Charles University in Prague

1st Faculty of Medicine

PhD Thesis summary

Clinical impact of cytochrome *c* oxidase disorders

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Prague 2008

Postgraduate studies of Biomedicine

Charles University in Prague and Academy of Sciencies of Czech Republic

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Opponents:

Abstract of PhD thesis sent:

Defense of PhD thesis: 2008 in front of the Biochemistry and Pathobiochemistry Commission.

The full text of the PhD thesis is available at the 1st Faculty of Medicine, Charles University in Prague.

The PhD thesis was elaborated during Postgraduate Studies in Biomedicine at the 1st Faculty of Medicine of Charles University in Prague and Academy of Sciences of the Czech Republic in years 2002-2008 and was supported by VZ MSM 0021620806 and GAČR 303/03/H065.

Contents

Souhrn

Práce vznikla v Laboratoři pro studium mitochondriálních poruch Kliniky dětského a dorostového lékařství 1. LF UK v Praze, která je diagnostickým centrem pro pacienty s mitochondriálním onemocněním z České republiky a ze Slovenska.

Ve spolupráci s The Division of Metabolic Diseases in Department of Paediatrics, The Children's Memorial Health Institute, Warsaw, Poland byla provedena retrospektivní multicentrická studie u 180 dětí s poruchou cytochrom c oxidázy (COX), která byla zaměřena na analýzu klinických projevů onemocnění, molekulární podstatu onemocnění a prognózu. U většiny pacientů mělo onemocnění rychle progredující průběh a prognóza byla většinou krajně nepříznivá. Genetické poradenství v postižených rodinách vyžaduje detailní objasnění molekulární podstaty poruchy COX. U 42 % dětí byly nalezeny mutace v mitochondriální DNA (mtDNA) nebo v nukleárně kódovaných genech pro proteiny surf1 a sco2, které se podílejí na assemblaci COX. Zatímco pacienti s mutacemi v *SURF1* a *SCO2* genech měli izolovanou poruchu COX, u pacientů s mutacemi v mtDNA byla porucha COX kombinována se snížením aktivit jednoho nebo více dalších komplexů dýchacího řetězce.

Ve druhé části práce byly stanoveny referenční hodnoty pro aktivity komplexů dýchacího řetězce v izolovaných trombocytech u 161 mužů a žen ve věku mezi 0,5 a 35 roky. Ukázali jsme, že izolované trombocyty lze použít pro diagnostiku mitochondriálních poruch. Současně bylo ve skupině 36 žen s mentální anorexií ve věku 18 až 35 let s BMI 15±1,7 zjištěno signifikantní zvýšení aktivit komplexů dýchacího řetězce I a II v porovnání s kontrolní skupinou 37 zdravých žen s BMI 21±2.2.

Třetí část práce byla zaměřena na studium aktivit a proteinového množství komplexů dýchacího řetězce a pyruvátdehydrogenázy (PDH) v mitochondriích izolovaných ze svalové tkáně získané při autopsii 19 nedonošených novorozenců. V porovnání s biochemickými analýzami u skupiny starších dětí byly nalezeny signifikantně nižší aktivity PDH a komplexů dýchacího řetězce III a IV.

Získané poznatky přispěly:

- k lepšímu pochopení přirozeného průběhu onemocnění („natural course of disease") a korelaci mezi genotypem a fenotypem u dětí s poruchou cytochrom c oxidázy

- získáním širokých věkových norem k možnosti používat izolované trombocyty ke studiu aktivit komplexů dýchacího řetězce u pacientů s primární nebo sekundární poruchou mitochondriálního energetického metabolismu

- k prohloubení znalostí o funkci mitochondriálního energetického metabolismu u nedonošených dětí v v časných fázích vývoje

Summary

This thesis has been worked out in The Laboratory for Study of Mitochondrial Disorders, Department of Paediatrics, 1st Faculty of Medicine, Charles University in Prague.

A retrospective multicentric study in 180 children with cytochrome *c* oxidase (COX) deficiency was designed in cooperation with the Division of Metabolic Diseases in Department of Paediatrics, The Children's Memorial Health Institute, Warsaw, Poland. The survey was focused on clinical manifestation, molecular background and prognosis of the disease and showed that COX deficiency in childhood represents a heterogeneous group of diseases with significantly unfavourable prognosis. Genetic counselling in affected families requires detailed characterization of COX deficiency at the molecular level. An underlying genetic defect was found in 42 % of patients by detection of mutation in mitochondrial DNA (mtDNA) or in nuclear coded genes for proteins surf1 and sco2 that contribute to COX assemblation. Isolated defects of COX were found in patients with mutations in *SURF1* or *SCO2* genes, whereas in the patients with mutations in mtDNA was the defect of COX combined with decreased activities of one or more other respiratory chain complexes.

In the second part of the work, activities of respiratory chain complexes in isolated platelets were analysed in large control group of 161 children and adults in age between 0.5 and 35 years. We showed that isolated platelets are suitable biological material for diagnostics of generalized mitochondrial disorders. In addition, a significant difference in mitochondrial energetic metabolism with increased activities of respiratory chain complexes I and II was found in a group of 36 females with anorexia nervosa and BMI 15±1.7 in comparison with the age related controls.

In the last part of the work, the activities and protein amount of respiratory chain complexes and pyruvate dehydrogenase (PDH) were analysed in 19 premature neonates in isolated muscle mitochondria obtained at autopsy. Significant age-related differences between premature neonates and older children were observed. Especially, the activities of PDH and respiratory chain complexes III and IV were significantly lower in premature neonates.

The results enabled:

- better understanding of the natural course of disease in children with cytochrome c oxidase deficiency with the respect to molecular background of the disease
- age related reference range for activities of respiratory chain complexes in isolated platelets in the group of 161 children and adults enable to improve biochemical diagnostics of mitochondrial disorders in patients with generalized tissues involvement
- better understanding of mitochondrial energy generating system in early stage of neonatal development

1. Introduction

The oldest known evidence of life was found by J.W.Schopf in 3.5 billion years old mineral from Apex chert at Marble bar, Western Australia (Schopf 1993). 2.5 billion years ago, the atmosphere in the Earth changed from reducing to oxidising. 2.1 billion years ago, bacteria assembling ATP using oxygen for production of energy were incorporated into the ancestor of eukaryotic cell (Han and Runnegar 1992). Somehow it happened that the two cells did not kill each other and instead of it, they began to cooperate and by the oxidative phosphorylation system (OXPHOS) they could very efficiently gain the energy from substrates they consumed. They were very successful and thus grew faster than their rivals. The eukaryotic cells originated and the incorporated ATP producing bacteria changed into mitochondria. In mammalian cells, more than 90% of ATP is produced by OXPHOS composed of four respiratory chain complexes (RC) and ATP synthase in the inner mitochondrial membrane.

Mitochondrial DNA in human cells lost most genes except mtDNA genes encoding several key structural proteins responsible for the main function of mitochondria – oxidative phosphorylation, and genes for mitochondrial tRNA and rRNA. Eukaryotic nuclear DNA provides the rest of OXPHOS genes and proteins. In total, more then 1000 proteins participate in mitochondrial functions and the cooperation between mitochondrial and nuclear DNA makes the mitochondrial metabolism very complex.

Of course, the complexity comes together with sensitivity to the malfunctions of parts of system. The mitochondrial disorders represent a heterogeneous group of diseases with unfavourable prognosis and they are considered as one of the most common group of metabolic diseases in humans. Our laboratory focuses on broad spectrum of mitochondrial diseases and the aim of my study as a paediatrician is to characterize mitochondrial diseases in children.

2. Aims of the study

Between 2000 and 2006 I was working in the Laboratory for studies of mitochondrial disorders, Faculty of Medicine, Charles University in Prague. I contributed in implementing and optimising new biochemical methods for studies of respiratory chain complexes and participated on diagnostics of mitochondrial diseases in patients from Czech Republic and Slovakia. I also visited the Division of Metabolic Diseases in Department of Paediatrics, The Children's Memorial Health Institute, Warsaw, Poland, where professor Pronicka gave me a chance to study her patients with cytochrome *c* oxidase deficiencies that is a most frequent group of mitochondrial disorders in children. Then I selected a group of our patients with cytochrome *c* deficiencies and described the clinical features of children with this specific characteristic of mitochondrial disorder.

This thesis is based on combined clinical, biochemical and molecular analyses of 180 children from Czech Republic, Slovakia and Poland with cytochrome *c* oxidase deficiency.

The specific aims of my study were:

1) to characterize a spectrum of clinical symptoms in children with cytochrome *c* oxidase deficiencies and to characterize a natural course of disease

2) a) to find out whether isolated platelets are usable biological material for the measurement of activities of respiratory chain complexes in humans

   b) to apply this method in patients with mitochondrial diseases and eventually, in patients with secondary defects of oxidative phosphorylation system

3) to analyze the activities and amounts of respiratory chain complexes and pyruvate dehydrogenase in premature neonates in comparison with older children

3. Material and metods

Material

1) The retrospective, multicentric study of cytochrome *c* oxidase deficiency included 180 COX-deficient children at the age of 1 month to 18 years from 147 families identified within last ten years in Poland, Czech Republic and Slovakia, representing in total a population of 53 million inhabitants. 58 boys and 55 girls were born in Poland (113 cases/38 million inhabitants) and 36 boys and 31 girls were born in the Czech Republic and Slovakia (67 cases/15 million inhabitants). The overall male/female ratio was 1.09. The medical reports from all 180 children at the time of COX deficiency diagnosis were available. After the diagnosis had been made, 138 children were followed on to assess clinical features, prognosis and molecular bases of the COX deficiency. No information about present status of the remaining 42 children was available due to lost contact with the families.

2) After the informed consent, activities of respiratory chain complexes were analysed in isolated platelets from

a) 161 people with no clinical suspition to mitochondrial disorders, 70 of them were males and 91 were females. According to age, four groups were made (32 children in age of 0.5-2 years, 50 children in age of 3-9 years, 39 teenagers in age 10-19 years and 40 young adults in age 20-35 years).

b) 36 unrelated female patients with anorexia nervosa at the age between 18 and 35 years with body mass index (BMI) 15 ± 1.7. The restrictive form of anorexia nervosa was present in 19 females, the other 17 females had purging form of anorexia nervosa. Blood samples for the enzymatic analyses were obtained in 14 females during the first week after admission to the hospital and in 22 females between the second and eighth week of hospitalisation. The age related control group consisted of 37 females with BMI 21 ± 2.2 at the age between 18 and 35 years.

3) Isolated muscle mitochondria were used in study of the function of oxidative phosphorylation system function in premature neonates. Samples of muscle tissues (m. triceps surae or m. tibialis anterior) from 19 premature neonates (12 boys and 7 girls, 2× twins) with birth weight 790±295 g (range 380–1460 g) and gestational age 25±3 weeks (range 23–35 weeks) were obtained 1–2 h after death and immediately frozen in liquid nitrogen and stored at −80 °C. Thirteen neonates were delivered by the Caesarean section, the other six vaginally. Seven neonates were born after premature rupture of membranes, eleven neonates due to mother's sepsis, chorioamniitis or initiation of premature parturition with unsuccessful tocolysis, one girl was delivered due to preeclampsia in the mother. The most common clinical complications were enteral sepsis or necrotizing enterocolitis after one or two weeks of relative good clinical conditions (9 neonates), severe hypoxic–ischemic encephalopathy (1 neonates), intracranial haemorrhage (5 neonates), apoplexia (2 neonates). Anaemia, thrombocytopenia, leucopenia as a consequence of bone marrow failure and low glucose tolerance resulting in hyperlactacidaemia were found in most patients. All neonates were treated at NICU including ventilatory support, antibiotics, catecholamines and others. Most neonates died within the first month of life, the oldest infant at the age of 62 days. Fourteen patients died due to multi-organ dysfunction syndrome, two due to cardiac failure, two due intracranial haemorrhage and one due to meningitis with systemic inflammatory response. Two control groups of children were established from 20 “disease free controls” at the age of 0.5–2 years and 26 “disease free” controls at the age of 3–18 years. These children were investigated due to clinical suspicion of neuromuscular diseases and were assigned retrospectively after no evidence of PDH deficiency and respiratory chain disorders were detected.

Metods

Clinical symptoms

In study of cytochrome *c* oxidase deficiencies, the following clinical symptoms relevant to the course of the disease were defined:
Failure to thrive as growth rate below 3 percentiles during infancy and early childhood, and in older children markedly decreased growth rate crossing two major growth percentiles (i.e., from above 75th to below 25th percentile)
Encephalopathy as functional impairment of the central nervous system with developmental delay and/or regression, repeated seizures, pathologic pyramidal, extrapyramidal or cerebellar symptoms and cortical and periventricular atrophy, hypotonic syndrome with generalized peripheral hypo- or hyperreflexia, Leigh syndrome with typical symmetric necrotic lesions in basal ganglia and/or in the brain stem using MRI or at autopsy
Cardiac involvement as "generally or partially hypertrophic" left ventricle in two-dimensional echo-Doppler investigation, changed dimension of the left ventricle posterior wall and/or the intra-ventricular septum in diastole measured by M-mode beyond 2 SD (hypertrophic cardiomyopathy), or isolated heart conduction abnormalities
Hepatopathy as acute and chronic liver disease (liver failure, fibrosis, cirrhosis, steatosis), or isolated persistent increase in serum alanine-aminotransferase and aspartate-aminotransferase without elevation of serum creatine kinase
Endocrinopathy, such as diabetes mellitus, hypothyroidism, pituitary dysfunction or adrenal insufficiency
Nephropathy, presenting as tubulopathy (proximal dysfunction of Fanconi type, hypermagnesiuria) or progressive renal failure.

Biochemical analyses

In study of cytochrome *c* oxidase deficiencies, in all patients the COX deficiency was analyzed biochemically in skeletal muscle and/or cultivated fibroblasts. In some patients the deficiency was detected also in other tissues. The COX deficiency was defined as a decreased activity <30% with decreased COX/CS (citrate synthase) ratio <30% of the mean of age related controls. The diagnostic procedures have been improved in the survey period. Firstly enzymatic assay in muscle homogenates was applied and/or exceptionally histochemical COX staining was performed. Later on cultivated fibroblasts and isolated muscle mitochondria were used for enzymatic analyses. Muscle mitochondria were isolated according to Makinen and Lee (Makinen and Lee 1968) without the use of protease.

In study of activities of respiratory chain complexes in women with anorexia nervosa, platelets isolated from peripheral blood were used for measurements. The plasma enriched of platelets was isolated from 9 ml of citrate-anticoagulated blood by centrifugation (20 minutes, 130g, 25 ^{o}C). Platelets were then purified by differential centrifugation according to Fox et al. without addition of prostacyclin (Fox et al. 1992). Only fresh samples were used for measurements of complexes I, II and I+III, activities of complexes III, IV and citrate synthase were measured in aliquotes stored frozen in -80 ^{o}C.

All spectrophotometric measurements were performed in 1 ml cuvettes (1 cm, 37°C) using double beam spectrophotometer Shimadzu UV-160. The activities of respiratory chain complexes, NADH-coenzyme Q_{10} oxidoreductase (complex I), succinate-coenzyme Q_{10} oxidoreductase (complex II), coenzyme Q_{10}-cytochrome *c* oxidoreductase (complex III), cytochrome *c* oxidase (complex IV) and NADH-cytochrome *c* reductase (complex I+III), and citrate synthase (CS) were measured spectrophotometrically (Rustin et al. 1994). Protein was determined by the method of Lowry (Lowry et al. 1951). The ratio between activity of individual respiratory chain complexes and citrate synthase (CS) was calculated to eliminate

possible effect of changes in number of mitochondria in patient cells. Quality of the enzymatic assays protocol was confirmed by 1st European laboratory ring external quality test (Dr Gellerich, Martin Luther University, Halle, Germany).

2-dimensional electrophoresis (BN-PAGE/SDS-PAGE) and/or Western blot analysis were applied for assessment of amount and composition of respiratory chain complexes.

Subunits of PDH were detected by Western blotting, using chicken polyclonal antibodies against the PDH holoenzyme. Immune complexes were detected with the aid of secondary peroxidaseconjugated anti-chicken IgG antibodies (Sigma) and enhanced chemiluminescence (Amersham, Little Chalfont, UK).

Molecular methods

In study of cytochrome *c* oxidase deficiencies, one or more molecular analyses were performed in 170 children. In most patients the PCR screening for large deletions in mtDNA and PCR-RFLP analyses for mtDNA mutations 3243A>G and 8344A>G were performed. Patients with negative screening were divided into smaller groups according to their clinical profiles. Southern blot analyses were used for identification of large-scale deletions in mtDNA or mtDNA depletion. With the use of cyclic sequencing, *SURF1, SCO2, SCO1, COX10* genes or whole mtDNA were analyzed in corresponding groups. Not all currently indicated and available DNA analyses were performed in each of the COX-deficient children due to inaccessibility or insufficient amounts of DNA samples.

Statistical analysis

In study of activities of respiratory chain complexes in women with anorexia nervosa, the enzyme activities were expressed as mean ± SD, median and lower – upper quartiles were calculated too. Statistical significance for comparison between groups was assessed using the Mann-Whitney test (p-value < 0.05 was considered to be significant). The Statistica 4.5 program for WINDOWS was used for the statistical analyses (Statsoft, Tulsa, OK, USA).

Ethics

The study was carried out in accordance with the Declaration of Helsinki of the World Medical Association and was approved by the Committees of Medical Ethics. The informed consent was obtained from parents before any blood, biopsies or molecular analyses taken.

4. Results

1) A spectrum of clinical symptoms in children with cytochrome *c* oxidase deficiencies was characterized and a natural course of diseases based on cytochrome *c* oxidase deficiencies was described

The age of the onset, the frequency of clinical symptoms and the course of the disease in 180 children with COX deficiency are shown in Tab. 1 and 2. The course of the disease was usually progressive and unfavorable, 66% of patients died. Many of the patients had normal birth weight and length and deteriorated after respiratory tract infection or other stress-bearing events. Generally, the first symptoms were failure to thrive and hypotony. Progressive encephalopathy was observed in most of the patients, but kind of neurological symptoms and its severity differed largely. As apparent from Tab. 1, Leigh syndrome was found in all SURF1 patients, none of SCO2 patients and only in 4 of 43 cases of isolated COX defect without known DNA mutation. In combined defects only 4 cases of 79 patients presented with Leigh syndrome, two of which harbored mtDNA mutation 8363G>A. Hypertrichosis was a common finding, especially in children with Leigh syndrome.

Increased blood-lactate was found in 85% and increased blood-alanine in 65% of patients. CSF-lactate level was elevated in 81% of examined cases. Lactic acidosis occurred regularly in patients with combined respiratory chain defects and known mtDNA mutations. In the children with Leigh syndrome increased lactate level was associated often with respiratory alkalosis and hypocapnia. There were some SURF1-deficient Leigh patients without lactic acidosis (10%), with normal lactate concentration in cerebrospinal fluid (8%).

An underlying genetic defect was detected in 56 from 101 patients with isolated COX deficiency and 19 from 79 patients with COX deficiency combined with disturbances of other respiratory chain complexes. The results of molecular analyses in probands are shown in Table 3. Mutations in the *SURF1* gene occurred exclusively in children with Leigh syndrome, the 2bp deletion 845-846delCT in *SURF1* gene was detected in 89% of independent alleles (Table 3). The mutations in the *SCO2* gene were identified in 9 children with encephalomyopathy and/or cardiomyopathy, the mutation 1541G>A in *SCO2* was found in 83% of independent alleles. MtDNA mutations were found in 19 children with combined COX deficiency. The heteroplasmic mtDNA mutation 3243A>G was present in 6 unrelated children, mtDNA mutation 8363G>A was found in two unrelated children with Leigh syndrome and mtDNA mutations 8344A>G and 9205-9206delTA in one child each. The same mtDNA mutations with various levels of heteroplasmy were detected in the patients' mothers and other maternal relatives (not included in the study). MtDNA large-scale deletions were found in 8 children with multi-organ involvement including encephalomyopathy, endocrinopathy and heart conduction impairment and in one child with Pearson syndrome (Tab. 3). All of them were sporadic and heteroplasmic deletions, in 5 cases we found single deletions of 4,9-5.6 kb, in one child deletion/insertion was found and 2 cases harbored multiple mtDNA deletions. MtDNA depletion was detected in a boy with Alpers-Huttenlocher syndrome.

Tab. 1. The frequency of clinical symptoms in 180 children with isolated or combined cytochrome *c* oxidase deficiency

COX deficiency	isolated (n = 101)			combined* (n = 79)		Total
mutations	in *SURF1* gene	in *SCO2* gene	DNA defect not known	in mtDNA	DNA defect not known	
Affected children	47	9	45	19	60	180
Affected families	35	6	35	19	52	147
Failure to thrive	40/46 (87%)	6/8 (75%)	22/38 (58%)	16/19 (84%)	22/48 (46%)	106/159 (67%)
Hypotony	41/46 (89%)	9/9 (100%)	27/39 (69%)	10/18 (56%)	30/48 (63%)	117/160 (73%)
Encephalopathy Leigh syndrome**	47/47 (100%) 47/47 (100%)	9/9 (100%) 0	36/43 (84%) 4/43 (9%)	15/19 (79%) 2/19 (11%)	49/55 (89%) 2/55 (4%)	156/173 (90%) 55/173 (31%)
Cardiac involvement	4/47 (9%)	4/9 (44%)	8/37 (22%)	11/19 (58%)	12/53 (23%)	39/165 (24%)
Hepatopathy	0/46	3/9 (33%)	17/39 (44%)	4/19 (21%)	16/54 (30%)	40/167 (24%)
Endocrinopathy	0/47	0/8	1/39 (3%)	6/19 (32%)	2/54 (4%)	9/167 (5%)
Fanconi syndrome	0/47	0/9	1/38 (3%)	0/19	1/37 (3%)	2/150 (1%)
Renal failure	0/47	0/9	1/38 (3%)	2/19 (11%)	4/55 (7%)	7/168 (4%)

** COX deficiency combined with disturbances of one or more other respiratory chain complexes*

*** patients with encephaloapthy and bilateral necrotic lesions in basal ganglia and/or in the brainstem recognized by MRI or at autopsy*

Tab. 2. The age of onset of clinical symptoms and the course of the disease in 180 children with isolated or combined cytochrome *c* oxidase deficiency

COX deficiency	isolated (n = 101)			combined* (n = 79)		Total
mutations	in *SURF1* gene	in *SCO2* gene	DNA defect not known	in mtDNA	DNA defect not known	
affected children	47	9	45	19	60	180
age of onset						
< 3 months	5/47 (11%)	5/9 (56%)	20/39 (51%)	5/17 (29%)	28/52 (54%)	63/164 (38%)
4-18 months	37/47 (79%)	4/9 (44%)	9/39 (23%)	4/17 (24%)	12/52 (23%)	66/164 (40%)
1½ -4 years	5/47 (11%)	0/9	7/39 (18%)	7/17 (41%)	7/52 (13%)	26/164 (16%)
> 14 years	0/47	0/9	3/39 (8%)	1/17 (6%)	5/52 (10%)	9/164 (5%)
died	33/40 (83%)	8/8 (100%)	16/30 (53%)	11/19 (58%)	23/41 (56%)	91/138 (66%)
died at the age						
< 3 months	0	1	3	0	8	
4-18 months	0	7	7	3	11	
1½-14 years	30	0	4	4	2	
> 14 years	3	0	2	4	2	
alive	7/40	0	14/30	8/19	18/41	47/138 (34%)
data not available	7	1	15	0	19	42/180 (23%)

** COX deficiency combined with disturbances of one or more other respiratory chain complexes*

Tab. 3. The frequency of mutations in *SURF1* and *SCO2* genes in 41 probands with isolated COX deficiency and the frequency of mtDNA mutations in 19 probands with combined COX deficiency *(data in siblings are not shown).*

	Gene	Probands	Mutation type	Frequency
nDNA	***SURF1****	35	845-846delCT/845-846delCT	18 x
			845-846delCT/?	6 x
			845-846delCT/312-321del10insAT	3 x
			845-846delCT/574C>T	1 x
			845-846delCT/756delCA	1 x
			845-846delCT/704T>C	1 x
			845-846delCT/821A>G	1 x
			821-838del18/821-838del18	1 x
			821-838del18/312-321del10insAT	1 x
			312-321del10insAT /?	1 x
			688C>T/688C>T	1 x
	SCO-2*	6	1541G>A/1541G>A	4 x
			1541G>A/1280C>T	1 x
			1541G>A/1518delA	1 x
mtDNA	point mutation***	10	3243A>G MELAS	6 x
			8344A>G MERRF	1 x
			8363G>A	2 x
			9205-9206delTA	1 x
	mtDNA deletion****	8	Single deletion (Southern blot analysis) 4977 bp-5.6 kb, heteroplasmy 70-80%	4 x
			Deletion/insertion 8035 bp, heteroplasmy 80 %	1 x
			Multiple deletion (Long-range PCR) heteroplasmy <10 %	2x
			PCR screening of common deletion 5.6 kb in region of mtDNA 8150-14276	1x
	mtDNA depletion	1	In liver and brain, the mtDNA amount was reduced to 11 and 15%	1 x

* *mutation in SURF1 gene was found in 35 probands and 12 affected siblings*

** *mutation in SCO2 gene was found in 6 probands and 3 affected siblings*

*** *mtDNA mutations were also found in mothers and other maternal relatives*

**** *mtDNA deletion/duplication was not found in parents.*

2a) Isolated platelets are usable biological material for the measurement of activities of respiratory chain complexes in humans

The mean activities of respiratory complexes I, II, III, IV and I+III and citrate synthase in platelets in whole group of 161 children and adults and in each of age groups are summarized in Tab. 4.

Tab. 4. Activities of respiratory chain complexes I, II, III, IV and I+III and citrate synthase in isolated platelets in 161 children and adults in four age groups.

Respiratory chain complexes	0,5 - 2 years (n = 32)	3 - 9 years (n = 50)	10 – 19 years (n = 39)	20 - 35 years (n = 40)	0,5 - 35 years (n = 161)
	mean ± SD (nmol/min/mg protein)				
NQR (complex I)	34,5 ± 16,2	35,6 ± 16,2	39,1 ± 15,7	36,4 ± 16,0	36,4 ± 15,9
SQR (complex II)	8,2 ± 2,9	9,6 ± 3,4	10,6 ± 3,3	9,4 ± 4,0	9,5 ± 3,5
QCCR (complex III)*	16,4 ± 6,6	14,5 ± 4,0	19,2 ±7,7	15,2 ± 5,3	16,0 ± 5,8
COX (complex IV)	21,6 ± 5,2	21,5 ± 5,4	21,6 ± 4,7	21,0 ± 4,7	21,4 ± 5,0
NCCR (complex I+III)	15,8 ± 7,2	17,6 ± 7,3	16,3 ± 4,7	15,6 ± 6,7	16,4 ± 6,6
CS (citrate synthase)	71,5 ± 13,4	76,4 ± 17,0	76,4 ± 15,6	81,7 ± 18,5	76,7 ± 16,6

NQR - NADH-coenzyme Q oxidoreductase
SQR - succinate-coenzyme Q oxidoreductase
QCCR - coenzyme Q-cytochrome c oxidoreductase
COX - cytochrome c oxidase
NCCR - NADH-cytochrome c oxidoreductase
** number of investigations of complex III in individual age groups n = 6 – 38.*

A significant relation between the activity of citrate synthase and age ($y = 72.9 + 0.31x$; $P < 0.05$) was found. There wasn't found any significant difference in activities of respiratory chain complexes I, II, III, IV and I+III and citrate synthase in isolated platelets related to gender of investigated persons. The relations between respiratory chain complexes and citrate synthase are shown in Tab. 5.

Tab. 5. The ratios between the activities of individual respiratory chain complexes and citrate synthase in 161 children and adults in four age groups (mean ± SD).

Ratio	0,5 - 2 years (n = 32)	3 - 9 years (n = 50)	10 – 19 years (n = 39)	20 - 35 years (n = 40)	0,5 - 35 years (n = 161)
NQR/CS	0,49 ± 0,22	0,48 ± 0,24	0,52 ± 0,20	0,47 ± 0,22	0,49 ± 0,22
SQR/CS	0,12 ± 0,04	0,13 ± 0,05	0,14 ± 0,06	0,12 ± 0,05	0,13 ± 0,05
QCCR/CS	0,26 ± 0,13	0,20 ± 0,05	0,24 ± 0,09	0,20 ± 0,08	0,22 ± 0,09
COX/CS	0,30 ± 0,06	0,28 ± 0,06	0,29 ± 0,08	0,26 ± 0,06	0,29 ± 0,06
NCCR/CS	0,22 ± 0,10	0,24 ± 0,13	0,23 ± 0,10	0,19 ± 0,08	0,22 ± 0,11

NQR/CS - ratio between activity of complex I and citrate synthase
SQR/CS - ratio between activity of complex II and citrate synthase
QCCR/CS - ratio between activity of complex III and citrate synthase
COX/CS - ratio between activity of complex IV and citrate synthase
NCCR/CS - ratio between activity of complex I+III and citrate synthase
** number of investigations of complex III in individual age groups n = 6 – 38.*

2b) The method of measurement of activities of respiratory chain complexes in isolated platelets was applied in group of women with anorexia nervosa

In the group of 36 females with anorexia nervosa, the activities of respiratory chain complexes I and II in isolated platelets were significantly higher ($p < 0.001$, $p < 0.05$) than in the group of 37 age related controls taken from previous study (Tab. 6). No differences between both groups were found in the activities of respiratory chain complexes IV and I+III and citrate synthase serving as the control enzyme. In addition, the relative activities of respiratory chain complexes I, II and IV expressed as the ratios between activity of individual respiratory chain complex and the activity of the citrate synthase were also significantly higher in females with anorexia nervosa in comparison with controls ($p < 0.05$- 0.001) (Tab. 6).

We did not find any significant differences between the group of 14 females with anorexia nervosa analysed early after admission to the ward and the group of 22 females investigated later between the second and eighth week after admission, when the weight of the patients was already stabilised or it even started to increase. We also did not observe any significant differences in the activities of individual respiratory chain complexes in the group of 19 females with restrictive form of anorexia nervosa and the group of 17 females with the purging form of anorexia nervosa, only the relative activity of respiratory chain complex I expressed as the ratio to the activity of the control enzyme citrate synthase was significantly higher in the group of females with restrictive type of anorexia nervosa (0,93 ± 0,50 nmol/min/mg protein) than in the group of females with purgative type of anorexia nervosa (0,66 ± 0,46 nmol/min/mg protein, $p < 0.05$).

Tab. 6. The activities of respiratory chain complexes in isolated platelets in 36 females with anorexia nervosa in comparison with 37 age related female controls. In addition, the relative activities of respiratory chain complexes are expressed as the ratios between specific enzyme activity and citrate synthase (CS) serving as the control enzyme.

Activities or respiratory chain complexes (nmol/min/mg protein)				
	Anorexia nervosa, BMI 15±1.7, n = 36		Controls, BMI 21±2.2, n = 37	
	mean ± SD	Median (lower - upper quartile)	mean ± SD	Median (lower - upper quartile)
C I	55,4 ± 19,0***	51,5 (43,9 - 66,6)	37,8 ± 15,9	32,9 (28,3 - 48,3)
C II	10,9 ± 3,2*	10,9 (8,9 - 12,2)	8,9 ± 3,6	8,5 (6,7 – 11,2)
C IV	24,0 ± 8,3	22,4 (19,7 - 25,3)	22,0 ± 5,3	21,4 (18,5 - 24,7)
C I+III	17,2 ± 6,1	15,9 (12,8 - 20,7)	16,1 ± 7,2	14,8 (12,3 - 18,5)
CS	78,7 ± 26,0	73,4 (67,5 - 83,5)	81,5 ± 18,8	79,7 (69,0 - 90,3)

Ratio between activity of individual complex and citrate synthase				
	Anorexia nervosa, BMI 15±1.7, n = 36		Controls, BMI 21±2.2, n = 37	
C I/CS	0,81 ± 0,50***	0,67 (0,50 - 0,86)	0,48 ± 0,21	0,44 (0,32 - 0,58)
C II/CS	0,14 ± 0,05**	0,14 (0,12 - 0,16)	0,11 ± 0,04	0,11 (0,08 - 0,14)
C IV/CS	0,31 ± 0,08*	0,30 (0,28 - 0,35)	0,28 ± 0,08	0,27 (0,23 - 0,31)
C I+III/CS	0,22 ± 0,06	0,21 (0,17 - 0,27)	0,20 ± 0,09	0,18 (0,14 - 0,26)

C I – complex I (NADH-coenzyme Q_{10} oxidoreductase), C II - complex II (succinate-coenzyme Q_{10} oxidoreductase), C IV – complex IV (cytochrome c oxidase), C I+III – complex I+III (NADH-cytochrome c reductase), CS - citrate synthase * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$*

3) The activities and amount of respiratory chain complexes and pyruvate dehydrogenase were described in premature neonates and compared with those in older children

Age-dependent differences in the activities of respiratory chain complexes and PDH were observed during childhood. In the premature neonates, the specific activities of respiratory chain complexes III, IV, PDH and CS in isolated muscle mitochondria were significantly lower in comparison with older children (Fig. 1). On the contrary, the activity of complex I was higher in premature neonates in comparison with older children. The activity of complex II was not significantly different from activity in older children (Fig. 1).

The relative activities of respiratory chain complexes and PDH expressed as the ratio between specific activity of individual enzymes and citrate synthase serving as the control mitochondrial matrix enzyme are shown in Tab. 7. In premature neonates, the ratios between respiratory chain complexes I, II and III and CS were significantly higher in comparison with both control groups of older children, whereas the ratio between PDH and CS was higher in premature neonates only in comparison with children at the age of 3–18 years and no age-dependent differences were found for complex IV (Tab. 7). The ratio between activities of respiratory chain complexes III, IV, PDH and complex II serving as the control mitochondrial membrane bound enzyme were lower in premature neonates in comparison with older children at the age of 3–18 years (Tab. 7). On the contrary, the ratio between complex I and complex II was higher in premature neonates in comparison to older children aged 3–18 years. In premature neonates, no significant correlation was found between individual activities of all analysed respiratory chain complexes and the birth weight or gestational age and the actual weight or postnatal age at the time of death. The PDH activity was significantly lower (3.9±2.2 nmol/min/mg protein) in the group of 10 premature neonates with severe hyperlactacidemia (>5.9 mmol/l, controls <2.3 mmol/l) in comparison with 9 premature neonates (6.1±2.4 nmol/min/mg protein, $p<0.05$) with normal or only mildly increased lactate. Blue-native electrophoresis of respiratory chain complexes in isolated muscle mitochondria in premature neonates revealed decreased protein amount of respiratory chain complexes I, III, IV and V in all 15 analysed premature neonates in comparison with controls. Data of four very premature neonates are shown in Fig. 2. Using Western blotting, a lower amount of E1-alfa, E1-beta, protein X and E2 subunits of pyruvate dehydrogenase (20–50% of control value) were found in all 9 analysed premature neonates in comparison with older controls. Data of three very premature neonates are shown in Fig. 3.

Tab. 7. The activities of respiratory chain complexes and pyruvate dehydrogenase expressed as the ratio between specific activity of individual enzymes and two control enzymes – citrate synthase or succinate-coenzyme Q_{10} oxidoreductase – in 19 premature neonates in comparison with older children at the age between 0.5 and 2 years or 3 and 18 years

Ratio	Group A Premature neonates BW: 380-1460 g GA: 23 wk n = 19	Group B Children Age 0.5-2 years n = 20	Group C Older children Age 3-18 years n = 26	p-values		
				A:B	A:C	B:C
NQR/CS	1.5±0.58	0.75±0.3	0.29±0.10	<0.001	<0.001	<0.001
SQR/CS	0.27±0.13	0.17±0.1	0.08±0.029	<0.02	<0.001	<0.001
QCCR/CS	0.92±0.5	0.56±0.3	0.68±0.2	<0.05	<0.05	NS
COX/CS	2.03±1.1	1.7±0.57	1.6±0.49	NS	NS	NS
PDH/CS	7.9±6.2	6±3.2	3.9±1.9	NS	<0.01	<0.02
NQR/SQR	0.03±0.02	0.02±0.01	0.018±0.008	NS	<0.02	NS
QCCR/SQR	4.1±2.8	4±2.1	8.8±3.7	NS	<0.001	<0.001
COX/SQR	10.7±10.3	13.2±8.4	21.8±8.6	NS	<0.001	<0.002
PDH/SQR	0.15±0.1	0.16±0.1	0.24±0.1	NS	<0.02	<0.02

BW: birth weight, GA: gestational age, NQR: NADH-coenzyme Q_{10} oxidoreductase (complex I), SQR: succinate-coenzyme Q_{10} oxidoreductase (complex II), QCCR: coenzyme Q_{10}-cytochrome c oxidoreductase (complex III), COX: cytochrome c oxidase (complex IV), PDH: pyruvate dehydrogenase, CS: citrate synthase, p-value of <0.05 was considered significant

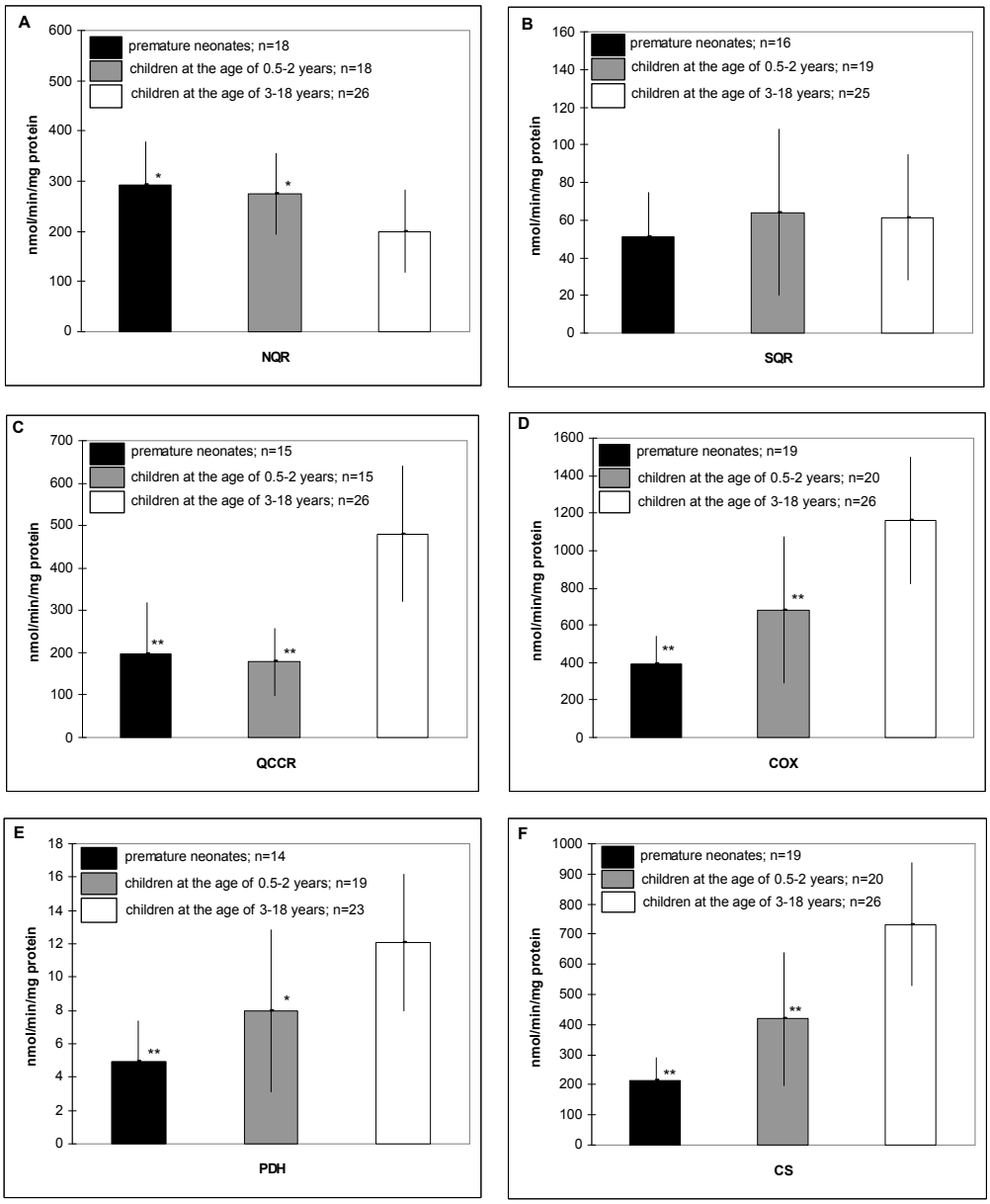

Fig. 1. *Activities of respiratory chain complexes and pyruvate dehydrogenase in isolated muscle mitochondria in 19 premature neonates in comparison with older children at the age between 0.5 and 2 years or 3 and 18 years. NQR: NADH-coenzyme Q_{10} oxidoreductase (complex I), SQR: succinate-coenzyme Q_{10} oxidoreductase (complex II), QCCR: coenzyme Q_{10}-cytochrome c oxidoreductase (complex III), COX: cytochrome c oxidase (complex IV), PDH: pyruvate dehydrogenase , CS: citrate synthase. The differences between age groups are marked with asterisk and the level of significance: *p < 0.01, **p < 0.001*

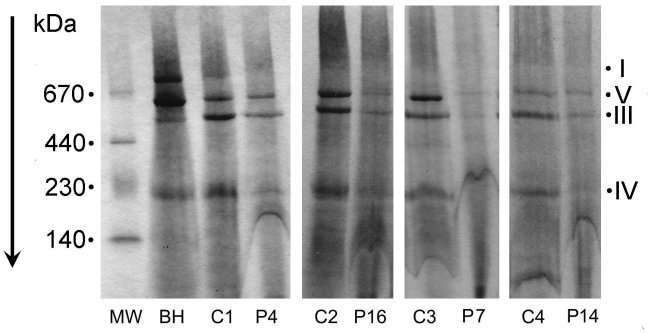

Fig. 2. *Blue-Native electrophoresis of respiratory chain complexes in isolated muscle mitochondria in four very premature neonates in comparison with controls. Protein aliquots of lauryl-maltoside-solubilised isolated mitochondria (15 µg) from bovine heart (BH), four very premature neonates (P4, P7, P14, P16) and four adult controls (C1-4) were analysed on a 5-10 % polyacrylamide gradient gel and stained with Coomassie Brilliant Blue R. The migration of molecular mass standards (MW) and the position of respiratory chain complexes I, III, IV and V are indicated*

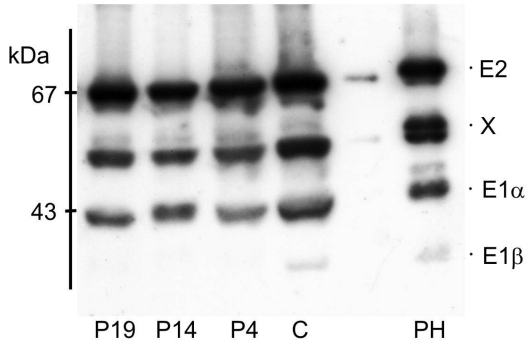

Fig. 3. *Western blot analysis of the pyruvate dehydrogenase complex in isolated muscle mitochondria. 15 µg protein aliquots of isolated mitochondria from three very premature neonates (P4, P14, P19), adult control (C) and 4.5 µg protein of isolated pyruvate dehydrogenase from porcine heart (PH) were analysed by SDS PAGE, transferred to nitrocellulose membrane and probed with anti-PDH antibodies. Positions of PDH subunits E2, X, E1α and E1β are indicated. PDH - pyruvate dehydrogenase complex, E1 - pyruvate decarboxylase, E2 - dihydrolipoamide transacetylase, X - subunit X (E3 binding protein).*

5. Discussion and conclusions

Ad 1) A spectrum of clinical symptoms in children with cytochrome *c* oxidase deficiencies and a natural course of diseases based on cytochrome *c* oxidase deficiencies

Clinical manifestation

The age of onset of clinical symptoms in patients with disturbances of oxidative phosphorylation system (OXPHOS) is extremely variable, but OXPHOS disorders in childhood are characterized by early onset of the disease in general (Sue et al. 2000). The true prevalence of mitochondrial disorders is uncertain, but the minimum prevalence of clinically affected adults with mtDNA point mutations or deletions in the North East of England is 1:10 000. The prevalence of those affected or at risk is even 2,9:10 000 (Schaefer et al. 2004).

Similarly to other studies (Scaglia et al. 2004) the most common clinical problems in group of children with COX deficiency are the functional impairments of the brain, muscle and heart. From clinical point of view, the major and most important discriminator for mitochondrial disorders with COX deficiency in childhood is especially encephalopathy characterized as a functional impairment of the central nervous system combined with failure to thrive and any impairment of any other tissue or even more tissues. Quite often the encephalopathy presents specifically with typical bilateral necrotic lesions in basal ganglia or brain stem characterized as Leigh syndrome that may discriminate subgroup of most severe COX defects. Interestingly, the Leigh syndrome was almost exclusively found in patients with mutations found in SURF1 gene, rarely in children with mtDNA mutations.

The first clinical symptoms developed early after the birth in more than one third of patients and 78 % of patients were clinically affected already within the first 18 months of life. Children with earlier onset of the disease, especially patients with Leigh syndrome due to mutations in the *SURF1* gene and patients with encephalopathy and cardiomyopathy due to mutations in the *SCO2* gene developed more severe course of the disease and died earlier than the children with combined respiratory chain defects. Similar observation was made by Sue (Sue et al. 2000).

Hypertrophic cardiomyopathy was found in 24 % of the patients, similarly to the observations of other authors (Sacconi et al. 2003). On the contrary to other reports (Sue et al. 2000), echocardiographic investigations revealed mild hypertrophic cardiomyopathy also in 4 of 47 children with Leigh syndrome due to mutations in *SURF1* gene, but cardiomyopathy developed only in children surviving more then ten years whereas absolute majority of children with Leigh syndrome died much earlier. One boy with late onset cardiomyopathy of unknown molecular background underwent heart transplantation before COX deficiency was established in his affected younger brother. Conduction heart defects, although common in adult patients with mitochondrial disorders, were found only in 8 children older than 7 years with large scale mtDNA deletion or common MELAS mutation. However, since recently, mitochondrial cardiomyopathy has been more often suspected by local physicians referring children with multi-organ involvement and hyperlactatemia for diagnostic purpose. In observed group of patients, ptosis and progressive external ophthalmoplegia (PEO) were exceptional.

The renal involvement in children with COX deficiency is quite rare. Chronic renal insufficiency was observed in less than 5 % of patients. One boy with Kearns-Sayre syndrome underwent kidney transplantation before the diagnosis of COX deficiency was made and

mtDNA deletion was recognized. Fanconi syndrome is also quite rare in children with mitochondrial disorders. Caruso et al. described Fanconi syndrome in 3 of 60 patients with respiratory chain defects (Caruso et al. 1996), in this study only in 2 of 180 children. However the results of this retrospective survey indicated that most of COX-deficient patients were not investigated properly to determine mild dysfunction of renal tubules.

The prognosis of children with COX deficiency is generally unfavourable, the course of the disease is usually progressive and the current therapeutic possibilities are very limited. Vitamin cocktails, coenzyme Q_{10} and sodium-dichloroacetate were tried in patients without any significant clinical improvement and 66 % of patients died in childhood, nearly half of them within the first 18 months of life. Similarly bad prognosis was observed in Italian patients with COX deficiency, 50 % of them died in early childhood (Caruso et al. 1996).

Biochemical analyses

In children with mitochondrial disorders, the clinical course of the disease may be associated with excessive production of lactic acid and development of metabolic acidosis. Hyperlactacidemia was found in more than 80 % of our children, usually together with increased lactate/pyruvate ratio. Biochemical diagnostics in children with the clinical suspicion on COX deficiency usually rely on the spectrophotometric or polarographic analyses of COX activity, especially in muscle biopsies or cultivated fibroblasts. The analyses in isolated muscle mitochondria from the fresh muscle biopsy are preferred, but analyses of muscle homogenate from frozen muscle biopsy have also a diagnostic value in routine practice. In accordance with other studies, the most profound decrease of COX activity both in muscle tissue and fibroblasts was found in all children with mutations in *SURF1* gene. In all patients with mutations in *SCO2* gene, the COX activity was low in muscle tissue but practically normal in the cultivated fibroblasts. In other cases, the spectrum of enzymatic changes was very broad and included the COX defects more pronounced in muscle as well as defects that dominated in fibroblasts.

DNA analyses

Significant percentage of consanguinity in affected families of COX-deficient children underlined in some reports (von Kleist-Retzow et al. 1998) indicates the major role of nDNA mutations in comparison with mtDNA mutation in this age group, but in observed group of patients the consanguinity was not a common finding. Genetic counselling in families with COX deficiency may be difficult, especially if the diagnosis in the proband was confirmed only at the enzymatic level. Molecular defect of COX deficiency is usually recognized in less then half of the patients.

An increasing number of mutations in genes encoding the COX assembly specific factors are recognized as a cause of isolated COX deficiency in childhood. They include mutations in the *SURF1* gene in children with Leigh syndrome (Williams et al. 2001), mutations in the *SCO1* gene in neonates with acute liver failure (Valnot et al. 2000), mutations in the *SCO2* gene in children with fatal hypertrophic cardiomyopathy and encephalopathy (Vesela et al. 2008), mutation in the *COX10* gene in children with tubulopathy (Valnot et al. 2000), mutation in the *COX15* gene in the child with cardiomyopathy (Antonicka et al. 2003) and mutations in the *LRPPRC* gene in the Leigh syndrome of French-Canadian type (Mootha et al. 2003).

In the group of children with isolated COX deficiency more than 45% of patients presented with Leigh syndrome and had mutations in *SURF1* gene. The deletion 845-846delCT in *SURF1* gene was prevalent. On the contrary, the deletion 312-321del10insAT in

SURF1 gene is prevalent in non-Slavonic population of children with Leigh syndrome and COX deficiency (Sue et al. 2000). In children with encephalopathy and cardiomyopathy with mutations in *SCO2* gene the mutation 1541G>A was found in all cases making this mutation also prevalent, at least in our Slavonic population. There wasn't found any mutation in the *COX10* gene analysed in small subgroup of patients with renal disease and no mutations in *SCO1* gene were found in another small subgroup of patients with liver disease.

The most common genetic abnormalities in children with combined COX deficiency are usually linked to the mutations in mtDNA. In the patients with combined COX deficiency, the most cases harboured the large scale mtDNA deletions and mtDNA point mutation 3243A>G. Altogether, mtDNA mutations or deletions were found in 24 % of patients with combined COX deficiency. These numbers are much lower than in any group of adult patients with mitochondrial disorders, where higher percentage of mtDNA mutations may be found (Olsen et al. 2003). The rising frequency of mtDNA mutations with respect to age may be explained by delayed onset of disease in the patients with mtDNA mutations in comparison with children with isolated COX deficiency caused by mutations in genes encoding the COX assembly factors. COX deficiency combined with other respiratory chain complex deficiencies may also arise from mutations in nuclear genes involved in mtDNA replication, translation and transcription. Mutations in nuclear DNA were found for example in the *POLG1* gene for mtDNA polymerase gamma (Naviaux et al. 1999) in children with Alpers-Huttenlocher syndrome and mtDNA depletion, in gene for deoxyguanosine kinase (Salviati et al. 2002) associated with hepatocerebral form of mtDNA depletion and in thymidine kinase-2 gene (Saada et al. 2001) associated with myopathy.

Conclusion

The results of this study suggest that COX deficiency in childhood is not rare in our Slavonic population. Understanding of molecular basis of COX deficiencies in childhood is of key importance not only for the non-invasive diagnostics, but first of all for the genetic counselling and prenatal diagnostics in affected families.

Ad 2a) The measurement of activities of respiratory chain complexes in isolated platelets

The necessity of muscle or liver biopsies in patients with suspicion to mitochondrial diseases leads to research of less invasive diagnostic methods. In patients with tissue-specific involvement, e.g. in those with mitochondrial myopathy, the muscle biopsy is still the only method leading to the right diagnosis. In patients with generalized disorder of mitochondrial energetic functions, where all cell types in organism are affected (with exception of erythrocytes), the diagnosis is often made by enzymatic and electrophoretic investigations in cultivated fibroblasts. The disadvantage for patient is not only the need of skin biopsy but also the long-term cultivation of large amount of fibroblasts that can take several months.

The first measurement of activities of respiratory chain complexes in isolated platelets (in patient with intermittent ataxia and lactate acidosis) was published in 1988 by Parker (Parker et al. 1988). Later, several authors studied the activities of respiratory chain complexes in isolated platelets in small groups of patients with Alzheimer, Huntington or Parkinson disease, their control groups were also small (Bosetti et al. 2002). However, in this group of diseases with affection of brain function the secondary disorder of energetic metabolism in platelets is anticipated.

Besides measuring activities of respiratory chain complexes, the ratios between activities of respiratory chain complexes and citrate synthase were calculated, because it is known that in some patients with mitochondrial myopathies are observed subsarcolemal multiplications of mitochondria. This phenomenon described as "ragged red fibres" (RRF) probably occurs as a particular compensatory mechanism of functional disorder of OXPHOS. However, with the increased amount of mitochondria grows also the absolute amount of respiratory chain complexes, therefore the most of authors recommend in biochemical analysis to correlate the activities of respiratory chain complexes with activities of control enzyme. The most often used is citrate synthase because its activity is generally correlated with "amount of mitochondrial mass" in cell.

In the observed group, the activity of cytochrome *c* oxidase didn't vary in relation with age of investigated persons between infants to age of 35 years, which is in accordance with results of measurement of activities of cytochrome *c* oxidase in muscle homogenate in 83 premenopausal females in age 23-47 years (Hunter et al. 2002). On the other hand, the activity of citrate synthase in platelets slightly increases in observed group of children and adults. Similar trend of increase of activity of citrate synthase in age up to 40 years was found in isolated lymphocytes, in the people older than 40 years the decrease of citrate synthase activity was observed (Capkova et al. 2002). On the contrary, in muscle homogenate in premenopausal females in age 23-47 years was observed a slight decrease of activity of citrate synthase (Hunter et al. 2002). The lower activity of citrate synthase in muscle homogenate was also found in males of age 58-68 years in comparison with males in age 21-33 years (Coggan et al. 1993).

Conclusion

Isolated platelets do not participate much on energetic turnover in the whole organism but in comparison with muscle fibers, neurons or cultivated fibroblasts represent an easily available material for investigation of respiratory chain complexes. The reference boundaries of activities of respiratory chain complexes in isolated platelets are used for diagnostics of some mitochondrial diseases, especially in patients with generalised types of respiratory chain disorders. In patients with tissue-specific mitochondrial disease, the results of enzymatic investigations are dependent on level of infliction of OXPHOS in platelets.

Ad 2b) The activities of respiratory chain complexes in isolated platelets in women with anorexia nervosa

The activities of complexes I and II are possibly influenced by regulation of function of fatty-acid beta-oxidation enzymes grouped in so-called "beta-oxidation metabolon". Although the exact mechanism and the direct evidence of such regulation are lacking, respiratory chain complexes I and II together with several dehydrogenases and trifunctional protein responsible for beta-oxidation of fatty acids were found in functional assemblies in gently sonicated porcine mitochondria (Bartlett and Eaton 2004).

The activity of supercomplex I+III was measured instead of the specific activity of respiratory chain complex III. No significant difference was observed in activity of supercomplex I+III between woman with anorexia nervosa and controls. It suggests normal activity of complex III in females with anorexia nervosa as the measured system is limited by the lower activity of the two complexes.

In contrast, when rats were fed by hypoenergetic diet for 7 days, the activities of mitochondrial respiratory chain complexes in skeletal muscle decreased and returned to normal values when rats were re-fed by protein diet (Briet and Jeejeebhoy 2001). It may be difficult to compare both studies, not only because of different kinds of tissues were examined but also because the short-term starvation in the animal study may have another impact on the mitochondrial functions in comparison with chronic starvation, which is typical for patients with anorexia nervosa.

Although there is no clear explanation for raised activity of OXPHOS in females with anorexia nervosa, there could be several factors possibly responsible for such results. Anorexia nervosa is a serious eating disorder characterised by the decreased caloric intake and resisting to body weight increase. The disease is often accompanied by endocrinologic complications, such as hypothalamic hypogonadism, hypercortisolaemia with normal concentration of adrenocorticotropic hormone (ACTH) and decreased concentrations of triiodothyronine (T3) and thyroxin (T4). The metabolic disturbances include higher synthesis of plasmatic lipoproteins with increased concentrations of triacylglyceroles and hypercholesterolaemia (Zak et al. 2003). In the group of investigated females with anorexia nervosa concentrations of triacylglyceroles and cholesterol did not differ in comparison with controls while free thyroxin appeared at the lower range of controls.

The metabolic changes in anorexia nervosa might also concern the efficiency of mitochondrial energy generating system. One possible way how to increase the efficiency of ATP production is the lower expression of uncoupling proteins (UCP), which would prevent the proton leak through the mitochondrial membrane. Apparently, the balance between ATP production and energy dissipation in the form of heat loss could be the key point of metabolic regulation in the state of low energy supply. This thought is supported by the fact that one of symptoms often connected with anorexia nervosa is hypothermia, which might be caused by preferential support of energy conservation into the molecule of ATP instead of heat production (Pirke 1996). Interestingly, in the study no females with anorexia nervosa showed hypothermia.

The differences in activities of respiratory chain complexes in anorexia nervosa might be also caused by changes in proportions between the function of OXPHOS and citric acid cycle and/or in mitochondrial compartments due to different cell demands in hypoenergetic state. It is supposed that, as a reaction to low energy supply, the cell may spare energy and use the metabolic pathways with higher efficacy, i.e. by increasing the amount of crucial OXPHOS enzymes and thus enlarging the inner mitochondrial surface relative to mitochondrial matrix volume.

Conclusion

In conclusion, the results of the study demonstrate the differences in the activities of respiratory chain complexes in females with anorexia nervosa and healthy controls and they support the view that mitochondrial oxidative capacity may rise in anorexia nervosa as the response to lower food supply. Moreover, the data suggest that isolated platelets may serve as an easily available material, which might be useful for further studies of the metabolic changes in patients with eating disorders.

Ad 3) The activities and amounts of respiratory chain complexes and pyruvate dehydrogenase in premature neonates in comparison with older children

After the birth, more than 90% of ATP is produced by mitochondrial ATP synthase in presence of oxygen. Postnatal switch from glycolytic to oxidative metabolism is important for successful adaptation of mammalian neonates to extrauterine life. The cellular capacity for energy provision relies on adequate biosynthesis of respiratory chain complexes.

The amount and activities of COX and mitochondrial creatine kinase increase in skeletal muscle between 28^{th} and 40^{th} week of gestation (Smeitink et al. 1992). The size and number of mitochondria per cell increase in the neonatal period in rats and during the first two months of life in dogs (Legato 1979). During first few weeks of life, the activities of PDH, citrate synthase and respiratory chain complexes in rat muscles increase to nearly adult values. Significant rat heart developmental differences in the activities of cytochrome *c* oxidase and citrate synthase were observed (Drahota et al. 2004). Increased activity of cytochrome *c* oxidase have also been noted in developing human heart (Moggio et al. 1989). In skeletal muscles of premature neonates, the activities of PDH and respiratory chain complexes III and IV and CS were significantly lower in comparison with controls of age 0.5–2 years (Wenchich et al. 2002).

In critically ill neonates, infection and oxidative stress may decrease ATP production. The low activities of RC complexes III and IV and pyruvate dehydrogenase in premature neonates muscle mitochondria observed in our study correspond to the low protein amount of these mitochondrial enzymes. The lower mitochondrial protein synthesis in premature individuals was also demonstrated in animal studies and scarce studies in human neonates (Sperl et al. 1992).

Only a part of the glucose is oxidised in premature neonates during the first days of life. The low activity of PDH in premature or critically ill neonates negatively influences the ATP production and may cause the low glucose tolerance and lactic acidosis. An increased level of lactate was found in neonates as a result of hypoxia, low blood perfusion, hepatic or renal failure, high glucose intake and also in children with various inherited metabolic disorders (Hutchesson et al. 1997). In our group of premature neonates with severe hyperlactacidemia we found lower activity of PDH in comparison with premature neonates in which lactic acidosis were not so accentuated. The activity of the PDH complex strongly depends on the actual ratio between the activities of PDH kinase (PDHK) and PDH phosphatase. The PDH activity is decreased in the skeletal muscle of rats during sepsis due to an increase of PDHK activity. In addition, cytokines may influence skeletal muscle protein metabolism during sepsis (Vary and Hazen 1999). In premature neonates, the infectious complications are very frequent but it is unlikely that the higher activity of PDHK is the only cause of the low activity of the PDH complex.

A group of premature neonates and two groups of older children of different age were observed in our study. The activities of PDH and several respiratory chain complexes tended to increase with age. The reason why reversed trend was observed for complex I (NQR) although electrophoretic analyses revealed a decreased protein amount of complex I in premature neonates in comparison to older children, is not clear. The physiological electron acceptor of complex I is a lipid-soluble endogenous coenzyme Q. The assay of complex I activity requires the use of artificial acceptors, because the physiological quinones, such as coenzyme Q_{10}, are too insoluble in water to be added as substrate to the assay media. One of the best electron acceptors for the study of the NQR activity is the commercially available synthetic analog decylubiquinone (DB). It was observed, that using DB as the electron acceptor results in non-linear kinetics in enzymatic assay. In addition, NADH oxidation by

DB is highly dependent on the amount of phospholipids (Estornell et al. 1993). Differently maturated muscle tissue can probably harbour various amount and composition of phospholipids which may influence the results of complex I activity measurement. Bruce described that phospholipid composition in human muscle varies from the fetal to middle age (Bruce 1974). Unfortunately, to standardise the content of phospholipids for optimal amount in all measured groups of our samples was not possible due to very limited amount of available tissue. On the other hand, complex I is the largest and the most unexplored enzyme complex, although it represents the key enzyme of the mitochondrial electron chain. Furthermore, since 7 out of the 13 polypeptides encoded by mtDNA belong to complex I, it is expected that complex I should be mostly affected by ageing. In addition, it was shown recently, that complex I becomes more rate controlling, over all others enzyme complexes of respiratory chain, during ageing (Ventura et al. 2002).

Citrate synthase is a soluble enzyme localized in the mitochondrial matrix, participating in Krebs cycle function. Activity of CS is used as a marker of mitochondria number per cell.We observed that the activity of RC complexes I, II, III and PDH normalized to CS were significantly higher in premature neonates in comparison with control groups of children. These data may indicate that during the intrauterine and early postnatal period the capacity of membrane-bound respiratory chain enzymes are completed sooner than that of auxiliary soluble matrix enzymes (CS) and/or that the enzyme activity of already synthesised enzyme complexes increases faster that the mitochondrial biogenesis. This hypothesis is supported by work of Drahota et al., who demonstrated different developmental kinetics of COX and CS in heart homogenates in rats at the age between 5 and 60 days (Drahota et al. 2004).

On the other hand, it is possible that any unstableness of membranes in extremely premature neonates may contribute to loss of integrity of inner mitochondrial membrane in obtained muscle during isolation and thereby cause higher leak of CS resulting in lower CS activities in premature neonates in comparison with term neonates. Detail data about sensitivity of muscle in very premature neonates in different stages of development to homogenization and information about influence of manipulation with isolated mitochondria obtained from premature neonates to stability of purified mitochondria is still lacking.

Conclusion

Most diseases in premature neonates are secondary to infection and immaturity of various organ systems. The results of our study document the age-dependent differences in activities of PDH and respiratory chain complexes in early childhood. Lower functional capacity of mitochondrial energy-providing system in critically ill premature neonates may be explained by combination of various factors including the delay in maturation of PDH and respiratory chain complexes in very premature neonates and increased degradation of mitochondrial proteins in connection with sepsis, tissue hypoperfusion or hypoxemia.

6. Selected references

Antonicka, H., Mattman, A., et al. (2003). "Mutations in COX15 produce a defect in the mitochondrial heme biosynthetic pathway, causing early-onset fatal hypertrophic cardiomyopathy." Am J Hum Genet **72**(1): 101-14.

Bartlett, K. and Eaton, S. (2004). "Mitochondrial beta-oxidation." Eur J Biochem **271**(3): 462-9.

Bosetti, F., Brizzi, F., et al. (2002). "Cytochrome c oxidase and mitochondrial F1F0-ATPase (ATP synthase) activities in platelets and brain from patients with Alzheimer's disease." Neurobiol Aging **23**(3): 371-6.

Briet, F. and Jeejeebhoy, K. N. (2001). "Effect of hypoenergetic feeding and refeeding on muscle and mononuclear cell activities of mitochondrial complexes I--IV in enterally fed rats." Am J Clin Nutr **73**(5): 975-83.

Bruce, A. (1974). "Skeletal muscle lipids. II. Changes in phospholipid composition in man from fetal to middle age." J Lipid Res **15**(2): 103-8.

Capkova, M., Houstek, J., et al. (2002). "Activities of cytochrome c oxidase and citrate synthase in lymphocytes of obese and normal-weight subjects." Int J Obes Relat Metab Disord **26**(8): 1110-7.

Caruso, U., Adami, A., et al. (1996). "Respiratory-chain and pyruvate metabolism defects: Italian collaborative survey on 72 patients." J Inherit Metab Dis **19**(2): 143-8.

Coggan, A. R., Abduljalil, A. M., et al. (1993). "Muscle metabolism during exercise in young and older untrained and endurance-trained men." J Appl Physiol **75**(5): 2125-33.

Drahota, Z., Milerova, M., et al. (2004). "Developmental changes of cytochrome c oxidase and citrate synthase in rat heart homogenate." Physiol Res **53**(1): 119-22.

Estornell, E., Fato, R., et al. (1993). "Assay conditions for the mitochondrial NADH:coenzyme Q oxidoreductase." FEBS Lett **332**(1-2): 127-31.

Fox, J. E. B., Reynolds, C. C., et al. (1992). "Studying the platelet cytoskeleton in Triton X-100 lysates." Methods Enzymol **215**: 42-47.

Han, T. M. and Runnegar, B. (1992). "Megascopic eukaryotic algae from the 2.1-billion-year-old negaunee iron-formation, Michigan." Science **257**(5067): 232-5.

Hunter, G. R., Newcomer, B. R., et al. (2002). "Age is independently related to muscle metabolic capacity in premenopausal women." J Appl Physiol **93**(1): 70-6.

Hutchesson, A., Preece, M. A., et al. (1997). "Measurement of lactate in cerebrospinal fluid in investigation of inherited metabolic disease." Clin Chem **43**(1): 158-61.

Legato, M. J. (1979). "Cellular mechanisms of normal growth in the mammalian heart. I. Qualitative and quantitative features of ventricular architecture in the dog from birth to five months of age." Circ Res **44**(2): 250-62.

Lowry, O. H., Rosebrough, N. J., et al. (1951). "Protein measurement with the Folin phenol reagent." J Biol Chem **193**(1): 265-75.

Makinen, M. W. and Lee, C. P. (1968). "Biochemical studies of skeletal muscle mitochondria. I. Microanalysis of cytochrome content, oxidative and phosphorylative activities of mammalian skeletal muscle mitochondria." Arch Biochem Biophys **126**(1): 75-82.

Moggio, M., Bresolin, N., et al. (1989). "Cytochrome c oxidase during human fetal development." Int J Dev Neurosci **7**(1): 5-14.

Mootha, V. K., Lepage, P., et al. (2003). "Identification of a gene causing human cytochrome c oxidase deficiency by integrative genomics." Proc. Natl. Acad. Sci. U.S.A. **100**(2): 605-610.

Naviaux, R. K., Nyhan, W. L., et al. (1999). "Mitochondrial DNA polymerase gamma deficiency and mtDNA depletion in a child with Alpers' syndrome." Ann Neurol **45**(1): 54-8.

Olsen, D. B., Langkilde, A. R., et al. (2003). "Muscle structural changes in mitochondrial myopathy relate to genotype." J Neurol **250**(11): 1328-34.
Parker, W. D., Jr., Frerman, F., et al. (1988). "Myxothiazol resistance in human mitochondria." Biochim Biophys Acta **936**(1): 133-8.
Pirke, K. M. (1996). "Central and peripheral noradrenalin regulation in eating disorders." Psychiatry Res **62**(1): 43-9.
Rustin, P., Chretien, D., et al. (1994). "Biochemical and molecular investigations in respiratory chain deficiencies." Clin Chim Acta **228**(1): 35-51.
Saada, A., Shaag, A., et al. (2001). "Mutant mitochondrial thymidine kinase in mitochondrial DNA depletion myopathy." Nat Genet **29**(3): 342-4.
Sacconi, S., Salviati, L., et al. (2003). "Mutation screening in patients with isolated cytochrome c oxidase deficiency." Pediatr Res **53**(2): 224-30.
Salviati, L., Sacconi, S., et al. (2002). "Mitochondrial DNA depletion and dGK gene mutations." Ann Neurol **52**(3): 311-7.
Scaglia, F., Towbin, J. A., et al. (2004). "Clinical spectrum, morbidity, and mortality in 113 pediatric patients with mitochondrial disease." Pediatrics **114**(4): 925-31.
Schaefer, A. M., Taylor, R. W., et al. (2004). "The epidemiology of mitochondrial disorders-past, present and future." Biochim Biophys Acta **1659**(2-3): 115-20.
Schopf, J. W. (1993). "Microfossils of the Early Archean Apex chert: new evidence of the antiquity of life." Science **260**: 640-6.
Smeitink, J., Ruitenbeek, W., et al. (1992). "Maturation of mitochondrial and other isoenzymes of creatine kinase in skeletal muscle of preterm born infants." Ann Clin Biochem **29 (Pt 3)**: 302-6.
Sperl, W., Sengers, R. C., et al. (1992). "Enzyme activities of the mitochondrial energy generating system in skeletal muscle tissue of preterm and fullterm neonates." Ann Clin Biochem **29 (Pt 6)**: 638-45.
Sue, C. M., Karadimas, C., et al. (2000). "Differential features of patients with mutations in two COX assembly genes, SURF-1 and SCO2." Ann Neurol **47**(5): 589-95.
Valnot, I., Osmond, S., et al. (2000). "Mutations of the SCO1 gene in mitochondrial cytochrome c oxidase deficiency with neonatal-onset hepatic failure and encephalopathy." Am J Hum Genet **67**(5): 1104-9.
Vary, T. C. and Hazen, S. (1999). "Sepsis alters pyruvate dehydrogenase kinase activity in skeletal muscle." Mol Cell Biochem **198**(1-2): 113-8.
Ventura, B., Genova, M. L., et al. (2002). "Control of oxidative phosphorylation by Complex I in rat liver mitochondria: implications for aging." Biochim Biophys Acta **1553**(3): 249-60.
Vesela, K., Hulkova, H., et al. (2008). "Structural analysis of tissues affected by cytochrome C oxidase deficiency due to mutations in the SCO2 gene." Apmis **116**(1): 41-9.
von Kleist-Retzow, J. C., Cormier-Daire, V., et al. (1998). "A high rate (20%-30%) of parental consanguinity in cytochrome-oxidase deficiency." Am J Hum Genet **63**(2): 428-35.
Wenchich, L., Zeman, J., et al. (2002). "Mitochondrial energy metabolism in very premature neonates." Biol Neonate **81**(4): 229-35.
Williams, S. L., Taanman, J. W., et al. (2001). "A novel mutation in SURF1 causes skipping of exon 8 in a patient with cytochrome c oxidase-deficient leigh syndrome and hypertrichosis." Mol Genet Metab **73**(4): 340-3.
Zak, A., Vecka, M., et al. (2003). "[Lipid metabolism in anorexia nervosa]." Cas Lek Cesk **142**(5): 280-4.

7. List of original articles

Böhm M., Pronicka E., Karczmarewicz E., Pronicki M., Piekutowska-Abramczuk D., Popowska E., Sykut-Cegielska J., Mierzewska H., Hansíková H., Veselá K., Tesařová M., Houšťková H., Houštěk J. and Zeman J.: Retrospective, multicentric study of 180 children with cytochrome *c* oxidase deficiency. Pediatr Res, 59 (1), 2006, p. 21-26; **IF**: 2.875

Böhm M., Wenchich L., Hansíková H., Papežová H. and Zeman J.: Activities of respiratory chain complexes in isolated platelets in female patients with anorexia nervosa, Int J Eat Disord. 2007 Nov;40(7):659-63; **IF**: 2.269

Honzík T., Drahota Z., Böhm M., Ješina P., Mráček T., Paul J., Zeman J., Houštěk J.: Specific properties of mitochondria from human-term placenta: glycerophosphate-dependent hydrogen peroxide production, Placenta, 27 (4-5), 2006, p. 348-356; **IF**: 2.969

Honzík T., Wenchich L., Böhm M., Hansíková H., Peiznochová M., Zapadlo M., Plavka R., Zeman J.: Activities of respiratory chain complexes and pyruvate dehydrogenase in isolated muscle mitochondria in premature neonates. Early Hum Dev. 2008 Apr;84(4):269-76. Epub 2007 Aug 14; **IF**: 1.850

Böhm M., Honzík T., Šnajperková A., Knopová S., Zeman J., Hansíková H.: Activities of respiratory chain complexes in isolated thrombocytes. Klin. Biochem. Metab., 11 (32), 2003, No.2, p. 97-101

Tesařová M., Hansíková H., Kytnarová J., Houšťková H., Böhm M., Černá L., Zeman J.: Clinical heterogeneity, tissue distribution, and integrational segregation of mtDNA mutation A3243G. Toxicology Mechanisms and Methods, 14, 2004, p. 79-84

Honzík T., Chrastina R., Hansíková H., Böhm M., Martincová O., Plavka R., Zapadlo M., Zeman J.: Carnitine concentrations in term and preterm newborns at birth and during the first days of life. Prague Med Rep. 106 (3), 2005, p. 297-306.