Investigations of carnitine transporters in primary carnitine deficiency and multifactorial disease

Ph.D. THESIS

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1. Summary

Carnitine, a ubiquitous water-soluble molecule exerts its primary physiological role in the transport of long-chain fatty acids into the mitochondria, to the place of their oxidation. Besides, carnitine is involved in various other metabolic and cellular processes. Humans obtain their proper carnitine levels mainly through the diet, although it is known that at some degree endogen synthesis of carnitine also takes place. Cells aquire carnitine through an active transport mechanism, the uptake takes place against a significant plasma/tissue concentration gradient.

Direct mutations of the high affinity human carnitine transporter gene (*SLC22A5*) lead to primary carnitine deficiency, which disease primarily affects the heart, liver and sceletal muscles, however, the phenotypic spectrum is very broad. We have investigated Hungarian Roma families with primary carnitine deficiency that showed variable phenotypic manifestations even within a family, and as a novelty, we found sudden infant death syndrome cases with verified *SLC22A5* mutations. The extensive histological examination of the organ manifestations added new information to the pathomorphology of primary carnitine deficiency.

Apart from its classical metabolic role additional cellular functions of carnitine were investigated through the analysis of the lymphoreticular tissues obtained from the SIDS cases. Our results showed an important contribution of carnitine in the humoral immune response.

As a high-throughput analytical and diagnostic tool tandem mass spectrometry was introduced in our laboratory 4 years ago. Taking advantage of this method we analysed the changes in carnitine homeostasis in homozygote and heterozygote primary carnitine deficiency.

Expanding our area of interest we investigated the role of carnitine transporter genes (*SLC22A5* és *SLC22A4*), their variants and regulator genes in the etilology of certain inflammatory multifactorial diseases. According to recent experimental and clinical studies we performed molecular genetic and tandem mass spectrometric metabolic investigations in Hungarian rheumatoid arthritis patient cohorts.

2. Introduction

2.1. The role of carnitine in human metabolism

A well-known biochemical property of L-carnitine (3-hydroxy-4-N,N,N-trimethylaminobutyrate) is its ability to form acyl-carnitine esters with organic acids by certain enzymatic reactions. Its classic physiological role can also be derived from this chemical property: carnitine is indipensable in the transport of activated long-chain fatty acids from the cytosol to the mitochondrial matrix, where β-oxidation takes place (**Fig.1.**). Cytosolic long-chain fatty acids are unable to cross the mitochondrial inner membrane even in their activated form as CoA esters, they are first transesterified to L-carnitine by the enzyme carnitine palmitoyltransferase I (CPTI) at the mitochondrial outer membrane, then transported over the inner membrane as long-chain acyl-carnitine esters via a specific carrier, carnitine-acylcarnitine translocase (CACT) and at the matrix side they are transesterified to intramitochondrial CoA via the enzyme carnitine-palmitoyltransferase II (CPTII). Carnitine is also involved in various other metabolic processes: it ensures a proper level of free CoA in the cell, which is indispensable in many metabolic routes, such as the citric acid cycle, ketogenesis, and gluconeogenesis, and carnitine can also modulate the toxic effects of poorly metabolised acyl-groups of various origin. By forming carnitine-esters with organic acids of xenobiotic origin (e.g. pivalic acid and valproate) and with those arising from various inborn errors of metabolism (e.g. propionic acid, methylmalonic acid), carnitine replenishes the free CoA pool of the cell and eliminates the toxic intermediates. Finally, carnitine is involved in the transport of products of the peroxisomal β-oxidation system from the peroxisomes into the mitochondria.

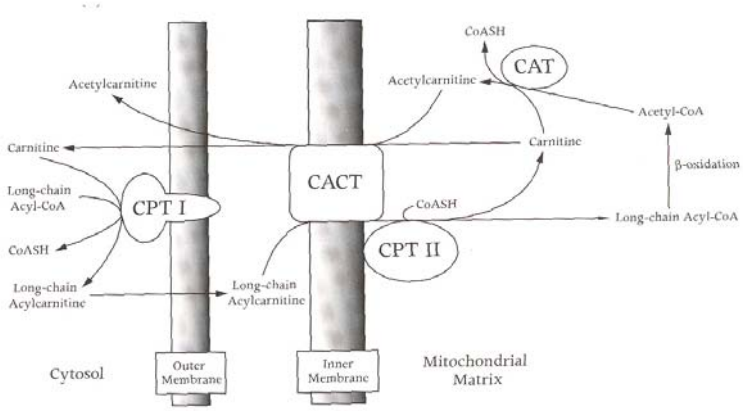

***Figure 1.**: The role of carnitine in the mitochondrial transport of long-chain fatty acids (Vaz et al., 2002).*

2.2. Other cellular functions of carnitine

Besides its classical metabolic role in energy-homeostasis, it has been proposed that carnitine is also functionally involved in many other cellular processes. Many experimental data stresses the involvement of carnitine in the immune response, also the clinical data of the increased susceptibility of primary carnitine deficient patients to infections leads to the assumption that carnitine might play a crucial role in the immune

processes. Besides extensive experimental data demonstrates the role of carnitine in both the extrensic and the intrinsic apoptotic pathways.

2.3. Primary carnitine deficiency

According to the etilogy primary and secondary carnitine deficiency can be differentiated in the literature. In primary systemic carnitine deficiency the cellular uptake of carnitine is mainly disturbed, while in secondary carnitine deficiency carnitine depletion is the secondary cause of other conditions or diseases.

Primary systemic carnitine deficiency (OMIM 212140), an autosomal recessive disorder, is caused by mutations of the *SLC22A5* gene leading to damage or loss of the function of the OCTN2 carnitine transporter protein. The genetically impaired or missing transporter is unable to take up carnitine from the circulation and transport it into the cells, thus the tissue carnitine stores are depleted. Since the renal reabsorption of carnitine was also shown to be OCTN2-dependent, the kidney is unable to reabsorp carnitine and therefore, the circulating carnitine concentration is also decreased. In circulating and tissue carnitine deficiency the mitochondrial utilization of long-chain fatty acids is impaired, energydepletion, and hypoketotic hypoglycaemia develop. Since the primary fuel for cardiac- and sceletal muscle cells as well as for hepatocytes and renal cells during starvation is provided by the long-chain fatty acids, the above mentioned organs are mainly affected in primary carnitine deficiency.

2.4. The human carnitine transporters

Since tissue carnitine concentration is 20-50 times higher as plasma carnitine levels, most tissues ensure their carnitine levels through active transport catalyzed by the high-affinity, Na^+-ion dependent carnitine transporter in the plasmamembrane, the organic cation transporter 2 protein (OCTN2). This transport system is also responsible for the tubular reabsorption of carnitine in the kidneys. The OCTN2 protein is composed of 557 aminoacids with a molecular mass of 63kDa. According to the aminoacid sequence analysis 12 putative transmembrane domains can be distinguished in its structure (**Fig. 2.**). *SLC22A5,* the gene encoding for OCTN2 is approximately 30 kb in size and is localized to the 5q31 chromosome region. The sequence of OCTN2 shows great homology (75,8%) to OCTN1 (encoded by the gene *SLC22A4*), one of the earlier discovered members of the organic cation transporter family, that also transports carnitine besides its primary substrate ergothioneine. Besides, another transporter, $ATB^{0,+}$ was discovered in the past decade, that was shown to transport carnitine as well. According to current data OCTN2 acts as the pimary physiological carnitine transporter, while OCTN1 and $ATB^{0,+}$ proteins can be regarded as aspecific carnitine transporters in the humans.

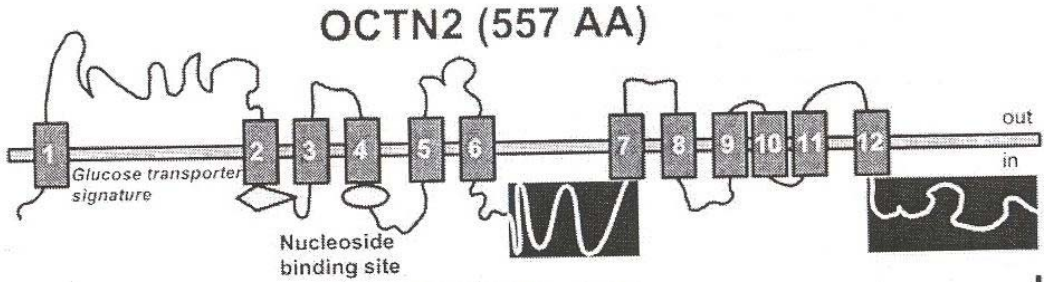

Figure 2.: The proposed structure of the OCTN2 protein, with N-terminal on the left, C-terminal on the right end. (Amat di San Filippo et al., 2003).

2.5. The clinical spectrum of OCTN2 mutations

Although the role of OCTN2 in carnitine uptake was first demonstrated in 1998 the condition of primary carnitine deficiency was already known in the literature. Currently primary carnitine deficiency (OMIM 212140) is regarded as the clinical syndrome associated with verified mutations in the *SLC22A5* gene.

In the cases reported so far in the literature the developing phenotype usually includes functional abnormality of the heart, skeletal muscle and the liver, however a wide phenotypic variability has already been reported. The appaerance of the clinical symptoms is usually between 1-6 years of age. The cardiac muscle and the liver are the two organs that are affected primarily and almost in all cases, or at a certain stage of the disease: in the full-blown picture usually dilative cardiomyopathy, in less severe forms subtle EKG- or ultrasound-abnormalities appear. The cardiomyopathy, as a potentially lethal manifestation of primary carnitine deficiency, shows a dramatic regression upon high-dose carnitine treatment: 100 mg/kg/day carnitine enables a quick clinical recovery and even EKG- and ultrasound abnormalities are corrected. The disrupted β-oxidation of FFA in the liver leads to hepathopathy, which can show in liver function elevations, in a more severe form it may lead to Reye-syndrome like episodes. Microvesicular lipid storage can also be observed in the sceletal muscle leading to hypotonia, excercise intolerance, muscle fatigue. Besides the typical hepatocardial and myopathic manifestations anaemia, mostly iron-deficient microcyter anaemia, can also occur in primary carnitine deficiency patients, as well as recurrent infections that respond very well to carnitine treatment. The phenotypic variability is very broad in OCTN2 defects, the developing phenotype can be quite different even wihin the same mutation and even within the same familiy. According to the cases reported so far in the literature no genotype-phenotype correlation can be predicted for the *SLC22A5* gene mutations.

On the molecular level the mutations detected so far can be simple, one basepair missense or nonsense mutations, insertions and deletions, or mutations involving several bases. The mutations affecting either the splicing mechanism or altering the proteinstructure can either lead to the synthesis of a functionally disturbed transporter or through the introduction of a premature stop codon no OCTN2 protein will be synthesised. There are reports that even heterozygote patients can show episodic heart manifestations, analysis of Japanese families showed an increased risk of adult- or late-onset cardiomyopathy in heterozygotes.

2.6. Carnitine transporter genes as susceptibility factors in multifactorial disease

More and more clinical syndromes and multifactorial diseases are shown to have a complex genetic background that contribute to its pathogenesis as susceptibility factors. An association between several inflammatory diseases, among them the inflammatory bowel disease and rheumatoid arthritis and the 5q31 cytokine cluster region has been proposed in recent years. This chromosome region contains several genes involved in immune- and inflammatory responses and it also harbors the genes for the two organic cation transporters (OCTN1 and OCTN2) that are involved in carnitine homeostasis.

Rheumatoid arthritis (RA) is a chronic inflammatory disease affecting 0.5%-1.0% of the world's population, including Hungary. Several studies have proposed that genetic susceptibility factors also contribute to its complex etiologic background. Combinations of the susceptibility alleles of various genes seem to be involved in the development of RA. A role for several non-HLA genes as susceptibility factors has recently been proposed, including the *TNFR2, PADI4, PTPN22, IL-1B, GSTM1, SLC22A4* and *RUNX1* genes. High-accuracy linkage disequilibrum mapping in the Japanese population demonstrated an association between RA and the *SLC22A4* gene, encoding OCTN1 the ergothioneine and aspecific carnitine transporter protein. In a case-control study a single nucleotide polymorphism called *slc2F2* (C6607T) was identified in intron 1 of the gene, which was associated with RA. In addition, another SNP denoted *runx1* (G24658C) in intron 6 of the gene that codes for the RUNX1 protein (Runt-related transcription factor-1), also showed a strong association with RA. The RUNX1 transcription factot was shown to repress the expression of the *SLC22A4* gene.

3. Objectives

1. Molecular genetic analysis of the *SLC22A5* gene from samples of Hungarian primary carnitine deficient patients, their relatives and their SIDS sibs.

2. Histological characterisation of the cardiac- and hepatic tissues from autopsy samples of SIDS cases with verified *SLC22A5* mutations.

3. Analysis of the carnitine homeostasis in OCTN2 deficient patients and heterozygote relatives with tandem mass spectrometric carnitine ester profile analysis.

4. Investigation of the role of carnitine in the immune response through the histopathologic analysis of immunetissues from autopsy samples of SIDS cases.

5. The role of susceptibility polymorphisms of the *SLC22A4* gene and of the regulating gene *RUNX1* in Hungarian rheumatoid arthritis patient cohorts.

6. As a reflection of the functional consequences of the proposed susceptibility polymorphisms of the *SLC22A4* és *RUNX1* genes the comparison of the circulating carnitine ester profile in rheumatoid arthritis patients and in controll groups.

4. Patients and methods

4.1. Patients

Several homozygote and heterozygote patients with OCTN2 deficinecy were diagnosed during the phenotypic, biochemical and genetic characterisation of primary carnitine deficiency. Our investigations focused on the analysis of the members of two extended Hungarian Roma families.

The first family we investigated is an extensive Hungarian Roma family from Eastern-Hungary (**Fig. 3.**). We could obtain DNA samples from 26 members of the family. Two SIDS cases (III/5 és III/8) and one perinatal death (III/11) was recorded in the family.

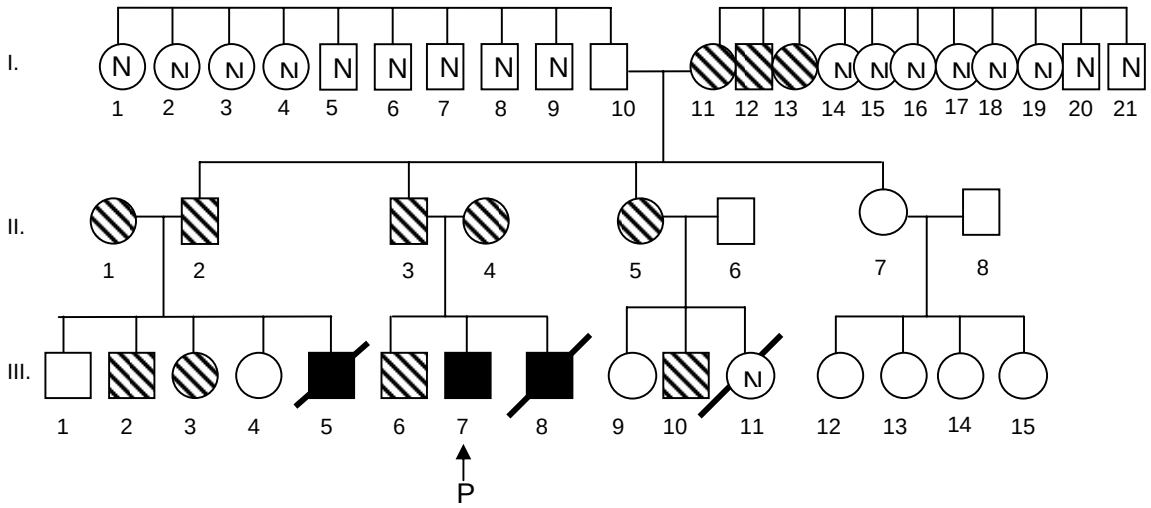

Figure 3.: Our first Hungarian family investigated with primary carnitine deficiency. Black symbols: homozygotes, white: normal, striped: heterozygotes, N: not examined. P: the proband currently under treatment

The second extended family (**Fig. 4**.) is not related to the first examined family, they have been living in another part of the country for at least three generations. In this family one verified SIDS case was noted. Consanguinity occured witihin the family.

Blood and serum samples of 209 patients with RA collected at the Department of Immunology and Rheumatology of the Medical Faculty of our university (169 females and 40 males; mean age ± SD: 57.3±14.6 years; 73% of all were rheumatoid factor positive) were used. All patients with RA were unrelated Caucasians and fulfilled the American College of Rheumatology criteria for RA. The control samples (n=217, 122 females and 95 males; mean age ± S.D., 56.5±10.4 years) were collected from healthy volunteers with no evidence or history of any systemic disease; special care was taken to exclude subjects with inflammatory arthritis from the control group. Patients and controls gave informed consent to all investigations performed in the study. The guidelines and regulations approved by the Ethical Committee of the Medical Faculty of our University were followed during all investigations.

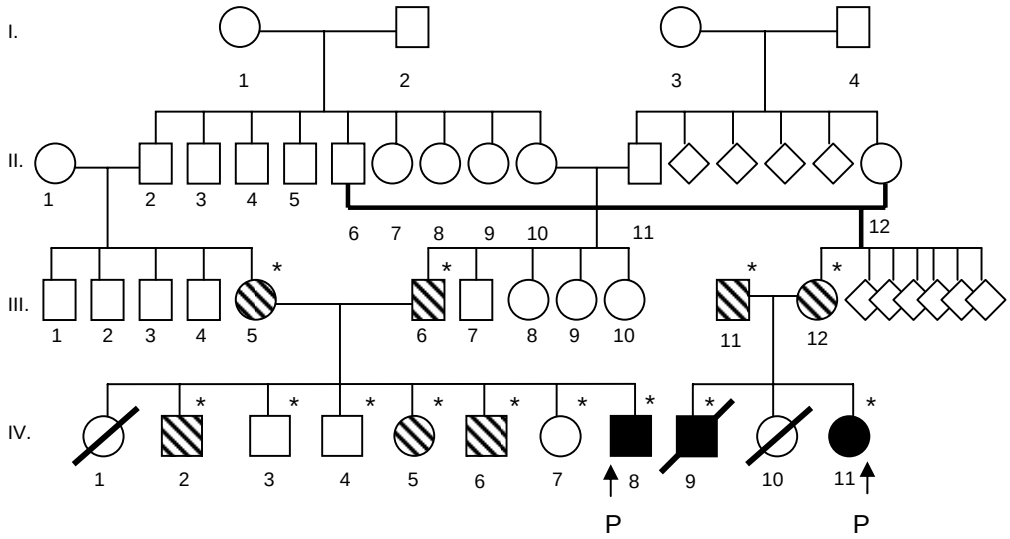

Figure 4.: The second family with primary carnitine deficiency.The asterisks indicate the familiy members examined.. Symbols see **Fig.3.**

4.2. Biochemical methods

Mass spectrometry analysis of the circulating carnitine esters

For carnitine ester profiling serum from each patient and control subject was collected after an overnight fasting period between 8:00 and 8:30 AM. Acylcarnitines were measured after methanolic extraction and derivatization as butyl esters by an isotope dilution mass spectrometry method using a Micromass Quattro Ultima ESI triple-quadrupole mass spectrometer. A Waters 2795 Alliance HPLC instrument was used for the solvent delivery maintaining a 0.1 ml/min stream of acetonitrile:water (80:20 v:v%). Ten µl of sample aliquots were injected into the flow at 4-min intervals. The free carnitine and all acylcarnitines were determined by ESI-MS/MS analysis using positive precursor ion scan of m/z 85; scan range was 200-550 m/z. The optimised capillary voltage, cone voltage and collision energy were 2.50kV, 55V and 26eV, respectively. Each sample was measured in triplicates starting with the injection step and the results are the means of the three determinations.

4.3. Molecular genetic methods

Analysis of the OCTN2 mutations and the OCTN1 and RUNX1 polymorphisms

Genomic DNA was extracted from peripheral blood by a routine desalting method. For the amplification of the 10 exons of the *SLC22A5* gene as well as for the analysis of the *SLC22A4* and *RUNX1* genes intron-based specific primer pairs were designed. We performed the polymerase chain reactions (PCR) on MJR PTC 200 thermal cyclers, similar conditions were applied to all exons. Our incubation mixture contained in 50 µl approx. 10-20 ng DNA, 3 pmol forward és reverse primers, 5 µl 10x reaction buffer, 2 U Taq polimerase enzyme, 0.2 mM of all four dNTPs and 1.5-2.5 mM $MgCl_2$. We applied standard reaction conditions, primer annealing was 1 minute on 58°C for the *SLC22A5*, and 1 minute on 60°C for the *RUNX1* and *SLC22A4* genes. The PCR products

were sequenced in both directions in an ABI Prism 310 Genetic Analyzer with a dye termination method (Applied Biosystems, Foster City, CA, USA). We also developed a RFLP method for quick genotyping of the samples: for the *SLC22A5* PCR products ***Bsl* I**, for the *RUNX1* ***BstN* I**, and for the *SLC22A4* PCR products ***Hph* I** enzyme digestion was used. The restriction fragments were separated by electrophoresis on 1% agarose gels containing ethidium bromide and visualized by UV transillumination.

4.4. Histopathologic methods

The haematoxylin-eosin (H&E) stained tissue sections from different lymphoreticular organs including bone marrow, spleen, tonsils, lymph node, and thymus were evaluated for histologic features and overall architecture.

Immunohistochemical analysis was performed with a DAKO autostainer (DAKO, Glostrup, Denmark) with use of a standard indirect avidin-biotin peroxidase detection method; the immunochemicals were also from DAKO.

Sections were immunostained for bcl-2 (1:50 dilution), IgD (1:200 dilution) and MIB-1 (1:150 dilution); for counterstainings Giemsa was used. The IgD was used as a specific indicator for mantle cells; the MIB-1 was used as a marker for proliferating and thereby functionally active cells inside the follicles. The anti- apoptotic bcl-2 immunoreaction was used for visualization of cells in the thymus. The immunohistochemical results were evaluated analyzing the dark brown positive signal distribution; location in the follicles or in the thymus cortex or medulla. The staining patterns of each antigen in our patient were compared with a normal control.

4.5. Statistical analysis

The distribution of the genotypes were compared using the χ^2 assay. For the comparison of the serum carnitine ester values the Student's *t* test for unpaired samples was used. The values are expressed as means ± SEM throughout, in three decimals for the carnitine esters with respect to the low levels of the long-chain carnitine esters.

5. Results

5.1. Molecular genetic results of the families with primary carnitine deficiency

The molecular genetic investigation of all three suspected patients (**Fig.3**.: III/7 and **Fig.4.**: IV/8 és IV/11) revealed a common mutation, a homozygous cytosin deletion in exon 5 of the *SLC22A5* gene (844 delC in the cDNA and 17081 delC in the genomic sequence). The mutation led to a frameshift and a premature stop codon in the 13th codon after the deletion site resulting in R282D aminoacid change or V295X truncation of the protein.

We could obtain DNA from 24 members of the first family (**Fig.3.**) and paraffin embedded blocks from two of the fatal cases. The two sudden death patients (III/5 and III/8) were also homozygous for the mutation, and a total of 12 carriers were verified (**Fig.3.**). In the second family we could obtain DNA from 12 members, both the parents

and 3 children were shown to be carriers of the common mutation. In the case of the sudden death patient (Fig.4. IV/9) DNA was extracted from a single block of lung tissue and the analysis verified the above mutation in a homozygous form.

5.2. Histologic features of the liver and heart tissues of the OCTN2 R282D homozygote patients

We performed the histopathologic examination of the liver, cardiac muscle and lung tissues obtained at autopsy from the SIDS cases in the investigated families.

Histology of tissues revealed lipid deposition vacuoles in the liver predominantly in the peripheral areas of the lobules, whereas the centro-lobular regions of the acini were less or not affected (**Fig. 5.**, H&E stainings in columnes A and B). The younger patient who died at 6 months of age (Family 1, III/5) had much more severe infiltration (**Fig. 5.** row 2) compared with the older patient (Family 1, III/8, **Fig.5.** row 1).

In the heart tissues different size of nuclei were seen providing microscopic evidence for muscle hypertrophy. In both patients (III/8 row 3 and III/5 row 4) focal lipid vacuoles were seen much less intensively than in the liver, the major lipid globules localized mainly into the subendocardial areas (**Fig. 5**. column A in rows 3 and 4). In other areas, like in the ventricular walls smaller aggregates of lipid droplets could be observed, the vacuoles were apparently separated from each other by membranes (**Fig. 5.**; column B, rows 3 and 4).

In the autopsy lung tissue of patient IV/9 in Family 2. (**Fig.4.**) only signs of emphysema was seen without any further characteristic pathologic signs (not shown). Staining with PAS (**Fig. 5.**, column C) showed severe exhaustion of the glycogen reserves. In age and sex matched controls who died after caloric depression PAS positive granulates could still be detected in the liver (inserts at the bottom right corners), while the control hearts did not differ significantly from the patient's tissues. In tissues of fed individuals a striking difference of PAS positive substance accumulation was seen (upper right corners).

5.3. Plasma carnitine ester profile in homozygous and heterozygous OCTN2 deficiency

In the investigated families we could identify three homozygous patients in whom carnitine supplementation was started in time. In all three patients we detected severely decreased free and total carnitine levels before treatment. In the treated homozygote patient of the first family (**Fig. 3.**: III/7) and in the treated girl of the second family (**Fig.4.**: IV/11) we were only able to perform plasma carnitine measurements with the radiochemical carnitine assay. Prior to treatment the postprandial levels of total and free carnitine were decreased, these values increased after the introduction of treatment, but did not reach control levels.

In the second family plasma carnitine ester profile of the affected patient (**Fig.4.**:IV/8) and the carrier family members was determined with tandem mass spectrometry, the results are shown in **Table 1. (**the results are in umol/l**)**. The controls were 6 healthy pediatric patients (4 males and 2 females matched pair wise by age to the three heterozygous sibs IV/2, 5 and 6). Prior to treatment we found a dramatic decrease of the free carnitine and all carnitine esters in the proband (**Fig.4.**:IV/8). The free carnitine levels of the 3 heterozygote siblings and of the parents were 62.2% of the

controls, while the levels of the individual carnitine esters were in average 48.4% of the control values. After two months of carnitine treatment in the proband the level of free carnitine and all individual esters increased, in average it reached 41.2% of the control levels. After 13 months of carnitine supplementation there was a further increase in the free carnitine level, as well as in the levels of the individual esters, however without reaching that of the age-matched controls. Before treatment the ratios of the carnitine esters were as follows: medium<long<short chain (in the controls: long<medium<short chain); while after 13 months of the treatment it changed to long<medium<short chain esters. The largest increase was seen for the acetylcarnitine after 2 months and for the butyrylcarnitine after 13 months of treatment.

5.4. Histopathologic abnormalities of the lymphoreticular tissues in primary carnitine deficiency

We performed the histologic and immunohistologic analysis of lymphoreticular tissues obtained from the autopsy sample of one of the SIDS cases in Family 1. (**Fig.3.:** III/8). Histopathological examination of the haematopoetic and immune organs revealed significant structural alterations being indicative of cellular dysfunction (**Fig. 6.**). The red bone marrow (**Fig. 6.**: row 1) harbored numerous enlarged secondary follicles that corresponded to definitely pathological ones (slide A1). The mantle zones were separated from the germinal centers of the follicles in all of the examined tissues: identifiable IgD positive cells were detected in the mantle zone of the bone marrow (B1), lymph node (B2) and in the spleen (B3). The germinal center compartment exhibited pathological hallmarks, which implied a decreased proliferation capacity in the bone marrow (C1), in the lymph nodes (C2), and also in the spleen (C3), as indicated by the decrease of the dark labels of the MIB-1 immunostainings.

Taking the spleen as an example for the coexisting morphological structures (A5 and B5) many follicles exhibited also a significantly increased apoptosis as evidenced by the large number of apoptotic bodies (basophil, diminished debris like materials; arrows in block B5). The extracellular location of the apoptotic bodies is indicative for a saturated phagocytic capacity of the intrafollicular macrophages (B5, arrowheads). This phenomenon was also detectable in numerous follicles of the other lymphoreticular tissues, such as in the lymph nodes, bone marrow and slightly in the tonsils (not shown). As a next stage of the cellular damage the proliferation and apoptosis diminished largely: in follicles captured in this stage hypocellularity as well as deposition of homogenous, eosinophil fibrinous material were seen (C5, arrows). This morphology was reminiscent to atrophic, so called “burned-out” follicles (rows 1-3 and slide C5); and was represented besides the follicles of the bone marrow (row 1 and slide C5) in the spleen, lymph nodes and at a much modest incidence rate in the tonsils (not shown).

The ultrastructure of the thymus did not show morphologically discernible alterations (**Fig. 6.**: row 6); normal architecture with H&E staining was seen (A6).

5.5. Investigation of the polymorphisms of the carnitine transporter and regulator genes as susceptibility factors in Hungarian rheumatoid arthritis patients

We investigated the *slc2F2* polymorphism (C6607T) of the first intron of the *SLC22A4* gene and the *runx1* polymorphism (G24658C) in the sixth intron of the *RUNX1* gene in blood samples of 209 rheumatoid arthritis patients and 217 controls. The distribution of different genotypes found in the patients and controls are shown in **Table 2.** All genotypes for both SNPs examined were in Hardy-Weinberg equilibrum in all groups. No significant differences could be found comparing the genotype prevalences of the patients and the controls, for either *slc2F2* or *runx1* SNP. The same result was obtained when we compared the distribution patterns of the particular alleles for each polymorphism in the two groups (C or T in *slc2F2* and G or C in *runx1*).

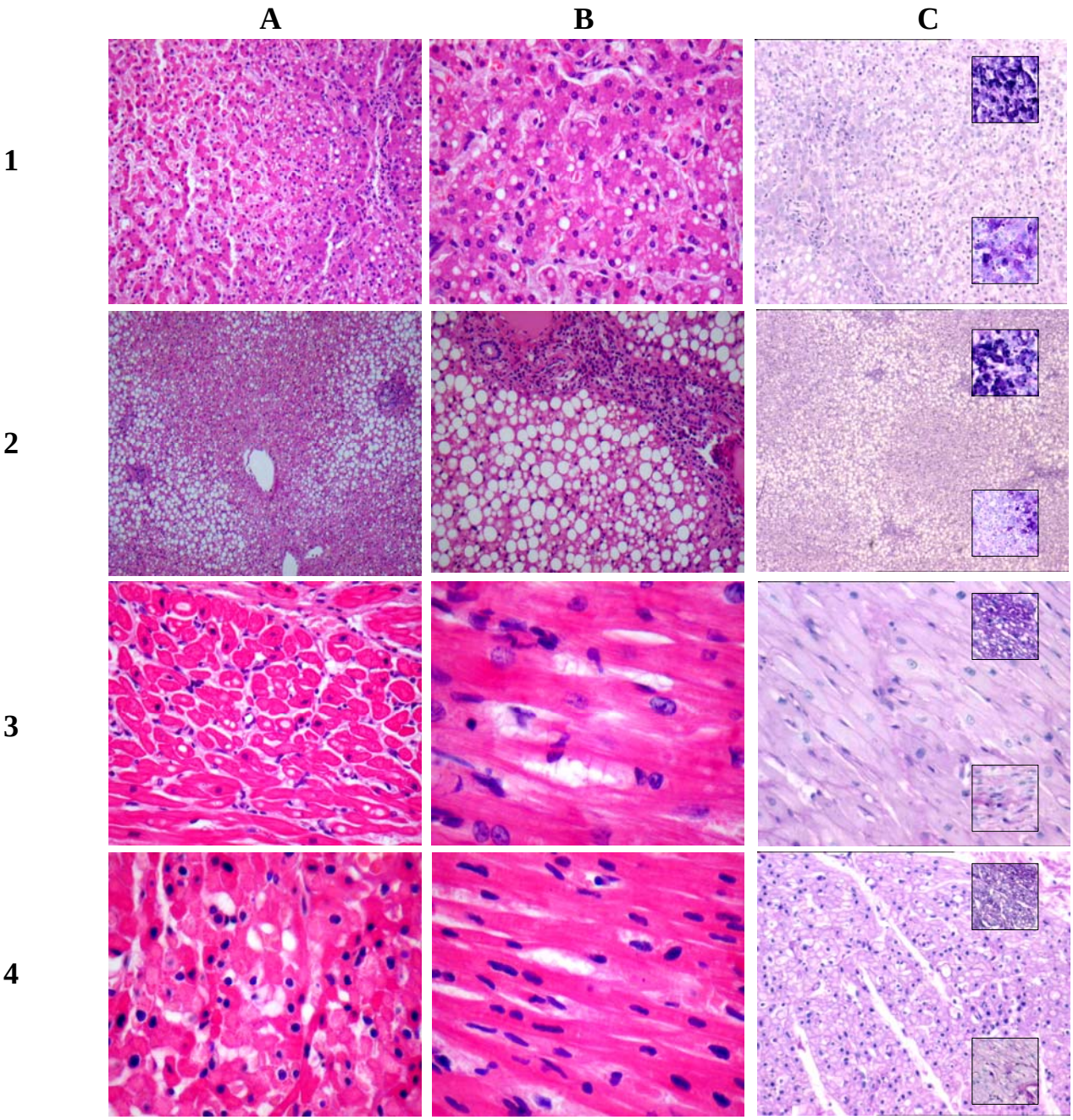

Figure 5.: Histology of liver and heart tissues in OCTN2 deficiency
Rows 1.(liver) and 3.(heart) are from Family 1. patient III/8 who died suddenly at age of 2 years and 9 months, below them rows 2. and 4. are blocks from patient III/5 who died as a typical SIDS victim at age of 6 months. (H&E stainings in columns A-B, PAS in column C). In PAS stained slides the inserts are from probably fed and from starved controls at the upper right and bottom right corners, respectively.

Table I. **Plasma carnitine esters in homozygote and heterozygote OCTN2 deficiency.** Values are µmol/l, means ± SEM for three separate determinations; in the % columns the values are expressed as percents of the controls.

	proband[1]						heterozygote		parents[2]		controls[2] (n=6)
	before treatment	%	2 months after treatment	%	13 months after treatment	%	siblings[2] (n=3)	%		%	100%
free carnitine	1,377 ± 0,050	4,2	12,790 ± 0,129	39,0	15,943 ± 0,233	48,6	20,390 ± 1,154	62,2	20,203 ± 2,230	61,63	32,782 ± 4,218
Short-chain acylcarnitines											
C2-carnitine	3,847 ± 0,083	17,9	19,753 ± 0,296	92,0	11,027 ± 0,324	51,3	10,072 ± 0,097	46,9	10,682 ± 1,045	49,74	21,477 ± 2,893
C3-carnitine	0,010 ± 0,001	3,1	0,133 ± 0,003	41,0	0,233 ± 0,009	71,7	0,248 ± 0,044	76,1	0,170 ± 0,007	52,22	0,326 ± 0,050
C4-carnitine	0,060 ± 0,012	30,8	0,153 ± 0,015	78,6	0,270 ± 0,026	138,5	0,059 ± 0,010	30,2	0,047 ± 0,027	23,93	0,195 ± 0,032
C5-carnitine	0,027 ± 0,007	6,8	0,053 ± 0,003	13,6	0,167 ± 0,003	42,7	0,194 ± 0,021	49,7	0,113 ± 0,030	28,98	0,391 ± 0,051
C5:1-carnitine	0,013 ± 0,003	26,4	0,013 ± 0,003	26,4	0,030 ± 0,000	59,3	0,029 ± 0,001	57,1	0,028 ± 0,002	56,04	0,051 ± 0,005
Medium-chain acylcarnitines											
C6-carnitine	0,037 ± 0,003	34,4	0,080 ± 0,010	75,0	0,117 ± 0,012	109,7	0,053 ± 0,003	50,0	0,052 ± 0,022	48,44	0,107 ± 0,007
C8-carnitine	0,010 ± 0,001	6,8	0,023 ± 0,003	15,9	0,040 ± 0,010	27,3	0,046 ± 0,006	31,1	0,078 ± 0,018	53,41	0,147 ± 0,022
C8:1-carnitine	0,007 ± 0,007	9,2	0,037 ± 0,003	50,8	0,023 ± 0,003	31,8	0,042 ± 0,001	58,5	0,045 ± 0,002	62,31	0,072 ± 0,009
C10-carnitine	0,007 ± 0,003	3,8	0,010 ± 0,001	5,8	0,027 ± 0,003	15,6	0,043 ± 0,008	25,0	0,087 ± 0,030	50,00	0,173 ± 0,038
C10:1-carnitine	0,057 ± 0,012	44,0	0,010 ± 0,000	7,7	0,017 ± 0,003	13,1	0,047 ± 0,015	36,1	0,088 ± 0,012	68,24	0,129 ± 0,020
C10:2-carnitine	0,003 ± 0,003	16,7	0,007 ± 0,003	33,3	0,010 ± 0,006	50,0	0,011 ± 0,002	55,6	0,012 ± 0,005	58,33	0,020 ± 0,002
C12-carnitine	0,020 ± 0,006	36,7	0,033 ± 0,003	61,2	0,033 ± 0,003	60,6	0,017 ± 0,002	30,6	0,032 ± 0,005	58,16	0,054 ± 0,010
C12:1-carnitine	0,010 ± 0,006	16,2	0,013 ± 0,003	21,6	0,023 ± 0,003	37,3	0,013 ± 0,004	21,6	0,027 ± 0,000	43,24	0,062 ± 0,015
Long-chain acylcarnitines											
C14-carnitine	0,010 ± 0,001	24,7	0,013 ± 0,003	32,9	0,020 ± 0,000	49,3	0,014 ± 0,001	35,6	0,017 ± 0,003	41,10	0,041 ± 0,004
C14:1-carnitine	0,020 ± 0,006	32,4	0,027 ± 0,003	43,2	0,017 ± 0,003	27,6	0,017 ± 0,005	27,0	0,027 ± 0,007	43,24	0,062 ± 0,013
C14:2-carnitine	0,010 ± 0,001	26,9	0,010 ± 0,001	26,9	0,013 ± 0,003	34,9	0,014 ± 0,005	38,8	0,020 ± 0,003	53,73	0,037 ± 0,008
C16-carnitine	0,040 ± 0,001	28,1	0,060 ± 0,006	42,2	0,067 ± 0,009	47,1	0,071 ± 0,008	50,0	0,083 ± 0,003	58,59	0,142 ± 0,019
C18-carnitine	0,033 ± 0,007	42,3	0,043 ± 0,009	54,9	0,043 ± 0,003	54,5	0,044 ± 0,001	56,3	0,057 ± 0,000	71,83	0,079 ± 0,009
C18:1-carnitine	0,050 ± 0,012	23,0	0,050 ± 0,006	23,0	0,040 ± 0,010	18,4	0,078 ± 0,005	35,7	0,115 ± 0,012	52,81	0,218 ± 0,034
C18:2-carnitine	0,020 ± 0,001	16,1	0,030 ± 0,006	24,1	0,013 ± 0,003	10,4	0,056 ± 0,005	44,6	0,095 ± 0,025	76,34	0,124 ± 0,015
total ester	4,290 ± 0,055	17,9	20,553 ± 0,357	86,0	12,230 ± 0,349	51,2	11,169 ± 0,201	47,3	11,873 ± 1,060	50,17	23,906 ± 3,113
total carnitine	5,667 ± 0,043	10,0	33,343 ± 0,464	58,8	28,173 ± 0,586	49,7	31,559 ± 1,343	55,8	32,077 ± 1,170	56,75	56,688 ± 6,792

1: mean±SEM of three parallel measurements

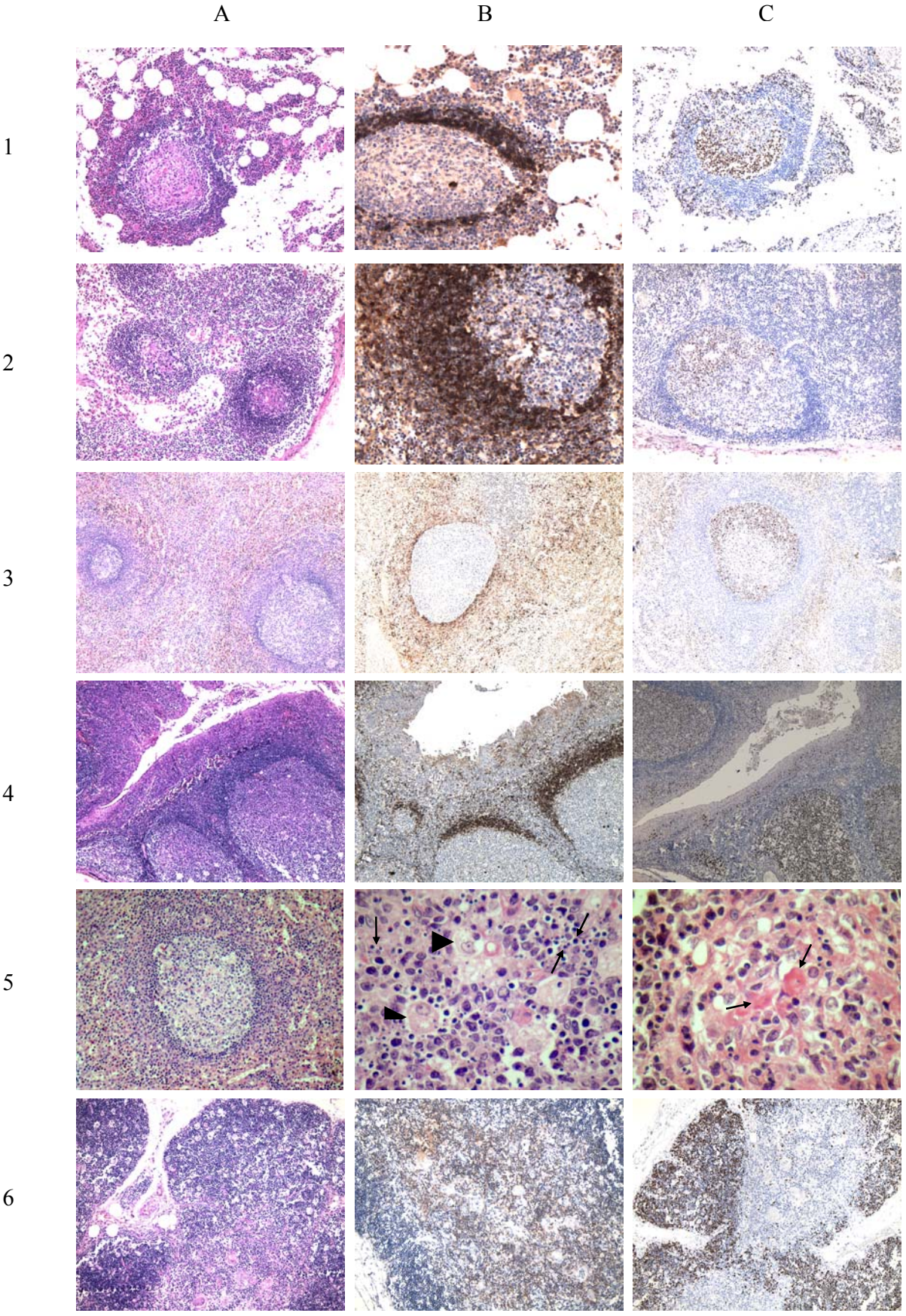
A
B
C
1
2
3
4
5
6

Figure 6.: Histopathology of the bone marrow, lymph nodes, spleen, tonsils and thymus in OCTN2 deficiency
row 1.: bone marrow, row 2.: lymph node, row 3.: spleen, row 4.: pharyngeal tonsil, row 5.: different stages of the pathologic germinal center reactions, A5 and B5: spleen, C5: bone marrow, row 6.: thymus (for details see description in text)
Stainings and immunohistochemical stainings: all of column A, B5 and C5 H&E staining. B1 – B4 slides: IgD; C1 – C4 and C6 slides: MIB-1; and block B6: bcl-2 immunoreaction. In all immunoreactions the positive signal is dark brown.
Magnification: all slides are 40x magnified, except A5 (100x) and B5 and C5 (400x).

Table 2. Distribution of the *slc2f2* and *runx1* genotypes and allele frequencies in the groups of patients with rheumatoid arthritis and controls.

SNP		patients n=209	controls n=217
slc2F2			
genotype	CC	180 (86.1%)	181 (83.4%)
	CT	28 (13.4%)	35 (16.1%)
	TT	1 (0.5%)	1 (0.5%)
alleles	C	92.8%	91.5%
	T	7.2%	8.5%
runx1			
genotype	GG	81 (38.8%)	98 (45.2%)
	GC	100 (47.8%)	94 (43.3%9
	CC	28 (13.4%)	25 (11.5%)
alleles	G	62.7%	66.8%
	C	37.3%	33.2%

6. Summary of the results and conclusions

1. The molecular genetic analysis of the *SLC22A5* gene encoding the high-affinity physiological carnitine transporter OCTN2 in two extended Hungarian Roma families revealed one common mutation, a 844delC mutation leading to R282D in the protein. The mutation causes systemic carnitine deficiency either through the formation of an unstable mRNA or through the rapid degradation of the synthesized truncated protein.

2. The primary clinical manifestation of the mutation involves the functional abnormality of the heart and liver with recurrent infections and iron-deficient anaemia, however, a variable phenotype can be seen even within a family. Oral carnitine supplementation results in dramatic regression of the symptoms.

3. We verified the above mentioned mutation in homozygote form in three SIDS cases of the investigated families. Clinically, we found a somewhat new phenotypic manifestation that corresponds to a lethal hepatocardial syndrome. Through the extensive histological examination of the autopsy tissues as a novelty we were the first to demonstrate lipiddeposition in cardiac muscle in primary carnitine deficiency.

4. Analysing the alterations of carnitine homeostasis in homozygote and hetrozygote primary carnitine deficiency, we found that in homozygotes besides the dramatic decrease in free carnitine all circulating carnitine esters are severely depleted, which probably results in serious metabolic disturbances. On the other hand, the detected decrease in the concentrations of the free and esterified carnitines in heterozygotes may reflect ongoing pathological changes on the cellular level.

5. The histologic examination of the lymphoreticular tissues of SIDS cases with verified *SLC22A5* mutations revealed cellular dysfunctions in the germinal centers of the lymphoid tissues reflecting a disturbed antigen driven affinity maturation of the B cells in primary carnitine deficiency. The results give morphological evidence of the functional contribution of carnitine in plasmacell maturation, however, the precise molecular mechanisms need still to be elucidated.

6. The common detected mutation in several non-consanguinous grandparent lines of our investigated families reflects the wide-spread incidence of the mutation in the Hungarian Roma poulation. The potentially lethal manifestations of primary carnitine deficiency (cardiomyopathy, SIDS), the available and effective treatment with oral carnitine supplementation underly the importance of the screening for this disease. Carnitine ester profile analysis by tandem mass spectrometry as developed in our institute provides an effective and rapid method for screening, with catious analysis of the samples even heterozygotes can be detected.

7. We performed molecular genetic studies in Hungarian rheumatoid arthritis patients for the determination of the role of proposed susceptibility polymorphisms of carnitine transporter genes and regulator genes in the development of multifactorial diseases. Our results do ot confirm the universal and population-independent susceptibility role of the *SLC22A4* C6607T and *RUNX1* G24658C variants for rheumatoid arthritis. Further studies on larger population samples are needed to clarify the real ethiopathologic role of a proposed susceptibility variant found in one population sample.

7. List of publications

The thesis is based on the following publications:

1. B. Melegh, J. Bene, G. Mogyorósy, V. Havasi, ***K. Komlósi***, L. Pajor, É. Oláh, Gy. Kispál, B. Sumegi, K. Méhes. Phenotypic Manifestations of the OCTN2 V295X Mutation: Sudden Infant Death and Carnitine-Responsive Cardiomyopathy in Roma Families. Am J Med Genet A. 2004;131A(2):121-6. IF: 3.659

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1. J.L. Környei, A. Oszter, K.A. Kovács, Z. Vértes, **K.M. Komlósi**, P.M. Gőcze, M.Vértes. Anti-mitogenic action of opioid peptides on epidermal growth factor-stimulated uterine cells. Eur. J. Pharmacol. 2001, 414: 159-167 IF:2.164

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Other congress abstracts and posters:

1. J.L. Környei, K.A. Kovács, A. Oszter, Z. Vértes, **K.M. Komlósi**, M. Vértes: Altered regulation of cell proliferation by opioid peptides in human uterine leiomyoma cells. Joint Meeting of The Physiological Society and the Hungarian Physiological Society, Hungarian Academy of Sciences, Budapest, Hungary**,** J. Physiol. (London) 2000, 526: 20P-21P IF:

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