Study of the carnitine metabolism in pregnancy, rheumatoid arthritis, systemic sclerosis and IBD by tandem mass spectrometry

Ph.D. thesis

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ABBREVIATIONS

CACT:	carnitine-acylcarnitine translocase
CAT:	carnitine acetyltransferase
CD:	Crohn's disease
CID:	collision induced decay
COT:	carnitine octanoyltransferase
CPT:	carnitine palmitoyltransferase
ESI:	electrospray ionisation
HPLC:	high-pressure liquid chromatography
IBD:	inflammatory bowel disease
LCAC:	long-chain acylcarnitine
MCAC:	medium-chain acylcarnitine
OCTN:	organic cation transporter
PS:	parent scan
RA:	rheumatoid arthritis
RFLP:	restriction fragment length polymorphism
SCAC:	short-chain acylcarnitine
SNP:	single nucleotide polymorphism
SSc:	systemic sclerosis
UC:	ulcerative colitis (colitis ulcerosa)

Abbreviations of carnitine esters: When characterising carnitine metabolism, the short names of the individual esters are often used instead of or besides the regular chemical or trivial names. Here „**C**" and a numeral denote the carbon atom number of the side chain esterified to the carnitine molecule, a colon and a numeral mark the amount of the unsaturated bonds independently of their positions, „**DC**" refers to double carboxylic acids (e.g. glutaric acid), and „**OH**" stands for hydroxylation. E.g. **C18:2-OH** indicates a side chain with 18 carbon atoms, two double bonds and one hydroxylation. Free carnitine is signed as **C0**.

1. INTRODUCTION

In eukaryotic cells the coenzyme stores of the organelles is usually sequestered, and their regulation is strictly separate. To connect these subcellular spaces such auxiliary molecules are needed that can carry substrates or atom groups in a shuttle-service manner. Carnitine (3-Hydroxy-4-N-trimethylammoniobutanoate; **Figure 1**) is also a compound of that kind, gaining its name from the Latin word *caro, carnis f*: 'meat', since it can be found at greatest level in the striated and cardiac muscle.

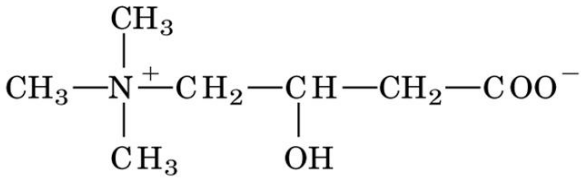


Figure 1. The carnitine molecule.

1.1. The carnitine in the metabolism

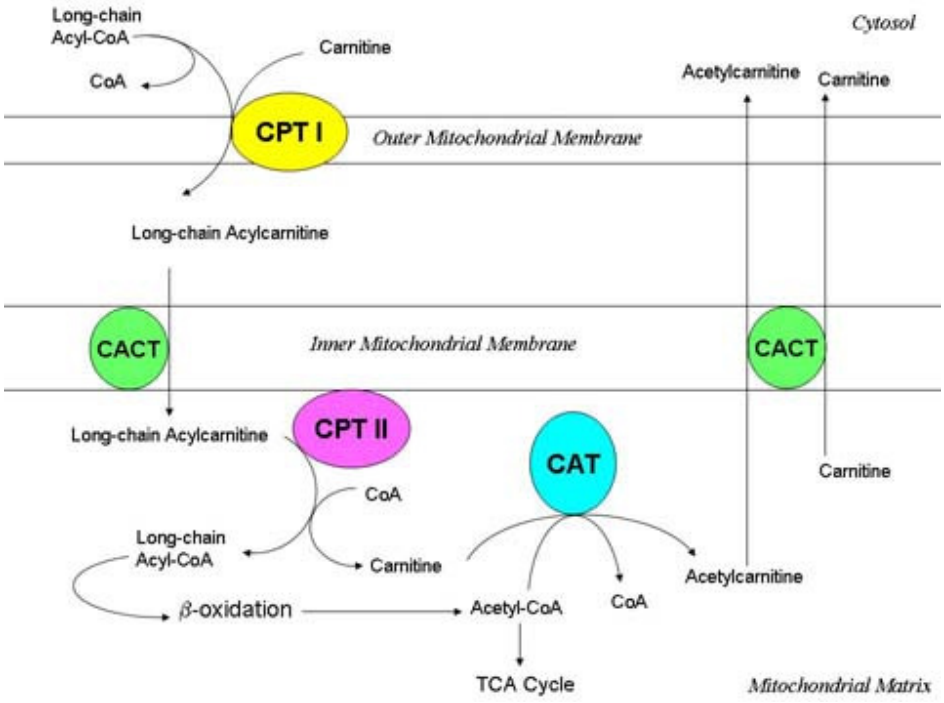
Carnitine is a vitamin-like nutrient that can be produced to a lesser extent by the living organism itself in the kidney and the liver, however, it is decisively taken up from the food, mainly by absorption in the small intestine. The largest stores are located in the liver and the muscles. The inward transport of carnitine in the human cells is accomplished by the products of the *slc22a* (solute carrier 22) gene family, the OCTN1 and OCTN2 carrier proteins. The primary high-affinity carrier is OCTN2, that is spanning the plasma membrane of all cell types involved in the traffic of carnitine: e.g. it performs the absorption in the intestinal epithelium, the very high-rate (>99%) recovery of the carnitine excreted with the filtrate in the renal tubules, and the uptake from the lymph into the muscle cells. OCTN2 was demonstrated in the placenta as well, which makes the carnitine traffic between the mother and the foetus quite probable.

Through its hydroxyl group carnitine is able to form esters with numerous carboxylic acid compounds. Its primary biochemical role is to carry the long-chain fatty acids from the cytoplasm into the mitochondrial matrix, linking the coenzyme A pools of these two functional spaces in the cell. The cytosolic fatty acids are first activated and attached to the free coenzyme by the acyl-coenzyme A synthetase. **Figure 2.** shows the subsequent steps of the process.

Carnitine has multiple additional functions in the cells, such as to prevent the mitochondrial coenzyme A pool from depletion, to form conjugates with toxic intermediates arising from enzyme defects or drug treatment and to promote their elimination, to participate in biosynthetic transformation of many lipids, or to interlink the metabolism of the peroxisome and the mitochondrion.

The esters formed by carnitine, just like free carnitine molecule, all appear in the blood and the urine, where they can be demonstrated and measured by several methods. A detailed carnitine profile, that is, the separate, reliable, qualitative and quantitative characterisation of the individual carnitine esters has only been possible since the introduction of the tandem mass spectrometry. The formerly used radioenzymatic methods made it available to determine only the level of the free carnitine and the cumulative amount of the carnitine esters (referred to as acylcarnitine, total ester), and furthermore, the sum of these two (total carnitine). Disorders of the fatty acid breakdown or other related biochemical processes can specifically modify the circulating carnitine. Mutations in the genes encoding the proteins of carnitine transport may also have impacts on the carnitine content of the blood.

Figure 2. The role of the carnitine in the mitochondrial beta-oxidation.



http://lpi.oregonstate.edu/infocenter/othernuts/carnitine/transport.html

1.2. Carnitine and pregnancy

The human newborn stores a great bulk of fat that accumulates mainly during the third trimester of the gestation. That time the *de novo* fatty acid synthesis in the foetal tissues is continuously increasing, which makes the developing organism substantially dependent on the nutrients arriving from the mother. During pregnancy the maternal metabolism gradually accommodates to the foetal requirements: fat depots accumulate from the food at first, then, in the late phase of gestation, the lipid breakdown becomes predominant. Although the level of circulating free fatty acids considerably increases, they can pass through the placenta only to limited extent, instead they are turned into ketone bodies in the liver, and reach the foetus in this way. Here they have a basic significance as energy carriers and raw materials needed to synthesise other lipids, since the foetal ketogenesis is of very low level.

Data published in the literature shows that the free carnitine level in pregnant women gradually decreases, while other studies describe similarly reduced or unchanged total ester concentrations. The free carnitine, total ester and total carnitine levels measured during and at the end of the gestation are significantly lower than those in non-pregnant women of similar age. A decisive part of this decrease takes place during the first trimester, and the corresponding blood levels reach again the values typical of non-pregnant women in a few weeks after labour. In the background of the changes several processes can be assumed, e.g. an increase in the volume of the body fluids during gestation, a higher need for carnitine (to eliminate toxic acyl groups) or more intensive transport towards the developing foetus. The larger acylcarnitine clearance that can be measured during pregnancy may also suggest a greater extent of the removal of noxious compounds.

Carnitine is essential also for the developing foetus, and in the final period of the pregnancy it is stored in the liver and muscles at increasing rates. In the placenta, the presence of both the enzymes involved in fatty acid oxidation and the OCTN2 carrier protein can be shown, which raises the issue of the carnitine utilisation of the placenta itself on the one hand, and active transport processes on

the other. In comparison of the carnitine levels found in the maternal and the umbilical cord blood, diverse and somewhat contradictory results have been published in the literature, regarding both the free and the esterified carnitine.

1.3. Inflammatory diseases and carnitine

Although the immunological diseases can show very different symptoms and pathomechanism, the idea that they may be related to the disorders of the carnitine metabolism arose in several cases. Inflammations affecting a considerable part or key organs of the body may disturb the carnitine homeostasis, on the other hand, an insufficient carnitine supply also might contribute to the development of pathological conditions.

Rheumatoid arthritis (RA). This is a chronic inflammatory illness primarily afflicting the hand joints, occurring in about 1% of the population with accumulated prevalence among women. It can be associated with many accessory symptoms, e.g. myopathies accompanied by a decrease of muscle strength. In a study on Japanese subjects an association was found between RA and an intronic polymorphism of the *slc22a4* gene (*slc2f2*; GenBank: **rs3792876**), which suggested a possible relationship with the carnitine system. This allelic variant is located in the recognition site of the RUNX1 transcription factor inhibiting the expression of the gene, and the regulator protein binds the mutant allele stronger. An intronic single nucleotide polymorphism (runx1; GenBank: **rs2268277**) in the gene of the RUNX1 protein was also shown to be significantly associated to the disease. The *slc22a4* and *slc22a5* genes contain several potential recognition sites for this transcription factor, which suggests that a modified carnitine transport might be involved in the pathological process.

Systemic sclerosis (SSc). It is an autoimmune disease of the connective tissue, generally accompanied by fibrosis, inflammation and occlusive or atrophic angiopathies. In the 70s beneficial effects of carnitine treatment on some symptoms of the systemic sclerosis were described. With a permanent administration, primarily the motility of the limb joints and the looseness of the skin were improved. Different disease terminology at that time, small case numbers and the lack of controls make these results, however, hard to be interpreted. To date, systemic sclerosis is distinguished as two clinically and serologically diverse subtypes: limited form (lSSc) affecting only the skin, and diffuse form (dSSc) extending over certain visceral organs, as well. A novel short publication reported on significantly lower free and total carnitine levels in patients, found only in the diffuse subtype, in contrast to the normal values in the lSSc subjects.

Inflammatory bowel disease (IBD).

This is the inflammation of the intestinal wall with various extents. Two clinical pictures belong here: Crohn's disease (CD) and ulcerative colitis (UC, colitis ulcerosa). Both inherited and environmental background play a role in the pathomechanism of these illnesses, they have common genetic susceptibility factors, and some overlaps may occur in their symptoms. The IBD5 locus of the 5q31 chromosomal region has been unambiguously and repeatedly proved to be associated with an increased risk for the development of CD. Some publications reported on similar results regarding UC. The *slc22a4* and *slc22a5* genes encoding the OCTN1 and OCTN2 proteins, respectively, are also located within the IBD5 region, which suggested the idea of a functional link between carnitine transport and IBD.

The C1672T and G-207C polymorphisms lying in the 9th exon of *slc22a4* and in the promoter region of *slc22a5*, respectively, together make a risk haplotype, and the occurrence of the TC genotype is significantly higher in the Crohn patients. Since then it has been confirmed by the most restudies, and some authors also published results on UC where they either approved or rejected the role of the TC haplotype. The relationship of these two variants and the IBD may depend on additional polymorphisms that create an extended risk haplotype. The elements of this haplotype,

like SNP IGR2230a_1 (GenBank: **rs17622208**) in an intron of the *slc22a5* gene, were shown to be significantly associated to Crohn's disease.

Little is still known about the relationship of the carnitine metabolism and the IBD. Traditional enzymatic radiochemical methods demonstrated lowered free and total carnitine levels in paediatric CD patients, and increased long-chain and total carnitine but decreased free carnitine concentrations in adults. The *slc22a* TC haplotype in fibroblasts may modify the expression and transport ability of OCTN proteins, which arises the possibility of a functional deficiency in the chronic inflammation state. Mass spectrometry carnitine profile studies yielded results showing lesser changes in the levels of a few esters in the blood plasma of CD patients, which were independent of the TC haplotype or its genetic elements.

The carnitine esters of the short-chain fatty acids make an important energy source for the colon epithelium, primarily butyryl-carnitine whose metabolism is disturbed in UC. Based upon clinical investigations, rectal irrigations containing short-chain fatty acids improved certain pathologic symptoms, and administration of propionyl-carnitine was also effective. The latter might have double beneficial impact, since propionic acid can serve as energy carrier, and carnitine itself is in turn a speed-limiting factor of the butyric acid breakdown, and, on the other hand, it can decrease the oxidative stress that is thought to play a role in the UC pathomechanism. In a rat model the experimentally evoked colitis decreased the OCTN2 expression and, consequently, the butyric acid oxidation, that were reversible to carnitine treatment. The high affinity transport protein for the absorption of the butyryl-carnitine in the intestine is no other than OCTN2, therefore changes affecting the *slc22a5* gene may influence this function. The results from mass spectrometry examinations in UC were similar as those in CD; the lower SCAC levels may reflect the limited availability of the corresponding fatty acids.

2. AIMS

Our work based on ESI tandem mass spectrometry, focussing on the following questions:

- How does the carnitine profile change in the pregnant mothers during the second half of gestation?
- What is the carnitine profile in the umbilical cord blood, compared to the mothers of the newborns and non-pregnant women?
- Whether is there a correlation between foetal and maternal carnitine ester or free carnitine levels?
- What conclusions can be drawn form the results regarding the connection of the foetal and maternal carnitine traffic?
- What effects does RA have on the carnitine profile of the patients and the healthy controls?
- Whether do the putative risk factors, the *slc2f2* and *runx1* polymorphisms, influence the carnitine metabolism in the RA patients and the controls?
- What impacts does systemic sclerosis exert on the carnitine profile of the patients when compared with healthy controls?
- Whether does the SSc subtype have significance about the carnitine metabolism?
- Whether does the IGR2230 genotype influence the carnitine profile in IBD patients or healthy control subjects?

3. SUBJECTS AND METHODS

3.1. Subjects.

The persons giving biological samples to our work were all avoid of primary carnitine deficiency, disease of the liver, kidney, endocrine organs, systemic disease of the circulatory or the nervous system, and – except for the patients examined – extended inflammation. The samples of patients suffering from rheumatoid arthritis or systemic sclerosis arrived from the Department of Immunology and Rheumatology of University of Pécs, and the samples of the pregnant mothers and their newborns from the Department of Paediatrics. The IBD patients were treated at the II. Department of Internal Medicine of the Semmelweis University, Budapest. Since current alimentation can substantially influence the levels of certain carnitine esters in the body fluids, blood after overnight fast was taken from every person in the morning hours, except for the mothers at labour and the umbilical cord blood, of course. All the subjects enrolled gave us informed consent to using their samples in the research. Everywhere in our work we followed the rules laid down by the Ethics Committee of the Medical Faculty of the University of Pécs and the prescriptions of authoritative international agreements.

Pregnant mother program. The 37 mothers taken into the experiments were the participants of international research collaboration (NUHEAL) addressed to the changes of the lipid metabolism and the effects of nutritional supplements. Their age was 29.0 ± 0.9 year (mean ± SEM), and their weight at 20th week of pregnancy was 68.8 ± 2.0 kg. The anthropometric parameters, clinical and laboratory results, important lifestyle and dietary habits of the participants were recorded in detail throughout gestation. The circumstances of the birth, the results of the physical and laboratory investigations of the newborns and their nutritional features were also noted down, up to the 24th week.

Births were free of complications: twenty boys (54%) and seventeen girls (46%) were born, with no twins (gestational age was 36.7 ± 0.3 weeks, body length 50 ± 1 cm, mass 3.24 ± 0.08 kg). Apgar scores were all normal. There were six premature births, but their gestational age also exceeded 35 weeks. We had umbilical blood sample of twenty children. The control group was 22 healthy, age-matched non-pregnant women of usual nutrition with no suspect of infertility. In the pregnant mother programme we applied the plasma as sample gained from anticoagulated blood.

Rheumatoid arthritis. We had DNA samples from 209 patients (169 women, 40 men; age 57.3 ± 1.0 year) diagnosed to have RA according to the criteria of the American College of Rheumatology. Seventy-three percent of the patients were positive for rheumatoid factor as well. The control group was 217 healthy, age-matched persons (122 women and 95 men; age 56.5 ± 0.7 year), whose medical history excluded any systemic diseases, especially joint inflammation. To perform the carnitine measurements we had serum samples from every patient and 142 control persons after overnight fast.

Systemic sclerosis. One hundred and seven patients (95 women and 12 men; age 53.8 ± 1.1 year) provided serum samples for the examinations; clinical and laboratory data resulting from the follow-up were recorded in a database. Patients were enrolled into localised and diffuse subgroups (78 and 29 persons, respectively). As control, the samples of 47 healthy people (32 women and 15 men; age 51.7 ± 2.1 year) selected as above were used.

IBD. Two-hundred CD (103 women and 97 men; age 39.4 ± 1.0 year) and 246 UC patients (138 women and 108 men; age 44.0 ± 1.0 year), whose diagnosis based on detailed clinical and histological investigations, and 187 healthy persons (81 women and 106 men; age 37.7 ± 0.8 year) provided DNA samples for our work. We had also after-fast plasma samples of 76 Crohn's disease and 43 UC patients and 45 control persons for the carnitine profile measurements.

3.2. Genotype determination

The IGR2230a_1 genotype of the IBD patients and their controls was established by a PCR-RFLP method. We amplified the sequence around the polymorphism with the 5' CAG AAG AAT GCC CTT GAT GTG 3' forward and 5' TCA GAA GCT GTC CAT CCC AC 3' reverse primers that flanked a product 438 bp long. The reaction mixture was 50 µl, and contained 2 units of Taq polymerase, 5 µl reaction buffer (100 mM Tris-HCl, pH 9.0; 500 mM KCl, 15 mM $MgCl_2$), 200 µM of each dNTP, 0.2 µM of each primer and 1 µg genomic DNA. In a MJ Research PTC-200 thermocycler we applied 35 cycles using the following program: predenaturation - 2 min 95°C, denaturation - 30 s 95°C, annealing - 30 s 54°C, extension - 30 s 72°C, final completion - 5 min 72°C. The resulting amplicon was digested with *DdeI* restriction enzyme at 37°C over night, and the fragments were run on 1 % agarose gel and stained with ethidium-bromid. Wild type 'G' allele yielded 122, 128 and 188 bp, while 'A' allele 128 and 310 bp bands.

3.3. Mass spectrometry

Serum or plasma was separated from blood by centrifugation (3.000 rpm, 15 min) and was stored at -80°C until use. Ten µl sample homogenised by vortexing was dropped on filter paper and let dry for 2 hours. The resulting spot was accurately excised and put into 200 µl methanol containing the following deuterium-labelled isotopes as internal standards: 0.76 µmol/L $^2H_3$-carnitine, 0.04 µmol/L $^2H_3$-propionyl-carnitine, 0.04 µmol/L $^2H_3$-oktanoyl-carnitine and 0.08 µmol/L $^2H_3$-palmitoyl-carnitine. While gently shaken the samples were incubated for 20 min at RT, then the supernatant was transferred into a new vial and the methanol was evaporated under N_2 stream at 40°C. One-hundred µl isobutanolic HCl (3M) was added and the sample was let stand for 15 min at 65°C, then the liquid phase was evaporated as before. The resulting derivatives were dissolved in 80%:20% mixture of acetonitrile and water.

A Waters 2795 HPLC instrument supplied the continuous flow of the 80%:20% acetonitrile:water eluent at 100 µl/min rate, where 10 µl sample was injected into. The measurements were performed with a Micromass Quattro Ultima tandem quadrupole mass spectrometer equipped with an electrospray ion source. The carnitine derivatives typically yield a positively charged fragment of 85Da molecular mass, thus 85 m/z parent scan was used in positive mode. Every measurement took 4 min and contained 78 independent scans, with the first analyser set on the range of 200-550 m/z, and the second one on the mass of the fragment mentioned. For measurements and calculations the MassLynx 4.0 software package was used. The temperature of the capillary was 100°C, the spray (70 L/min) and the evaporation (400 L/min, 350°C) of the eluent was carried out with N_2, while the collision gas was argon. The optimised capillary voltage, cone voltage and collision energy was 2.5 kV, 55 V and 26 eV, respectively.

3.4. Statistics

Each sample was measured in triplicates, and the average was considered as one measurement result. To compare the carnitine levels of different groups the unpaired *t*-test of Student was used, while for the results at different times of the same persons the paired test was applied. The limit of significance was 0.01. The correlation analysis was carried out with Pearson's bivariate test, and the examination of the genotype distribution with χ^2 (Chi-square) test. The limit of significance was set as 0.05 in both cases. The calculations were performed using Excel or SPSS 11.5 software.

4. RESULTS AND DISCUSSION

4.1. Carnitine in pregnancy

4.1.1. Carnitine profile in the second half of pregnancy

We found a significant decrease in the concentration of the free carnitine between the 20th and 30th weeks, that did not change in the next interval. The level of medium-chain acylcarnitines showed a slight, while that of the long-chain ones a greater increase; the changes predominantly occurred between the 30th week and the delivery. Acetylcarnitine had a similar behaviour, but the other short-chain esters remained unaltered. As a result of these processes the total ester concentration also significantly increased in the last ten weeks, and the total carnitine first decreased then the original level was restored.

The free and esterified carnitine levels were almost all significantly lower during birth than those found in the non-pregnant women's blood plasma, except for the long-chain esters that were higher in the mothers. The total ester and total carnitine concentrations were also significantly lower in the mothers.

The numerical data of the carnitine profile and the statistical differences can be seen in **Table 1**.

	Pregnant mother (n=37)			Control	Newborn
	20th week	30th week	at term	(n=22)	(n=20)
total carnitine	27.27 ± 1.46 †	24.38 ± 1.35 §	27.17 ± 1.20	43.93 ± 2.12 §	33.18 ± 2.59 § *
C0	19.61 ± 1.25 § †	16.72 ± 0.93	16.75 ± 0.90	27.90 ± 1.42 §	20.00 ± 1.30 *
acylcarnitine	7.66 ± 0.36 §	7.67 ± 0.59 §	10.42 ± 0.54	16.04 ± 0.88 §	13.19 ± 1.54
SCAC					
C2	6.24 ± 0.32 §	6.30 ± 0.56 §	8.48 ± 0.49	13.76 ± 0.80 §	11.24 ± 1.56
C3	0.11 ± 0.01	0.10 ± 0.01	0.10 ± 0.01	0.20 ± 0.01 §	0.22 ± 0.02 §
C4	0.26 ± 0.01	0.24 ± 0.01	0.24 ± 0.01	0.33 ± 0.01 §	0.32 ± 0.02 §
C5	0.07 ± 0.01	0.07 ± 0.01	0.07 ± 0.01	0.14 ± 0.01 §	0.14 ± 0.02 §
C6	0.06 ± 0.01 §	0.07 ± 0.01	0.07 ± 0.01	0.10 ± 0.01 §	0.11 ± 0.01 §
MCAC					
C8:1	0.06 ± 0.01	0.07 ± 0.01	0.08 ± 0.01	0.08 ± 0.01	0.08 ± 0.01
C8	0.05 ± 0.01 §	0.05 ± 0.01	0.07 ± 0.01	0.13 ± 0.01 §	0.05 ± 0.01 § *
C10:1	0.04 ± 0.01 §	0.05 ± 0.01	0.06 ± 0.01	0.13 ± 0.01 §	0.04 ± 0.01 § *
C10	0.05 ± 0.01 §	0.06 ± 0.01	0.07 ± 0.01	0.15 ± 0.01 §	0.04 ± 0.01 § *
LCAC					
C16	0.07 ± 0.01 §	0.07 ± 0.01 §	0.13 ± 0.01	0.12 ± 0.01	0.15 ± 0.01
C18:2	0.08 ± 0.01 §	0.08 ± 0.01 §	0.16 ± 0.01	0.11 ± 0.01 §	0.12 ± 0.01 §
C18:1	0.11 ± 0.01 §	0.11 ± 0.01 §	0.24 ± 0.01	0.15 ± 0.01 §	0.12 ± 0.01 §
C18	0.07 ± 0.01 § †	0.05 ± 0.01 §	0.09 ± 0.01	0.08 ± 0.01	0.07 ± 0.01

†: p< 0,01 vs. 30th week §: p< 0,01 vs. at term *: p< 0,01 newborn vs. control

Table 1: Plasma carnitine profile in mothers at different times of pregnancy, in non-pregnant women and in the umbilical cord blood (µmol/L; mean ± SEM)

Based on the earlier radioenzymatic methods a simplified picture arose where the levels of carnitine metabolites showed a uniform trend of decrease in the pregnancy. Our results proved the processes more complex, and carnitine metabolism specific to gestation can be described. During pregnancy the free and esterified carnitine gradually reduce, but in the last phase the long-chain esters and the acetylcarnitine increase again, or at least they are higher at birth than in the 30th week. In the last weeks of the gestation lipolysis and fatty acid breakdown is enhanced in the maternal organism, which may be associated with an enlarged demand for carnitine, keeping the level of free carnitine lower. Our results agree with this hypothesis, as at the end of pregnancy it is just the level of the long-chain fatty acid oxidation related carnitine esters that increases. We think therefore, that the changes of the carnitine profile during gestation may reflect the specific rearrangement of the carnitine metabolism where the enhanced fatty acid utilisation of the maternal organism probably has its role. Besides, it is also known that during labour, with declining insulin level, the concentration of free fatty acids along with that of glucose significantly raises in the mother's blood. The intensive and long lasting muscle performance of the womb contractions may also be involved in the enhanced fatty acid breakdown, and, consequentially, the specific carnitine profile.

4.1.2. Carnitine profile in newborns

The levels of free carnitine and short-chain esters in the umbilical cord were significantly higher, and the medium- and long-chain esters predominantly lower than in the mothers. We found an insignificant decrease of acetylcarnitine and a significant reduction of free and total esters and medium-chain esters, and, furthermore, roughly similar short- and long-chain ester levels in the newborns compared with non-pregnant women (**Table 1**). Thus the newborn carnitine profile can be considered as a highly specific pattern, which was already proven also from whole blood investigated in different age groups.

We investigated as well the correlation of the individual carnitine metabolite levels between the mothers and their own newborns. The strongest relationship was found with free carnitine, besides there was significant positive correlation for C8:1, C18:1 and C18:2, too. The most publications in the literature report on the demonstration of positive correlation for free and total carnitine and total esters, or only free carnitine, but some studies resulted in no significant connection.

Carnitine is also involved in the lively traffic of metabolites between the pregnant mother and the developing foetus. Regarding the carnitine content of the maternal and umbilical blood, the literature is contradictory in many respects, which renders the evaluation of data more difficult. Similarly, the traffic of carnitine between the mother and her foetus, and the foetal or placental carnitine metabolism are still far from being unravelled in every detail. Some researchers did not find different free or esterified carnitine levels in the arterious and venous blood of the umbilical cord, while others report on higher free carnitine in the artery than in the vein. These results more favour the thought that the intensity of the metabolism linked to carnitine may not be high in the foetus (at least right around the time of delivery).

It was also underpinned by the widely accepted former opinion that the foetus can primarily utilise glucose as fuel, and the breakdown of fatty acids is not significant before birth. Since then it has been revealed that the enzymes of the fatty acid oxidation are expressed in the foetal, and even in the embryonic tissues, although their activity remains below adult levels, and that carnitine esters are present in the foetal circulation, but in lower concentrations than after delivery. These results, however, must be interpreted with great caution for the present, because they came from the examination of few and *post mortem* samples.

Leastwise, it seems certain that shortly after birth the fats accumulated during the pregnancy become the main energy source of the newborn, and the organism switches from glucose breakdown to the oxidation of fatty acids. Since food intake in this period is often little, the

appropriate carnitine provision of the newborn becomes of key importance, and the carnitine profile undergoes specific changes. The level of numerous carnitine esters significantly increases in the blood, then it falls again in a few weeks. At the time of delivery we did not find signs of enhanced fatty acid breakdown in the foetal carnitine profile, which can mean that the liver of the newborn needs time to switch over the utilisation of fatty acids, in parallel with the depletion of the carbohydrate stores in the liver. This is also supported by the observation that the concentration of unesterified fatty acids increases very soon – in a few hours – after birth in the newborn deprived of his continuous food supply, but this is followed only later (1/2-1 day) by the elevation of the ketone body levels.

The provision of carnitine is already essential for the foetus, and carnitine itself accumulates in increasing amount in the foetal liver and muscles during the last period of the pregnancy. The foetus and the newborn probably has a quite limited inherent capacity of synthesising carnitine, which is also reinforced by the fact that by total parenteral nutrition the plasma carnitine level significantly drops. The ability of carnitine synthesis presumably is confined to certain foetal tissues and the placenta. According to this, one can suppose that the carnitine levels in the foetus and the newborn at birth greatly depend on the maternal values, which was confirmed by correlation examinations on free carnitine. The exact mechanism of the transport in not yet fully known, but the high affinity carnitine transporter OCTN2 protein is expressed also in the placenta. Additionally, the ATB(0,+) transporter protein with broad substrate range can also come into question. These facts seem to verify the theory that the primary carnitine source of the foetus is the mother, and, with the level of foetal and placental carnitine synthesis being low, the carnitine can pass into the foetal circulation by an active transport process.

The presence of OCTN2 in mice is necessary for the accumulation of carnitine in the placenta and the foetus. One of the basic energy source of the placenta is the fatty acids, and the enzyme system of the beta-oxidation can be found also here in an active state. This may suggest that carnitine esters returning from the placenta can also contribute to the concentration increase observed in the maternal blood. The differences between the maternal and the foetal carnitine ester levels are significant but heterogeneous, and they practically do not correlate with each other. The problem is made more difficult by that the carnitine concentrations to be measured in the blood are determined by the different metabolic conditions in the pregnant woman, the placenta and the foetus, together with the bidirectional traffic of the carnitine and its esters. The amount, significance and pathologic role of the carnitine esters getting into the maternal circulation from the foetus is as well nowadays under lively scientific debate.

4.2. Carnitine and immunologic diseases

4.2.1. Rheumatoid arthritis

Comparing the carnitine profiles in the entire groups of the patients and the controls we found that the former showed a slight decrease in the level of the free carnitine and several esters, with C3, C4, C8:1 and C18:2 reaching the level of statistical significance. The *slc2f2* and *runx1* SNP genotype results of the patients and the controls were also available. Between the acylcarnitine profiles of the patient genotype groups we observed one single difference: carriers of the *runx1* mutant allele had significantly higher free carnitine level as the non-carriers. The controls showed a converse picture: the homozygous carriers had lower free carnitine level. These results indicate that we did not find differences specific to the alleles examined in the carnitine ester profile. The patients showed negligibly decreased ester levels compared with the controls. Other authors reported on similarly small reduction or increase of carnitine esters in RA.

4.2.2. Systemic sclerosis

In our study we examined the carnitine profile in serum samples of patients belonging to both disease subtypes and that of age-matched healthy controls (**Table 2.**). We found statistically significant decrease in C2, C8, C10:1 and C10 levels, while increase in C5 and C6 levels, compared with controls. The total ester and total carnitine concentrations showed also a significant reduction in the patients. The free carnitine level did not differ significantly between any groups. The total amount of the medium-chain esters (C8-C14) was significantly lower in the patients. The same held for the long-chain esters (C16-C18) to a lesser extent.

The amount of saturated and modified medium- and long-chain acylcarnitines decreased in the serum of patients, which suggests that SSc exerts some effect on the transport function of carnitine. One of the possible targets is CPT-I enzyme that is a rate limiting factor of the fatty acid breakdown. In systemic inflammatory conditions CPT-I is inhibited in the cardiac muscle, for example in sepsis or ischemia/reperfusion injuries.

The change of the concentrations was in the range that rules it out as primary factor in the background of the pathomechanism. Shifts of acylcarnitine levels in such directions and degrees, however, have not been found yet in any known and studied pathologic or physiologic conditions, so the observed profile can be considered disease-specific. The normal level of free carnitine shows that the transport and availability of the carnitine has not been damaged. Our results can not support

	All patients (n=107)	Limited SSc (n=78)	Diffuse SSc (n=29)	Control (n=47)
total carnitine	46.31 ± 0.74	46.91 ± 0.88	44.67 ± 1.42	48.78 ± 1.46
C0	33.41 ± 0.59	33.64 ± 0.67	32.75 ± 1.27	31.81 ± 0.99
acylcarnitine	12.90 ± 0.27 §	13.27 ± 0.33 §	11.92 ± 0.39 §	16.97 ± 0.64
SCAC				
C2	9.96 ± 0.23 §	10.30 ± 0.29 §	9.04 ± 0.34 §	13.78 ± 0.56
C3	0.27 ± 0.01	0.28 ± 0.01	0.26 ± 0.01	0.31 ± 0.01
C4	0.47 ± 0.01	0.46 ± 0.02	0.50 ± 0.03	0.43 ± 0.02
C5	0.34 ± 0.01 §	0.34 ± 0.01 §	0.33 ± 0.02	0.29 ± 0.01
C6	0.19 ± 0.01 §	0.19 ± 0.01 §	0.19 ± 0.01 §	0.16 ± 0.01
MCAC				
C8:1	0.07 ± 0.01	0.07 ± 0.01	0.06 ± 0.01	0.08 ± 0.01
C8	0.12 ± 0.01 §	0.13 ± 0.01 §	0.12 ± 0.01 §	0.17 ± 0.01
C10:1	0.10 ± 0.01 §	0.11 ± 0.01 §	0.09 ± 0.01 §	0.14 ± 0.01
C10	0.14 ± 0.01 §	0.14 ± 0.01 §	0.13 ± 0.01 §	0.20 ± 0.01
LCAC				
C16	0.12 ± 0.01	0.12 ± 0.01	0.12 ± 0.01	0.14 ± 0.01
C18:2	0.11 ± 0.01	0.11 ± 0.01	0.10 ± 0.01 §	0.13 ± 0.01
C18:1	0.21 ± 0.01	0.20 ± 0.01	0.21 ± 0.01	0.23 ± 0.01
C18	0.08 ± 0.01	0.08 ± 0.01	0.08 ± 0.01	0.09 ± 0.01
total MCAC	0.63 ± 0.03 §	0.66 ± 0.03 §	0.57 ± 0.04 §	0.85 ± 0.05
total LCAC	0.55 ± 0.01 §	0.55 ± 0.02 §	0.54 ± 0.03 §	0.65 ± 0.03

§: $p < 0.01$ vs. control

Table 2: Serum carnitine profile of SSc patients and controls (µmol/L; mean ± SEM)

the conception that the disease and the carnitine metabolism would be connected by the absorption disorders so frequent in systemic sclerosis, or, that a lack of free carnitine would cause reduced antioxidant protection raising then the amount of free radicals, or, that the same lack would disturb the effect of cytokines and the activation of lymphocytes.

A separate examination of the disease subtypes led to similar conclusions, additionally, we observed a significant decrease of C18:2 in the diffuse SSc group. It is also conspicuous that in most cases the concentrations of lSSc patients were closer to the control results than those of the dSSc patients. The cumulative level of medium-chain acylcarnitines with limited sclerosis was closer again to the control value than with diffuse SSc, but this difference in long-chain acylcarnitines was negligible. Although the two subtypes represent clinically diverse forms of the disease, their carnitine profile was essentially quite similar, which suggests that the origin of the changes can be common. Therefore, our results do not support the idea that in the diffuse SSc the levels of free and total carnitine significantly decrease, while they remain unchanged in the limited sclerosis.

It is well known that systemic sclerosis occurs 8-10 times more frequently in women, but in men the disease expressivity is often much more severe. Our examinations did not reveal any gender-specific difference in the carnitine profile of the patients. Similarly, we did not find characteristic changes in those patients with oesophagus, lung or heart involvement.

Since it is not about a carnitine deficiency condition, it is hard to estimate the potential advantages of the carnitine treatment. The preliminary studies are encouraging in that the exogenous excess carnitine alone might turn certain processes into the desired direction, but really significant results concerning the essentials of the disease are questionable.

4.2.3. Inflammatory bowel disease (IBD)

We performed the determination of the IGR2230a_1 genotype in 200 CD and 246 UC patients and 187 healthy control persons from the adult Hungarian population. The prevalence data are in concordance with results published elsewhere. All the three groups was in Hardy-Weinberg equilibrium. The incidence and carriage of the mutated allele *A* was higher among the patients than in the controls, the rate of the homozygotes, in turn, was higher in the UC subjects. The statistical analysis did not reveal significant differences in any groups using the comparisons as follows (**Table 3**):

Wild-type allele (G) vs. mutated allele (A)

Carriers (AG + AA) vs. non-carriers (GG)

Wild-type homozygotes (GG) vs. mutated homozygous (AA)

IGR2230 genotype	CD patients (n = 200)		UC patients (n = 246)		Controls (n = 187)	
	n	%	n	%	n	%
GG	47	23.5	70	28.4	59	31.6
AG	112	56.0	120	48.8	89	47.6
AA	41	20.5	56	22.8	39	20.8
A allele prevalence		48.5		47.1		44.6

Table 3: s*lc22a5* IGR2230a_1 genotypes in Crohn and UC patients, and control persons

Several causes may underlie the negative results, offering possible explanations. Since the frequency values of the identical categories between the individual groups do not differ significantly, we can suggest that the association of the genotype and the disease is weak, and it might need a study with greater case numbers to demonstrate it. IBD itself has a composite background and pathomechanism, in addition, the efforts to functionally map the IBD5 region and to attribute it to the phenotype in a more delicate way did not yield consistent and uncontradicted results yet. This can partially be explained by that ethnic or geographic differences may largely bias the outcome of the experiments. The situation is further complicated because both CD and UC are heterogeneous and overlapping clinical pictures of several phenotypes, which may lead to misspecification in the diagnosis, especially with colon involvement. It probably has the background that the individual patients have distinct at-risk allele combinations, which may substantially influence the appearance of the disease. This was the base of the opinion that the IBD5 region associates to Crohn's disease in a 'phenotype-specific' manner, which can be further modified by additional genetic variants.

We examined by tandem mass spectrometry the carnitine ester profile of CD and UC patients and healthy control persons stratified by their IGR230a_1 genotype. We wanted to know if this SNP has an impact on the carnitine metabolism, by influencing the function of the *slc22a5* gene. We found one single statistically significant difference: in the UC carriers the C2 level was decreased. The free carnitine concentration was normal everywhere, showing appropriate carnitine supply. We conclude that the IGR2230a_1 polymorphism does not represent a substantive risk factor for the IBD in the Hungarian population, but the validity of this assessment should be examined with a larger sample size. Moreover, we can state that the IGR2230a_1 genotype has no significant effect on the carnitine metabolism either in IBD patients or in healthy control persons.

4.2.4. The relationship of the carnitine and the immunological diseases

The connection between the carnitine metabolism and the operation of the immune system was suggested long before, but our knowledge is still sparse and uncertain. Formerly, efforts were made to reveal the relationships using enzymatic radiochemical determination and genetic or clinical information. The effect of the carnitine is probably exerted at multiple points; for instance, it basically concerns the energy utilisation from long-chain fatty acids of the immune cells, which is a key to maintain the structure of the plasma membrane and the cellular viability. Reduced triglyceride and increased unesterified fatty acid levels were found in rheumatoid arthritis and IBD patients among others, which raised the possibility of the dysfunction of the lipid metabolism and the involvement of the carnitine system. In rat experiments the carnitine decreased the generation of reactive free radicals in the immune cells, and restored the declining activity of the neutrophil granulocytes and macrophages in the old animals. The carnitine treatment was able to enhance the stimulated proliferation and the chemotactic activity of certain leukocytes. The carnitine content of the peripheral mononucleate cells is lower in AIDS patients than in healthy persons. Inter alia, it was proved in clinical studies that the carnitine treatment decreased the apoptosis of the lymphocytes and the oxidative stress, inhibited the expression of inflammatory cytokines, Fas/FasL and caspase-1. The examinations based on mass spectrometry measurements showed characteristic changes of the carnitine profile in some inflammatory diseases of the intestinal tract.

It is highly important to clarify the role of the carnitine in the immune processes or in the development of certain immunological disorders, moreover, to reveal its potential therapeutic applications and significance, regarding especially the counteraction to the continuous decline of the immune response in aging. The results to date suggest that the carnitine may have some modifying effect on certain immunological events, otherwise, it can reduce the amount of the oxidative by-products formed in acute inflammations and the extent of the apoptosis of both the

immune cells and the parenchymal cells. This must be handled, however, with increased caution since the low number of *in vitro* and clinical data allow us to draw limited conclusions about the actual and *in vivo* processes in the human organism. We can not exclude the possibility that a multiply (absorption, synthesis) disturbed carnitine supply secondarily causes a general deterioration of the mitochondrial function and the oxidative metabolism, further aggravating the state of the immune system. A favourable effect of the carnitine treatment on these events may seem as if it beneficially influenced certain properties of the basic disease.

5. SUMMARY

- The level of acetylcarnitine and the medium- and long-chain esters significantly increased between the 30th week of the pregnancy and delivery, while that of the free carnitine reduced between the 20th and 30th weeks. The ester and free carnitine concentrations of the pregnant mothers were significantly lower than those of the non-pregnant women of similar age, except for the long-chain esters where the opposite was the case. The details show a composite and dynamic view about the carnitine metabolism during gestation, which was not available by the formerly used methods. In our opinion the change of the carnitine profile reflects the more intensive utilisation of the carnitine stores, and the enhancement of the maternal fatty acid breakdown in the second half of the pregnancy and the delivery on the other hand.
- In the umbilical blood of the newborns a unique carnitine profile was found that differed from that of both the mothers and the non-pregnant women, such as from other known profiles. The free carnitine was in significant positive correlation between the mothers and their babies. The higher value of the foetal than the maternal free carnitine, together with the positive correlation, suggest that, since the foetal carnitine synthesis is probably of low intensity, the mother ensures the carnitine provision of the foetus by active transport mechanisms. The role of the OCTN2 protein seems here conspicuous.
- As the carnitine profile of the umbilical cord markedly differs from that of newborns a few days old, the directions of the changes allows us to conclude that after delivery a metabolic shift takes place from the carbohydrate breakdown to the oxidation of fatty acids.
- Rheumatoid arthritis generally causes a small decrease in the concentrations of the patients' carnitine metabolites compared with the healthy controls. The extent of these changes is by no means proportional to evoking a severe pathological event. The carriers of the individual SNP genotypes did not show any difference in their carnitine profiles again, therefore the effect of the allelic variants examined on the carnitine metabolism was not to be proven
- Patients suffering from systemic sclerosis had a specific and yet undescribed carnitine profile that is characterised by the lower levels of primarily the acetylcarnitine and medium-chain esters, and, to a lesser extent, the long-chain esters, compared with the healthy controls. The concentration of the free carnitine was maintained, which did not prove that its transport disorder or a shortage of carnitine caused by severe malabsorption would have a role in the pathomechanism. Reduced ester levels may be related to the moderation of the fatty acid oxidation.
- No significant differences were found between the carnitine profiles of the both SSc subtypes. Therefore, we assume the common origin of the changes, and we believe, on the other hand, that the clinical subtype has no essential influence on the carnitine metabolism.
- It was not verified that IGR2230a_1 genotype influences the carnitine profile in either CD and UC patients or healthy controls, hence this SNP seems to have no effect mediated by the OCTN2 protein on the carnitine.

6. LIST OF PUBLICATIONS

The thesis is based on the following publications

1. **Talián GC**, Komlósi K, Decsi T, Koletzko B, Melegh B. Determination of carnitine ester patterns during the second half of pregnancy, at delivery, and in neonatal cord blood by tandem mass spectrometry: complex and dynamic involvement of carnitine in the intermediary metabolism. *Pediatr Res* 2007 Jul; 62(1):88-92 Impact factor: 2,839

2. Komlósi K, **Talián CG**, Faragó B, Magyari L, Cserép V, Kovács B, Bene J, Havasi V, Kiss CG, Czirják L, Melegh B No influence of SLC22A4 C6607T and RUNX1 G24658C genotypic variants on the circulating carnitine ester profile in patients with rheumatoid arthritis. *Clin Exp Rheumatol* 2008 Jan-Feb; 26(1):61-6 Impact factor: 2,270 (2007)

3. **Talián CG**, Kiss CG, Melegh B, Czirják L Features of serum carnitine ester profile in systemic sclerosis. (Magyar Immunológia)

4. **G Talián**, J Bene, L Magyari, K Komlósi, K Horváth, B Gasztonyi, P Miheller, M Figler, G Mózsik, Z Tulassay, B Melegh Plasma carnitine ester profiles in Crohn's disease and ulcerative colitis patients with different IGR2230a_1 genotypes. (International Journal of Immunogenetics) Impact factor: 1,279

Other publications:

1. Csikós G, Molnár K, Borhegyi NH, **Talián GC**, Sass M.Insect cuticle, an in vivo model of protein trafficking. *Journal of Cell Science* 1999 Jul; 112 (Pt 13):2113-24. Impact factor: 6,044

2. Lõw P, **Talián GC**, Sass M Up- and downregulated genes in muscles that undergo developmentally programmed cell death in the insect Manduca sexta. *FEBS Letters* 2005; 579: 4943–4948 Impact factor: 3,415

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6. Bene J, Magyari L, **Talián G**, Komlósi K, Gasztonyi B, Tari B, Várkonyi A, Mózsik G, Melegh B. Prevalence of SLC22A4, SLC22A5 and CARD15 gene mutations in Hungarian pediatric patients with Crohn's disease. *World J Gastroenterol* 2006 Sep; 14;12(34):5550-3

7. Bene J, Komlósi K, Havasi V, **Talián G**, Gasztonyi B, Horváth K, Mózsik G, Hunyady B, Melegh B, Figler M. Changes of plasma fasting carnitine ester profile in patients with ulcerative colitis. *World J Gastroenterol* 2006; 12:110-113

8. Bene J, Komlósi K, Magyari L, **Talián G**, Horváth K, Gasztonyi B, Miheller P, Figler M, Mózsik G, Tulassay Z, Melegh B. Plasma carnitine ester profiles in Crohn's disease patients characterized for SLC22A4 C1672T and SLC22A5 G-207C genotypes. Br J Nutr 2007; 98(2):345-50. Impact factor: 2,339

9. Magyari L, Bene J, Komlósi K, **Talián G**, Faragó B, Csöngei V, Járomi L, Sáfrány E, Sipeky C, Lakner L, Varga M, Gasztonyi B, Melegh B.Prevalence of SLC22A4 1672T and SLC22A5 -207C combination defined TC haplotype in Hungarian ulcerative colitis patients. Pathol *Oncol Res* 2007; 13(1):53-6. Impact factor: 1,272

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Cumulative impact factor: 30,213

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