neurol*, **35,** 482-7.

De Witt, P., Deias, R., Muck, S., Galletti, B., Meloni, D., Celletti, P. & Marzo, A. (1994). High-performance liquid chromatography and capillary electrophoresis of L- and D-carnitine by precolumn diastereomeric derivatization. *J Chromatogr B Biomed Appl*, **657,** 67-73.

DeVane, C.L. (2003). Pharmacokinetics, drug interactions, and tolerability of valproate. *Psychopharmacol Bull*, **37 Suppl 2,** 25-42.

Donnelly, C.T., Hameed, A.B., Abdenur, J.E. & Wing, D.A. (2007). Carnitine deficiency in pregnancy. *Obstet Gynecol*, **110,** 480-2.

Dubelaar, M.L., Lucas, C.M. & Hulsmann, W.C. (1991). Acute effect of L-carnitine on skeletal muscle force tests in dogs. *Am J Physiol*, **260,** E189-93.

Duranti, G., Boenzi, S., Rizzo, C., Rava, L., Di Ciommo, V., Carrozzo, R., Meschini, M.C., Johnson, D.W. & Dionisi-Vici, C. (2008). Urine acylcarnitine analysis by ESI-MS/MS: a new tool for the diagnosis of peroxisomal biogenesis disorders. *Clin Chim Acta*, **398,** 86-9.

Eaton, S. & Pierro, A. (2005). Carnitine and Fatty Acid Oxidation in Sepsis. *Monatshefte Chemie*, **136,** 1483-1492.

El-Hattab, A.W., Li, F.Y., Shen, J., Powell, B.R., Bawle, E.V., Adams, D.J., Wahl, E., Kobori, J.A., Graham, B., Scaglia, F. & Wong, L.J. (2010). Maternal systemic primary carnitine deficiency uncovered by newborn screening: clinical, biochemical, and molecular aspects. *Genet Med*, **12,** 19-24.

Engel, A.G. & Angelini, C. (1973). Carnitine deficiency of human skeletal muscle with associated lipid storage myopathy: a new syndrome. *Science*, **179,** 899-902.

Eubanks, A.L., Aguirre, B. & Bourgeois, J.A. (2008). Severe acute hyperammonemia after brief exposure to valproate. *Psychosomatics*, **49,** 82-3.

Evans, A.M. & Fornasini, G. (2003). Pharmacokinetics of L-carnitine. *Clin Pharmacokinet*, **42,** 941-67.

Famularo, G., De Simone, C., Trinchieri, V. & Mosca, L. (2004). Carnitines and its congeners: a metabolic pathway to the regulation of immune response and inflammation. *Ann N Y Acad Sci*, **1033,** 132-8.

Farkas, V., Bock, I., Cseko, J. & Sandor, A. (1996). Inhibition of carnitine biosynthesis by valproic acid in rats--the biochemical mechanism of inhibition. *Biochem Pharmacol*, **52,** 1429-33.

FDA, U. (2001). *Guidance for Industry: Bioanalytical Method Validation*. Rockville, MD, USA.

Fingerhut, R., Roschinger, W., Muntau, A.C., Dame, T., Kreischer, J., Arnecke, R., Superti-Furga, A., Troxler, H., Liebl, B., Olgemoller, B. & Roscher, A.A. (2001). Hepatic carnitine palmitoyltransferase I deficiency: acylcarnitine profiles in blood spots are highly specific. *Clin Chem*, **47,** 1763-8.

Fortin, G., Yurchenko, K., Collette, C., Rubio, M., Villani, A.C., Bitton, A., Sarfati, M. & Franchimont, D. (2009). L-carnitine, a diet component and organic cation transporter OCTN ligand, displays immunosuppressive properties and abrogates intestinal inflammation. *Clin Exp Immunol*, **156,** 161-71.

Fraenkel, G. (1948). Bt, a new vitamin of the B-group and its relation to the folic acid group, and other anti-anaemia factors. *Nature*, **161,** 981-3.

Fraenkel, G. (1954). The distribution of vitamin BT (carnitine) throughout the animal kingdom. *Arch Biochem Biophys*, **50,** 486-95.

Friedman, S. (1958). Determination of carnitine in biological materials. *Arch Biochem Biophys*, **75,** 24-30.

Friolet, R., Hoppeler, H. & Krahenbuhl, S. (1994). Relationship between the coenzyme A and the carnitine pools in human skeletal muscle at rest and after exhaustive exercise under normoxic and acutely hypoxic conditions. *J Clin Invest*, **94,** 1490-5.

Fritz, I. (1955). The effect of muscle extracts on the oxidation of palmitic acid by liver slices and homogenates. *Acta Physiol Scand*, **34,** 367-85.

Fritz, I.B. & Marquis, N.R. (1965). The role of acylcarnitine esters and carnitine palmityltransferase in the transport of fatty acyl groups across mitochondrial membranes. *Proc Natl Acad Sci U S A*, **54,** 1226-33.

Galloway, S. & Broad, E. (2005). Oral L-Carnitine Supplementation and Exercise Metabolism. *Monatshefte Chemie*, **136,** 1391-1410.

Genuth, S.M. & Hoppel, C.L. (1981). Acute hormonal effects on carnitine metabolism in thin and obese subjects: responses to somatostatin, glucagon, and insulin. *Metabolism*, **30,** 393-401.

Ghoshal, A.K., Guo, T., Soukhova, N. & Soldin, S.J. (2005). Rapid measurement of plasma acylcarnitines by liquid chromatography-tandem mass spectrometry without derivatization. *Clin Chim Acta*, **358,** 104-12.

Greenhaff, P.L., Campbell-O'Sullivan, S.P., Constantin-Teodosiu, D., Poucher, S.M., Roberts, P.A. & Timmons, J.A. (2002). An acetyl group deficit limits mitochondrial ATP production at the onset of exercise. *Biochem Soc Trans*, **30,** 275-80.

Groot, P., Scholte, H. & Hülsmann, W. (1976). Fatty acid activation: specificity, localization, and function. *Hoppe-Seyler's Zeitschrift für physiologische Chemie*, **14,** 75-126.

Gulewitsch, W. & Krimberg, R. (1905). Zur Kenntnis der Extraktionsstoffe der Muskeln. 2. Mitteilung über das Carnitin. *Hoppe-Seylers Zeitschrift für physiologische Chemie*, **45**.

Hamed, S.A. & Abdella, M.M. (2009). The risk of asymptomatic hyperammonemia in children with idiopathic epilepsy treated with valproate: Relationship to blood carnitine status. *Epilepsy Res*.

Harris, R.C., Hultman, E. & Nordesjo, L.O. (1974). Glycogen, glycolytic intermediates and high-energy phosphates determined in biopsy samples of musculus quadriceps femoris of man at rest. Methods and variance of values. *Scand J Clin Lab Invest*, **33,** 109-20.

Haschke, M., Vitins, T., Lude, S., Todesco, L., Novakova, K., Herrmann, R. & Krahenbuhl, S. (2010). Urinary excretion of carnitine as a marker of proximal tubular damage associated with platin-based antineoplastic drugs. *Nephrol Dial Transplant*, **25,** 426-33.

Hawkins, J.M., Jones, W.E., Bonner, F.W. & Gibson, G.G. (1987). The effect of peroxisome proliferators on microsomal, peroxisomal, and mitochondrial enzyme activities in the liver and kidney. *Drug Metab Rev*, **18,** 441-515.

Heinig, K. & Henion, J. (1999). Determination of carnitine and acylcarnitines in biological samples by capillary electrophoresis-mass spectrometry. *J Chromatogr B Biomed Sci Appl*, **735,** 171-88.

Heuberger, W., Berardi, S., Jacky, E., Pey, P. & Krahenbuhl, S. (1998). Increased urinary excretion of carnitine in patients treated with cisplatin. *Eur J Clin Pharmacol*, **54,** 503-8.

Hirose, S., Mitsudome, A., Yasumoto, S., Ogawa, A., Muta, Y. & Tomoda, Y. (1998). Valproate therapy does not deplete carnitine levels in otherwise healthy children. *Pediatrics*, **101,** E9.

Hoppel, C.L., Brass, E.P., Gibbons, A.P. & Turkaly, J.S. (1986). Separation of acylcarnitines from biological samples using high-performance liquid chromatography. *Anal Biochem*, **156,** 111-7.

Horie, S. & Suga, T. (1985). Enhancement of peroxisomal beta-oxidation in the liver of rats and mice treated with valproic acid. *Biochem Pharmacol*, **34,** 1357-62.

Huang, Z.H., Gage, D.A., Bieber, L.L. & Sweeley, C.C. (1991). Analysis of acylcarnitines as their N-demethylated ester derivatives by gas chromatography-chemical ionization mass spectrometry. *Anal Biochem*, **199,** 98-105.

Hultman, E., Greenhaff, P.L., Ren, J.M. & Soderlund, K. (1991). Energy metabolism and fatigue during intense muscle contraction. *Biochem Soc Trans*, **19,** 347-53.

Indiveri, C., Iacobazzi, V., Tonazzi, A., Giangregorio, N., Infantino, V., Convertini, P., Console, L. & Palmieri, F. (2011). The mitochondrial carnitine/acylcarnitine carrier: function, structure and physiopathology. *Mol Aspects Med*, **32,** 223-33.

Inoue, F., Terada, N., Nakajima, H., Okochi, M., Kodo, N., Kizaki, Z., Kinugasa, A. & Sawada, T. (1999). Effect of sports activity on carnitine metabolism. Measurement of free carnitine, gamma-butyrobetaine and acylcarnitines by tandem mass spectrometry. *J Chromatogr B Biomed Sci Appl*, **731,** 83-8.

Jakobs, B.S. & Wanders, R.J. (1995). Fatty acid beta-oxidation in peroxisomes and mitochondria: the first, unequivocal evidence for the involvement of carnitine in shuttling propionyl-CoA from peroxisomes to mitochondria. *Biochem Biophys Res Commun*, **213,** 1035-41.

Jeppesen, J. & Kiens, B. (2012). Regulation and limitations to fatty acid oxidation during exercise. *J Physiol*, **590,** 1059-68.

Johnson, D.W. (1999). Inaccurate measurement of free carnitine by the electrospray tandem mass spectrometry screening method for blood spots. *J Inherit Metab Dis*, **22,** 201-2.

Kamimori, H., Hamashima, Y. & Konishi, M. (1994). Determination of carnitine and saturated-acyl group carnitines in human urine by high-performance liquid chromatography with fluorescence detection. *Anal Biochem*, **218,** 417-24.

Karpati, G., Carpenter, S., Engel, A.G., Watters, G., Allen, J., Rothman, S., Klassen, G. & Mamer, O.A. (1975). The syndrome of systemic carnitine deficiency. Clinical, morphologic, biochemical, and pathophysiologic features. *Neurology*, **25,** 16-24.

Kato, Y., Sugiura, M., Sugiura, T., Wakayama, T., Kubo, Y., Kobayashi, D., Sai, Y., Tamai, I., Iseki, S. & Tsuji, A. (2006). Organic cation/carnitine transporter OCTN2 (Slc22a5) is responsible for carnitine transport across apical membranes of small intestinal epithelial cells in mouse. *Mol Pharmacol*, **70,** 829-37.

Kerner, J. & Bieber, L.L. (1983). A radioisotopic-exchange method for quantitation of short-chain (acid-soluble) acylcarnitines. *Anal Biochem*, **134,** 459-66.

Kerner, J. & Hoppel, C. (2000). Fatty acid import into mitochondria. *Biochim Biophys Acta*, **1486,** 1-17.

Kiessig, S. & Vogt, C. (1997). Separation of carnitine and acylcarnitines by capillary electrophoresis. *Journal of Chromatography A*, **781,** 475-479.

Koepsell, H., Lips, K. & Volk, C. (2007). Polyspecific organic cation transporters: structure, function, physiological roles, and biopharmaceutical implications. *Pharm Res*, **24,** 1227-51.

Koizumi, T., Nikaido, H., Hayakawa, J., Nonomura, A. & Yoneda, T. (1988). Infantile disease with microvesicular fatty infiltration of viscera spontaneously occurring in the C3H-H-2(0) strain of mouse with similarities to Reye's syndrome. *Lab Anim*, **22,** 83-7.

Krähenbühl, S. (1996). Carnitine metabolism in chronic liver disease. *Life Sci*, **59,** 1579-99.

Krahenbuhl, S., Brass, E.P. & Hoppel, C.L. (2000). Decreased carnitine biosynthesis in rats with secondary biliary cirrhosis. *Hepatology*, **31,** 1217-23.

Krahenbuhl, S. & Reichen, J. (1997). Carnitine metabolism in patients with chronic liver disease. *Hepatology*, **25,** 148-53.

Krimberg, R. (1908). Zur Kenntnis der Extraktivstoffe der Muskeln. X. Mitteilung. Über die Identität des Novains mit dem Carnitin. *Hoppe-Seyler's Zeitschrift für physiologische Chemie*, **55,** 466-480.

Kuntzer, T., Reichmann, H., Bogousslavsky, J. & Regli, F. (1990). Emetine-induced myopathy and carnitine deficiency. *J Neurol*, **237,** 495-6.

Kutscher, F. (1905). *Z. Untersuch Nähr. u. Genussm.*, **10,** 528-537.

Kuwajima, M., Kono, N., Horiuchi, M., Imamura, Y., Ono, A., Inui, Y., Kawata, S., Koizumi, T., Hayakawa, J., Saheki, T. & et al. (1991). Animal model of systemic carnitine deficiency: analysis in C3H-H-2 degrees strain of mouse associated with juvenile visceral steatosis. *Biochem Biophys Res Commun*, **174,** 1090-4.

Lambert, B.D., Dobson, C.M., Cherry, N.M. & Sanderford, M.G. (2009). Chemical form of dietary L-carnitine affects plasma but not tissue carnitine concentrations in male Sprague-Dawley rats. *J Anim Physiol Anim Nutr (Berl)*, **93,** 174-80.

Lamhonwah, A.M., Ackerley, C.A., Tilups, A., Edwards, V.D., Wanders, R.J. & Tein, I. (2005). OCTN3 is a mammalian peroxisomal membrane carnitine transporter. *Biochem Biophys Res Commun*, **338,** 1966-72.

Lee, K., Kerner, J. & Hoppel, C.L. (2011). Mitochondrial carnitine palmitoyltransferase 1a (CPT1a) is part of an outer membrane fatty acid transfer complex. *J Biol Chem*, **286,** 25655-62.

Lerner, A., Gruener, N. & Iancu, T.C. (1993). Serum carnitine concentrations in coeliac disease. *Gut*, **34,** 933-5.

Lewin, L.M., Peshin, A. & Sklarz, B. (1975). A gas chromatographic assay for carnitine. *Anal Biochem*, **68,** 531-6.

Li, S.F.Y. (1992). Capillary Electrophoresis - Principles, Practice and Applications. *Journal of Chromatography Library*, **52**.

Liu, Z., Mutlib, A.E., Wang, J. & Talaat, R.E. (2008). Liquid chromatography/mass spectrometry determination of endogenous plasma acetyl and palmitoyl carnitines as potential biomarkers of beta-oxidation in mice. *Rapid Commun Mass Spectrom*, **22,** 3434-42.

Lombard, K.A., Olson, A.L., Nelson, S.E. & Rebouche, C.J. (1989). Carnitine status of lactoovovegetarians and strict vegetarian adults and children. *Am J Clin Nutr*, **50,** 301-6.

Longo, A., Bruno, G., Curti, S., Mancinelli, A. & Miotto, G. (1996). Determination of L-carnitine, acetyl-L-carnitine and propionyl-L-carnitine in human plasma by high-performance

liquid chromatography after pre-column derivatization with 1-aminoanthracene. *J Chromatogr B Biomed Appl*, **686,** 129-39.

Lowes, S. & Rose, M.E. (1990). Simple and unambiguous method for identifying urinary acylcarnitines using gas chromatography--mass spectrometry. *Analyst*, **115,** 511-6.

Madiraju, P., Pande, S.V., Prentki, M. & Madiraju, S.R. (2009). Mitochondrial acetylcarnitine provides acetyl groups for nuclear histone acetylation. *Epigenetics*, **4,** 399-403.

Maeda, T., Wakasawa, T., Funabashi, M., Fukushi, A., Fujita, M., Motojima, K. & Tamai, I. (2008). Regulation of Octn2 transporter (SLC22A5) by peroxisome proliferator activated receptor alpha. *Biol Pharm Bull*, **31,** 1230-6.

Maeda, Y., Ito, T., Suzuki, A., Kurono, Y., Ueta, A., Yokoi, K., Sumi, S., Togari, H. & Sugiyama, N. (2007). Simultaneous quantification of acylcarnitine isomers containing dicarboxylic acylcarnitines in human serum and urine by high-performance liquid chromatography/electrospray ionization tandem mass spectrometry. *Rapid Commun Mass Spectrom*, **21,** 799-806.

Mancinelli, A., Evans, A.M., Nation, R.L. & Longo, A. (2005). Uptake of L-carnitine and its short-chain ester propionyl-L-carnitine in the isolated perfused rat liver. *J Pharmacol Exp Ther*, **315,** 118-24.

Mardones, C., Rios, A., Valcarcel, M. & Cicciarelli, R. (1999). Enantiomeric separation of D- and L-carnitine by integrating on-line derivatization with capillary zone electrophoresis. *J Chromatogr A*, **849,** 609-16.

Marquis, N.R. & Fritz, I.B. (1964). Enzymological Determination of Free Carnitine Concentrations in Rat Tissues. *J Lipid Res*, **5,** 184-7.

Marson, A.G., Al-Kharusi, A.M., Alwaidh, M., Appleton, R., Baker, G.A., Chadwick, D.W., Cramp, C., Cockerell, O.C., Cooper, P.N., Doughty, J., Eaton, B., Gamble, C., Goulding, P.J., Howell, S.J., Hughes, A., Jackson, M., Jacoby, A., Kellett, M., Lawson, G.R., Leach, J.P., Nicolaides, P., Roberts, R., Shackley, P., Shen, J., Smith, D.F., Smith, P.E., Smith, C.T., Vanoli, A. & Williamson, P.R. (2007). The SANAD study of effectiveness of valproate, lamotrigine, or topiramate for generalised and unclassifiable epilepsy: an unblinded randomised controlled trial. *Lancet*, **369,** 1016-26.

Martinuzzi, A., Vergani, L., Rosa, M. & Angelini, C. (1991). L-carnitine uptake in differentiating human cultured muscle. *Biochim Biophys Acta*, **1095,** 217-22.

Marzo, A., Arrigoni Martelli, E., Mancinelli, A., Cardace, G., Corbelletta, C., Bassani, E. & Solbiati, M. (1991). Protein binding of L-carnitine family components. *Eur J Drug Metab Pharmacokinet*, **Spec No 3,** 364-8.

Matsuda, K., Yuasa, H. & Watanabe, J. (1998). Fractional absorption of L-carnitine after oral administration in rats: evaluation of absorption site and dose dependency. *Biol Pharm Bull*, **21,** 752-5.

Matsumoto, K., Ichitania, Y., Ogasawaraa, N., Yukia, H. & Imai, K. (1994). Precolumn fluorescence derivatization of carnitine and acylcarnitines with 4-(2-aminoethylamino)-7-nitro-2,1,3-benzoxadiazole prior to high-performance liquid chromatography. *Journal of Chromatography A*, **678** 241–247.

Matsumoto, K., Yamada, Y., Takahashi, M., Todoroki, T., Mizoguchi, K., Misaki, H. & Yuki, H. (1990). Fluorometric determination of carnitine in serum with immobilized carnitine dehydrogenase and diaphorase. *Clin Chem*, **36,** 2072-6.

McGarry, J.D. & Foster, D.W. (1976). An improved and simplified radioisotopic assay for the determination of free and esterified carnitine. *J Lipid Res*, **17,** 277-81.

McGarry, J.D., Mills, S.E., Long, C.S. & Foster, D.W. (1983). Observations on the affinity for carnitine, and malonyl-CoA sensitivity, of carnitine palmitoyltransferase I in animal and human tissues. Demonstration of the presence of malonyl-CoA in non-hepatic tissues of the rat. *Biochem J*, **214,** 21-8.

Melegh, B., Kerner, J. & Bieber, L.L. (1987). Pivampicillin-promoted excretion of pivaloylcarnitine in humans. *Biochem Pharmacol*, **36,** 3405-9.

Millington, D.S., Bohan, T.P., Roe, C.R., Yergey, A.L. & Liberato, D.J. (1985). Valproylcarnitine: a novel drug metabolite identified by fast atom bombardment and thermospray liquid chromatography-mass spectrometry. *Clin Chim Acta*, **145,** 69-76.

Millington, D.S., Kodo, N., Norwood, D.L. & Roe, C.R. (1990). Tandem mass spectrometry: a new method for acylcarnitine profiling with potential for neonatal screening for inborn errors of metabolism. *J Inherit Metab Dis*, **13,** 321-4.

Millington, D.S., Norwood, D.L., Kodo, N., Roe, C.R. & Inoue, F. (1989). Application of fast atom bombardment with tandem mass spectrometry and liquid chromatography/mass spectrometry to the analysis of acylcarnitines in human urine, blood, and tissue. *Anal Biochem*, **180,** 331-9.

Millington, D.S., Roe, C.R. & Maltby, D.A. (1984). Application of high resolution fast atom bombardment and constant B/E ratio linked scanning to the identification and analysis of acylcarnitines in metabolic disease. *Biomed Mass Spectrom*, **11,** 236-41.

Minkler, P.E., Brass, E.P., Hiatt, W.R., Ingalls, S.T. & Hoppel, C.L. (1995). Quantification of carnitine, acetylcarnitine, and total carnitine in tissues by high-performance liquid chromatography: the effect of exercise on carnitine homeostasis in man. *Anal Biochem*, **231,** 315-22.

Minkler, P.E., Ingalls, S.T. & Hoppel, C.L. (1990). High-performance liquid chromatographic separation of acylcarnitines following derivatization with 4'-bromophenacyl trifluoromethanesulfonate. *Anal Biochem*, **185,** 29-35.

Minkler, P.E., Stoll, M.S., Ingalls, S.T., Yang, S., Kerner, J. & Hoppel, C.L. (2008). Quantification of carnitine and acylcarnitines in biological matrices by HPLC electrospray ionization-mass spectrometry. *Clin Chem*, **54,** 1451-62.

Möder, M., Kießling, A. & Löster, H. (2005). Current methods for determination of L-Carnitine and Acylcarnitines. *Monatshefte Chemie*, **136,** 1279-1291.

Morand, R., Todesco, L., Donzelli, M., Fischer-Barnicol, D., Mullen, PJ., Krähenbühl, S. (2012). Effect of short- and long-term treatment with valproate on carnitine homeostasis in humans. *Ther Drug Monit*, **(accepted manuscript)**.

Mueller, P., Schulze, A., Schindler, I., Ethofer, T., Buehrdel, P. & Ceglarek, U. (2003). Validation of an ESI-MS/MS screening method for acylcarnitine profiling in urine specimens of neonates, children, adolescents and adults. *Clin Chim Acta*, **327,** 47-57.

Murakami, K., Sugimoto, T., Woo, M., Nishida, N. & Muro, H. (1996). Effect of L-carnitine supplementation on acute valproate intoxication. *Epilepsia*, **37,** 687-9.

Muro, H., Tatsuhara, T., Sugimoto, T., Woo, M., Nishida, N., Murakami, K. & Yamaguchi, Y. (1995). Determination of urinary valproylcarnitine by gas chromatography-mass spectrometry with selected-ion monitoring. *J Chromatogr B Biomed Appl*, **663,** 83-9.

Nanni, G., Pittiruti, M. & Castagneto, M. (1983). Carnitine plasma levels during total parenteral nutrition. *Am J Clin Nutr*, **38,** 339-41.

Negrao, C.E., Ji, L.L., Schauer, J.E., Nagle, F.J. & Lardy, H.A. (1987). Carnitine supplementation and depletion: tissue carnitines and enzymes in fatty acid oxidation. *J Appl Physiol*, **63,** 315-21.

Nezu, J., Tamai, I., Oku, A., Ohashi, R., Yabuuchi, H., Hashimoto, N., Nikaido, H., Sai, Y., Koizumi, A., Shoji, Y., Takada, G., Matsuishi, T., Yoshino, M., Kato, H., Ohura, T., Tsujimoto, G., Hayakawa, J., Shimane, M. & Tsuji, A. (1999). Primary systemic carnitine deficiency is caused by mutations in a gene encoding sodium ion-dependent carnitine transporter. *Nat Genet*, **21,** 91-4.

Ohashi, R., Tamai, I., Yabuuchi, H., Nezu, J.I., Oku, A., Sai, Y., Shimane, M. & Tsuji, A. (1999). Na(+)-dependent carnitine transport by organic cation transporter (OCTN2): its pharmacological and toxicological relevance. *J Pharmacol Exp Ther*, **291,** 778-84.

Ohnishi, S., Okamura, N., Sakamoto, S., Hasegawa, H., Norikura, R., Kanaoka, E., Takahashi, K., Horie, K., Sakamoto, K. & Baba, T. (2008). Role of Na+/L-carnitine transporter (OCTN2) in renal handling of pivaloylcarnitine and valproylcarnitine formed during pivalic acid-containing prodrugs and valproic acid treatment. *Drug Metab Pharmacokinet*, **23,** 293-303.

Ohtani, Y., Endo, F. & Matsuda, I. (1982). Carnitine deficiency and hyperammonemia associated with valproic acid therapy. *J Pediatr*, **101,** 782-5.

Okamura, N., Ohnishi, S., Shimaoka, H., Norikura, R. & Hasegawa, H. (2006). Involvement of recognition and interaction of carnitine transporter in the decrease of L-carnitine concentration induced by pivalic acid and valproic acid. *Pharm Res*, **23,** 1729-35.

Olsen, C. (1971). An enzymatic fluorimetric micromethod for the determination of acetoacetate, -hydroxybutyrate, pyruvate and lactate. *Clin Chim Acta*, **33,** 293-300.

Oyono-Enguelle, S., Freund, H., Ott, C., Gartner, M., Heitz, A., Marbach, J., Maccari, F., Frey, A., Bigot, H. & Bach, A.C. (1988). Prolonged submaximal exercise and L-carnitine in humans. *Eur J Appl Physiol Occup Physiol*, **58,** 53-61.

Pace, J.A., Wannemacher, R.W., Jr. & Neufeld, H.A. (1978). Improved radiochemical assay for carnitine and its derivatives in plasma and tissue extracts. *Clin Chem*, **24,** 32-5.

Parvin, R. & Pande, S.V. (1977). Microdetermination of (-)carnitine and carnitine acetyltransferase activity. *Anal Biochem*, **79,** 190-201.

Peluso, G., Barbarisi, A., Savica, V., Reda, E., Nicolai, R., Benatti, P. & Calvani, M. (2000). Carnitine: an osmolyte that plays a metabolic role. *J Cell Biochem*, **80,** 1-10.

Pesta, D. & Gnaiger, E. (2012). High-resolution respirometry: OXPHOS protocols for human cells and permeabilized fibers from small biopsies of human muscle. *Methods Mol Biol*, **810,** 25-58.

Peterson, G.M. & Naunton, M. (2005). Valproate: a simple chemical with so much to offer. *J Clin Pharm Ther*, **30,** 417-21.

Pons, R., Carrozzo, R., Tein, I., Walker, W.F., Addonizio, L.J., Rhead, W., Miranda, A.F., Dimauro, S. & De Vivo, D.C. (1997). Deficient muscle carnitine transport in primary carnitine deficiency. *Pediatr Res*, **42,** 583-7.

Poorthuis, B.J., Jille-Vlckova, T. & Onkenhout, W. (1993). Determination of acylcarnitines in urine of patients with inborn errors of metabolism using high-performance liquid chromatography after derivatization with 4'-bromophenacylbromide. *Clin Chim Acta*, **216,** 53-61.

Pormsila, W., Morand, R., Krahenbuhl, S. & Hauser, P.C. (2011). Capillary electrophoresis with contactless conductivity detection for the determination of carnitine and acylcarnitines in clinical samples. *J Chromatogr B Analyt Technol Biomed Life Sci*, **879,** 921-6.

Primassin, S., Ter Veld, F., Mayatepek, E. & Spiekerkoetter, U. (2008). Carnitine supplementation induces acylcarnitine production in tissues of very long-chain acyl-CoA dehydrogenase-deficient mice, without replenishing low free carnitine. *Pediatr Res*, **63,** 632-7.

Rashed, M.S., Ozand, P.T., Bennett, M.J., Barnard, J.J., Govindaraju, D.R. & Rinaldo, P. (1995). Inborn errors of metabolism diagnosed in sudden death cases by acylcarnitine analysis of postmortem bile. *Clin Chem*, **41,** 1109-14.

Rebouche, C.J. (2004). Kinetics, pharmacokinetics, and regulation of L-carnitine and acetyl-L-carnitine metabolism. *Ann N Y Acad Sci*, **1033,** 30-41.

Rebouche, C.J. & Chenard, C.A. (1991). Metabolic fate of dietary carnitine in human adults: identification and quantification of urinary and fecal metabolites. *J Nutr*, **121,** 539-46.

Rebouche, C.J., Lombard, K.A. & Chenard, C.A. (1993). Renal adaptation to dietary carnitine in humans. *Am J Clin Nutr*, **58,** 660-5.

Rebouche, C.J. & Paulson, D.J. (1986). Carnitine metabolism and function in humans. *Annu Rev Nutr*, **6,** 41-66.

Ringseis, R. & Eder, K. (2009). Influence of pharmacological PPARalpha activators on carnitine homeostasis in proliferating and non-proliferating species. *Pharmacol Res*, **60,** 179-84.

Ringseis, R., Keller, J. & Eder, K. (2012). Role of carnitine in the regulation of glucose homeostasis and insulin sensitivity: evidence from in vivo and in vitro studies with carnitine supplementation and carnitine deficiency. *Eur J Nutr*, **51,** 1-18.

Ringseis, R., Posel, S., Hirche, F. & Eder, K. (2007). Treatment with pharmacological peroxisome proliferator-activated receptor alpha agonist clofibrate causes upregulation of organic cation transporter 2 in liver and small intestine of rats. *Pharmacol Res*, **56,** 175-83.

Rodriguez-Segade, S., de la Pena, C.A., Tutor, J.C., Paz, J.M., Fernandez, M.P., Rozas, I. & Del Rio, R. (1989). Carnitine deficiency associated with anticonvulsant therapy. *Clin Chim Acta*, **181,** 175-81.

Roe, C.R., Millington, D.S., Maltby, D.A. & Wellman, R.B. (1987). Post-mortem recognition of inherited metabolic disorders from specific acylcarnitines in tissue in cases of sudden infant death. *Lancet*, **1,** 512.

Roepstorff, C., Halberg, N., Hillig, T., Saha, A.K., Ruderman, N.B., Wojtaszewski, J.F., Richter, E.A. & Kiens, B. (2005). Malonyl-CoA and carnitine in regulation of fat oxidation in human skeletal muscle during exercise. *Am J Physiol Endocrinol Metab*, **288,** E133-42.

Rose, E.C., di San Filippo, C.A., Ndukwe Erlingsson, U.C., Ardon, O., Pasquali, M. & Longo, N. (2011). Genotype-phenotype correlation in primary carnitine deficiency. *Hum Mutat*, **33,** 118-23.

Roth, K. (2009). Medium-chain acyl-CoA dehydrogenase deficiency. ed. Reference, M.

Schiaffino, S. & Reggiani, C. (2011). Fiber types in mammalian skeletal muscles. *Physiol Rev*, **91,** 1447-531.

Schmidt-Sommerfeld, E., Zhang, L., Bobrowski, P.J. & Penn, D. (1995). Quantitation of short- and medium-chain acylcarnitines in plasma by radioisotopic exchange/high-performance liquid chromatography. *Anal Biochem*, **231,** 27-33.

Schurch, R., Todesco, L., Novakova, K., Mevissen, M., Stieger, B. & Krahenbuhl, S. (2010). The plasma carnitine concentration regulates renal OCTN2 expression and carnitine transport in rats. *Eur J Pharmacol*, **635,** 171-6.

Sierra, A.Y., Gratacos, E., Carrasco, P., Clotet, J., Urena, J., Serra, D., Asins, G., Hegardt, F.G. & Casals, N. (2008). CPT1c is localized in endoplasmic reticulum of neurons and has carnitine palmitoyltransferase activity. *J Biol Chem*, **283,** 6878-85.

Silva, M.F., Selhorst, J., Overmars, H., van Gennip, A.H., Maya, M., Wanders, R.J., de Almeida, I.T. & Duran, M. (2001). Characterization of plasma acylcarnitines in patients under valproate monotherapy using ESI-MS/MS. *Clin Biochem*, **34,** 635-8.

Spaniol, M., Brooks, H., Auer, L., Zimmermann, A., Solioz, M., Stieger, B. & Krahenbuhl, S. (2001). Development and characterization of an animal model of carnitine deficiency. *Eur J Biochem*, **268,** 1876-87.

Spiekerkoetter, U., Huener, G., Baykal, T., Demirkol, M., Duran, M., Wanders, R., Nezu, J. & Mayatepek, E. (2003). Silent and symptomatic primary carnitine deficiency within the same family due to identical mutations in the organic cation/carnitine transporter OCTN2. *J Inherit Metab Dis*, **26,** 613-5.

Stadler, D.D., Bale, J.F., Jr., Chenard, C.A. & Rebouche, C.J. (1999). Effect of long-term valproic acid administration on the efficiency of carnitine reabsorption in humans. *Metabolism*, **48,** 74-9.

Stanley, C.A. (2004). Carnitine deficiency disorders in children. *Ann N Y Acad Sci*, **1033,** 42-51.

Stanley, C.A., Hale, D.E., Berry, G.T., Deleeuw, S., Boxer, J. & Bonnefont, J.P. (1992). Brief report: a deficiency of carnitine-acylcarnitine translocase in the inner mitochondrial membrane. *N Engl J Med*, **327,** 19-23.

Steiber, A., Kerner, J. & Hoppel, C.L. (2004). Carnitine: a nutritional, biosynthetic, and functional perspective. *Mol Aspects Med*, **25,** 455-73.

Stephens, F.B., Constantin-Teodosiu, D. & Greenhaff, P.L. (2007a). New insights concerning the role of carnitine in the regulation of fuel metabolism in skeletal muscle. *J Physiol*, **581,** 431-44.

Stephens, F.B., Constantin-Teodosiu, D., Laithwaite, D., Simpson, E.J. & Greenhaff, P.L. (2006). Insulin stimulates L-carnitine accumulation in human skeletal muscle. *Faseb J*, **20,** 377-9.

Stephens, F.B., Constantin-Teodosiu, D., Laithwaite, D., Simpson, E.J. & Greenhaff, P.L. (2007b). A threshold exists for the stimulatory effect of insulin on plasma L-carnitine clearance in humans. *Am J Physiol Endocrinol Metab*, **292,** E637-41.

Stephens, F.B., Evans, C.E., Constantin-Teodosiu, D. & Greenhaff, P.L. (2007c). Carbohydrate ingestion augments L-carnitine retention in humans. *J Appl Physiol*, **102,** 1065-70.

Stieger, B., O'Neill, B. & Krahenbuhl, S. (1995). Characterization of L-carnitine transport by rat kidney brush-border-membrane vesicles. *Biochem J*, **309 (Pt 2),** 643-7.

Stratton, S.L., Horvath, T.D., Bogusiewicz, A., Matthews, N.I., Henrich, C.L., Spencer, H.J., Moran, J.H. & Mock, D.M. (2010). Plasma concentration of 3-hydroxyisovaleryl carnitine is an early and sensitive indicator of marginal biotin deficiency in humans. *Am J Clin Nutr*, **92,** 1399-405.

Stratton, S.L., Horvath, T.D., Bogusiewicz, A., Matthews, N.I., Henrich, C.L., Spencer, H.J., Moran, J.H. & Mock, D.M. (2011

). Urinary excretion of 3-hydroxyisovaleryl carnitine is an early and sensitive indicator of marginal biotin deficiency in humans. *J Nutr*, **141,** 353-8.

Stumpf, D.A., Parker, W.D. & Haas, R. (1983). Carnitine deficiency with valproate therapy. *J Pediatr*, **103,** 175-6.

Sun, D., Cree, M.G., Zhang, X.J., Boersheim, E. & Wolfe, R.R. (2006). Measurement of stable isotopic enrichment and concentration of long-chain fatty acyl-carnitines in tissue by HPLC-MS. *J Lipid Res*, **47,** 431-9.

Takeyama, N., Takagi, D., Adachi, K. & Tanaka, T. (1986). Measurement of free and esterified carnitine in tissue extracts by high-performance liquid chromatography. *Anal Biochem*, **158,** 346-54.

Tamai, I., China, K., Sai, Y., Kobayashi, D., Nezu, J., Kawahara, E. & Tsuji, A. (2001). Na(+)-coupled transport of L-carnitine via high-affinity carnitine transporter OCTN2 and its subcellular localization in kidney. *Biochim Biophys Acta*, **1512,** 273-84.

Tamai, I., Ohashi, R., Nezu, J., Yabuuchi, H., Oku, A., Shimane, M., Sai, Y. & Tsuji, A. (1998). Molecular and functional identification of sodium ion-dependent, high affinity human carnitine transporter OCTN2. *J Biol Chem*, **273,** 20378-82.

Tamai, I., Ohashi, R., Nezu, J.I., Sai, Y., Kobayashi, D., Oku, A., Shimane, M. & Tsuji, A. (2000). Molecular and functional characterization of organic cation/carnitine transporter family in mice. *J Biol Chem*, **275,** 40064-72.

Tein, I., DiMauro, S., Xie, Z.W. & De Vivo, D.C. (1993). Valproic acid impairs carnitine uptake in cultured human skin fibroblasts. An in vitro model for the pathogenesis of valproic acid-associated carnitine deficiency. *Pediatr Res*, **34,** 281-7.

Theodoulou, F.L., Holdsworth, M. & Baker, A. (2006). Peroxisomal ABC transporters. *FEBS Lett*, **580,** 1139-55.

Thom, H., Carter, P.E., Cole, G.F. & Stevenson, K.L. (1991). Ammonia and carnitine concentrations in children treated with sodium valproate compared with other anticonvulsant drugs. *Dev Med Child Neurol*, **33,** 795-802.

Todesco, L., Bodmer, M., Vonwil, K., Haussinger, D. & Krahenbuhl, S. (2009). Interaction between pivaloylcarnitine and L-carnitine transport into L6 cells overexpressing hOCTN2. *Chem Biol Interact*, **180,** 472-7.

Todesco, L., Bur, D., Brooks, H., Torok, M., Landmann, L., Stieger, B. & Krahenbuhl, S. (2008). Pharmacological manipulation of L-carnitine transport into L6 cells with stable overexpression of human OCTN2. *Cell Mol Life Sci*, **65,** 1596-608.

Tomita, M. & Sendju, Y. (1927). Über die Oxyaminoverbindungen, welche die Biuretreaktion zeigen. III. Spaltung der g-Amino-b-oxybuttersäure in die optisch aktiven Komponenten. *Hoppe-Seylers Zeitschrift für physiologische Chemie*, **169**.

Tortorelli, S., Hahn, S.H., Cowan, T.M., Brewster, T.G., Rinaldo, P. & Matern, D. (2005). The urinary excretion of glutarylcarnitine is an informative tool in the biochemical diagnosis of glutaric acidemia type I. *Mol Genet Metab*, **84,** 137-43.

Tsuchida, H., Anzai, N., Shin, H.J., Wempe, M.F., Jutabha, P., Enomoto, A., Cha, S.H., Satoh, T., Ishida, M., Sakurai, H. & Endou, H. (2010). Identification of a novel organic anion transporter mediating carnitine transport in mouse liver and kidney. *Cell Physiol Biochem*, **25,** 511-22.

Van Hove, J.L., Chace, D.H., Kahler, S.G. & Millington, D.S. (1993). Acylcarnitines in amniotic fluid: application to the prenatal diagnosis of propionic acidaemia. *J Inherit Metab Dis*, **16,** 361-7.

van Kempen, T.A. & Odle, J. (1992). Quantification of carnitine esters by high-performance liquid chromatography. Effect of feeding medium-chain triglycerides on the plasma carnitine ester profile. *J Chromatogr*, **584,** 157-65.

van Loon, L.J., Greenhaff, P.L., Constantin-Teodosiu, D., Saris, W.H. & Wagenmakers, A.J. (2001). The effects of increasing exercise intensity on muscle fuel utilisation in humans. *J Physiol*, **536,** 295-304.

Van Veldhoven, P.P., Just, W.W. & Mannaerts, G.P. (1987). Permeability of the peroxisomal membrane to cofactors of beta-oxidation. Evidence for the presence of a pore-forming protein. *J Biol Chem*, **262,** 4310-8.

Vaz, F.M., Melegh, B., Bene, J., Cuebas, D., Gage, D.A., Bootsma, A., Vreken, P., van Gennip, A.H., Bieber, L.L. & Wanders, R.J. (2002a). Analysis of carnitine biosynthesis metabolites in urine by HPLC-electrospray tandem mass spectrometry. *Clin Chem*, **48,** 826-34.

Vaz, F.M. & Wanders, R.J. (2002b). Carnitine biosynthesis in mammals. *Biochem J*, **361,** 417-29.

Verhoeven, N.M., Roe, D.S., Kok, R.M., Wanders, R.J., Jakobs, C. & Roe, C.R. (1998). Phytanic acid and pristanic acid are oxidized by sequential peroxisomal and mitochondrial reactions in cultured fibroblasts. *J Lipid Res*, **39,** 66-74.

Vernez, L., Dickenmann, M., Steiger, J., Wenk, M. & Krahenbuhl, S. (2006). Effect of L-carnitine on the kinetics of carnitine, acylcarnitines and butyrobetaine in long-term haemodialysis. *Nephrol Dial Transplant*, **21,** 450-8.

Vernez, L., Hopfgartner, G., Wenk, M. & Krahenbuhl, S. (2003). Determination of carnitine and acylcarnitines in urine by high-performance liquid chromatography-electrospray ionization ion trap tandem mass spectrometry. *J Chromatogr A*, **984,** 203-13.

Vernez, L., Thormann, W. & Krahenbuhl, S. (2000). Analysis of carnitine and acylcarnitines in urine by capillary electrophoresis. *J Chromatogr A*, **895,** 309-16.

Vernez, L., Wenk, M. & Krahenbuhl, S. (2004). Determination of carnitine and acylcarnitines in plasma by high-performance liquid chromatography/electrospray ionization ion trap tandem mass spectrometry. *Rapid Commun Mass Spectrom*, **18,** 1233-8.

Vielhaber, S., Feistner, H., Weis, J., Kreuder, J., Sailer, M., Schroder, J.M. & Kunz, W.S. (2004). Primary carnitine deficiency: adult onset lipid storage myopathy with a mild clinical course. *J Clin Neurosci*, **11,** 919-24.

Visser, W.F., van Roermund, C.W., Ijlst, L., Waterham, H.R. & Wanders, R.J. (2007). Metabolite transport across the peroxisomal membrane. *Biochem J*, **401,** 365-75.

Voet, D. & Voet, J. (2004). *Biochemistry*.

Wachter, S., Vogt, M., Kreis, R., Boesch, C., Bigler, P., Hoppeler, H. & Krahenbuhl, S. (2002). Long-term administration of L-carnitine to humans: effect on skeletal muscle carnitine content and physical performance. *Clin Chim Acta*, **318,** 51-61.

Wagner, C.A., Lukewille, U., Kaltenbach, S., Moschen, I., Broer, A., Risler, T., Broer, S. & Lang, F. (2000). Functional and pharmacological characterization of human Na(+)-carnitine cotransporter hOCTN2. *Am J Physiol Renal Physiol*, **279,** F584-91.

Wall, B.T., Stephens, F.B., Constantin-Teodosiu, D., Marimuthu, K., Macdonald, I.A. & Greenhaff, P.L. (2011). Chronic oral ingestion of L-carnitine and carbohydrate increases muscle carnitine content and alters muscle fuel metabolism during exercise in humans: the dual role of muscle carnitine in exercise metabolism. *J Physiol*.

Wanders, R.J., Ferdinandusse, S., Brites, P. & Kemp, S. (2010). Peroxisomes, lipid metabolism and lipotoxicity. *Biochim Biophys Acta*, **1801,** 272-80.

Wen, G., Kuhne, H., Rauer, C., Ringseis, R. & Eder, K. (2011). Mouse gamma-butyrobetaine dioxygenase is regulated by peroxisome proliferator-activated receptor alpha through a PPRE located in the proximal promoter. *Biochem Pharmacol*, **82,** 175-83.

Wennberg, A., Hyltander, A., Sjoberg, A., Arfvidsson, B., Sandstrom, R., Wickstrom, I. & Lundholm, K. (1992). Prevalence of carnitine depletion in critically ill patients with undernutrition. *Metabolism*, **41,** 165-71.

Werner, T., Treiss, I., Kohlmueller, D., Mehlem, P., Teich, M., Longin, E., Gerstner, T., Koenig, S.A. & Schulze, A. (2007). Effects of valproate on acylcarnitines in children with epilepsy using ESI-MS/MS. *Epilepsia*, **48,** 72-6.

Wittels, B. & Bressler, R. (1965). Two-Dimensional Thin-Layer Chromatographic Isolation of Fatty Acyl Carnitines. *J Lipid Res*, **6,** 313-4.

Wolff, J., Muller, D.M. & Strack, E. (1971). [Toxicity of L(-)-carnitine and various 0-acylcarnitines]. *Acta Biol Med Ger*, **26,** 1237-42.

Wu, X., Huang, W., Prasad, P.D., Seth, P., Rajan, D.P., Leibach, F.H., Chen, J., Conway, S.J. & Ganapathy, V. (1999). Functional characteristics and tissue distribution pattern of organic cation transporter 2 (OCTN2), an organic cation/carnitine transporter. *J Pharmacol Exp Ther*, **290,** 1482-92.

Yokogawa, K., Yonekawa, M., Tamai, I., Ohashi, R., Tatsumi, Y., Higashi, Y., Nomura, M., Hashimoto, N., Nikaido, H., Hayakawa, J., Nezu, J., Oku, A., Shimane, M., Miyamoto, K. & Tsuji, A. (1999). Loss of wild-type carrier-mediated L-carnitine transport activity in hepatocytes of juvenile visceral steatosis mice. *Hepatology*, **30,** 997-1001.

Yoshida, T., Aetake, A., Yamaguchi, H., Nimura, N. & Kinoshita, T. (1988). Determination of carnitine by high-performance liquid chromatography using 9-anthryldiazomethane. *J Chromatogr*, **445,** 175-82.

Ziegler, H.J., Bruckner, P. & Binon, F. (1967). O-acylation of dl-carnitine chloride. *J Org Chem*, **32,** 3989-91.

Zuniga, A. & Li, L. (2011). Ultra-high performance liquid chromatography tandem mass spectrometry for comprehensive analysis of urinary acylcarnitines. *Anal Chim Acta*, **689,** 77-84.

Zytkovicz, T.H., Fitzgerald, E.F., Marsden, D., Larson, C.A., Shih, V.E., Johnson, D.M., Strauss, A.W., Comeau, A.M., Eaton, R.B. & Grady, G.F. (2001). Tandem mass spectrometric analysis for amino, organic, and fatty acid disorders in newborn dried blood spots: a two-year summary from the New England Newborn Screening Program. *Clin Chem*, **47,** 1945-55.

11. CURRICULUM VITAE

PERSONAL INFORMATION

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July 11th 1982 in Fribourg
Swiss nationality

EDUCATION

Since 10/2008	PhD thesis, Division for Clinical Pharmacology and Toxicology, University Hospital Basel (Prof. Stephan Krähenbühl) "Carnitine: analytical and physiological aspects"
08/2008	Federal Pharmacist Diploma
2005-2008	Master in Pharmacy, University of Basel
2003-2005	Bachelor of Pharmacy, University of Fribourg and Basel
2000-2001	High school diploma, Miller, South Dakota, USA
1998-2003	Ste-Croix College, Fribourg

TRAININGS AND CONTINUING EDUCATION

05/2010-11/2011	WIN – Women into Industry, mentorship program Novartis-University of Basel
02/2009	Introductory course in Laboratory Animal Science: LTK Modul 1, Institut für Labortierkunde, University of Zürich
12/2008	Mass spectrometry training, AB Sciex, Rotkreuz

WORKING EXPERIENCES

Since 04/2009	Pharmacist, Pharmacie de la Belle-Croix, Romont
08/2008	Organization member, FIP Congress 2008 in Basel
09/2007-06/2008	Pharmacist-in-training, Pharmacie Dr. C. Repond, Bulle
07/2005, 2006, 2007	Office work (holiday replacement), AS Désinfection, Villarlod
2000-2007	Cashier in a grocery store (Coop), Romont

LANGUAGES

French	mother tongue
English	fluent (spoken and written)
German	fluent (spoken and written)
Swiss German	fluent (spoken)

TECHNICAL SKILLS

Mass spectrometry (API 4000)
Diverse molecular biology techniques (Cell culturing, Western Blotting, PCR)
GraphPad Prism, Endnote
MS office

PUBLICATIONS

Rapid quantification of plasma carnitine and acylcarnitines by high performance liquid chromatography-tandem mass spectrometry using online solid phase extraction.
Morand R, Donzelli M, Haschke M, Krähenbühl S.
(submitted)

Effect of short- and long-term treatment with valproate on carnitine homeostasis in humans.
Morand R, Todesco L, Fischer-Barnicol D, Donzelli M, Mullen P, Krähenbühl S.
Ther Drug Monit 2012 (accepted manuscript)

Determination of creatine and phosphocreatine in muscle biopsy samples by capillary electrophoresis with contactless conductivity detection.
See HH, Schmidt-Marzinkowski J, Pormsila W, Morand R, Krähenbühl S, Hauser PC.
Anal Chim Acta. 2012 May 21;727:78-82.

Capillary electrophoresis with contactless conductivity detection for the determination of carnitine and acylcarnitines in clinical samples.
Pormsila W, Morand R, Krähenbühl S, Hauser PC.
J Chromatogr B Analyt Technol Biomed Life Sci. 2011 Apr 15;879(13-14):921-6.

Quantification of plasma lactate concentrations using capillary electrophoresis with contactless conductivity detection.
Pormsila W, Morand R, Krähenbühl S, Hauser PC.
Electrophoresis. 2011 Apr;32(8):884-9.

The role of CYP3A4 in amiodarone-associated toxicity on HepG2 cells.
Zahno A, Brecht K, Morand R, Maseneni S, Török M, Lindinger PW, Krähenbühl S.
Biochem Pharmacol. 2011 Feb 1;81(3):432-41.

HOBBIES

Scuba diving (D** CMAS), swimming, cycling, reading, playing the piano