Developmental, pathobiochemical and molecular aspects of selected inborn errors of metabolism

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ABBREVIATIONS

Apo C-III: Apolipoprotein C-III
ATP: Adenosine Triphosphate
BCVA: Best corrected Visual Acuity
CDG: Congenital Disorders of Glycosylation
COX: Cytochrome c Oxidase
CPEO: Chronic Progressive External Ophthalmoplegia
CS: Citrate Synthase
DOA: Dominant Optic Atrophy
ERN: European Reference Network
GSD: Glycogen Storage Diseases
GW: Gestational Week
LCA2: Leber Congenital Amaurosis 2
LIH: Liver Haematopoiesis
LHON: Leber Hereditary Optic Neuropathy
LPL: Lipoprotein Lipase
MD: Mitochondrial Disorders
MELAS: Mitochondrial myopathy, Encephalopathy, Lactic Acidosis and Stroke-like Episodes
MON: Mitochondrial Optic Neuropathy
mtDNA: Mitochondrial DNA
nDNA: Nuclear DNA
OXPHOS: Oxidative Phosphorylation System
RGC: Retinal Ganglion Cells
RNFL: Retinal Nerve Fibre Layer
SD-OCT: Spectral-Domain Optical Coherence Tomography
SD-OCT-A: Spectral-Domain Optical Coherence-Angiography
SLE: Stroke-Like Episodes
SQR: Succinate Coenzyme Q Reductase
ABSTRAKT

Dědičné poruchy metabolismu představují heterogenní skupinu vzácných onemocnění, jež obvykle postihují méně než 1 na 100 000 žive narozených dětí. Vzhledem k jejich nízké prevalenci zůstávají často na okraji zájmu základního i aplikovaného výzkumu farmaceutických společností. Studium vzácných onemocnění představuje jediný způsob jak zlepšit prognózu pacientů.

Předkládaná disertační práce se věnuje především problematice poruch mitochondriálního metabolismu. Jejím zámkem bylo charakterizovat mitochondriální biogenezi během fetálního období a v průběhu dětského i dospělého věku. Další část se zabývá studiem mitochondriálních neuropatií optiku na klinické, biochemické a molekulárně–genetické úrovni.

Tato práce významně podpořila dřívější nález, že 22. gestační týden je kritickou periodou pro viabilitu plodu, což je nutno zvážit především při tvorbě doporučení pro resuscitaci nedonošených novorozenců. Další přínosem byla charakterizace velkého souboru pacientů s neuropatiemi optiku na podkladě mitochondriální dysfunkce. Podařilo se nám charakterizovat více než 200 pacientů na biochemické a molekulárně–genetické úrovni, kteří byli popsáni jak v kazustických sděleních (LHON/MELAS překryvný syndrom), tak i v mezinárodních kohortových studiích (MELAS, DOA, extraokulární příznaky LHON). Hlavním přínosem této práce je především aplikace teoretických poznatků v klinické praxi. V roce 2013 se nám podařilo založit Národní centrum pro pacienty s mitochondriálními neuropatiemi optiku s cílem poskytnout nejlepší péči pro naše pacienty, optimalizovat diagnosticko-terapeutické postupy a (pre-)klinický výzkum těchto onemocnění.

Klíčová slova: mitochondrie, biogeneze, vývoj, mitochondriální neuropatie optiku, Leberova hereditární neuropatie optiku (LHON)
ABSTRACT

Inborn errors of metabolism represent a heterogenous group of rare conditions, most having an incidence of less than 1 in 100,000 births. Because of their low prevalence, they are on the margin of attention of general research and even more so of large pharmaceutical companies. Study of rare diseases is the only way to design therapeutic options in order to improve quality of life of affected patients.

Present Thesis particularly focuses on disturbances in mitochondrial energy metabolism. The main goals were the characterization of mitochondrial biogenesis within foetal development, as well as in childhood and adulthood. Another aim was to define clinical, biochemical and molecular aspects of mitochondrial optic neuropathies in childhood and adulthood.

This work supported the earlier observations that gestational week 22 is the edge of viability, which has to be taken into account in upcoming discussions about guidelines on resuscitation of preterm neonates. Secondly, over last four years, we managed to examine and describe large cohort of patients with optic neuropathies based on a mitochondrial dysfunction. We have managed to characterize the biochemical and molecular–genetic background in more than 200 patients, and both selected cases (LHON/MELAS overlap syndrome) and cohort studies (MELAS, DOA, LHON plus) were published, bringing novel phenotype and genotype findings. Finally, the main contribution of this Thesis was the application of our results and experience in clinical practice. In 2013, we succeeded in creating a National Center for Patients with Mitochondrial Optic Neuropathies in order to bring the best care to patients with rare mitochondrial optic diseases, to improve diagnostic tools and to harmonize (pre–)clinical research.

Key words: mitochondrion, biogenesis, development, mitochondrial optic neuropathies, Leber Hereditary Optic Neuropathy (LHON)
1 INTRODUCTION

1.1 Mitochondria, Oxidative Phosphorylation System (OXPHOS)
Mitochondria are tubular-shaped organelles responsible for providing the major portion of Adenosine Triphosphate (ATP) for cell energy requirements. This is achieved by OXPHOS organized in four enzymatic Complexes I - IV and ATP synthase. In line with the theory of chemi-osmotic coupling, metabolites generated through breakdown of carbohydrates, proteins and fatty acids are fuelled into the citric acid cycle and subsequently to OXPHOS to generate a proton gradient released through ATP synthase to produce ATP. Two genomes, the Nuclear DNA (nDNA) and Mitochondrial DNA (mtDNA), control the generation of respiratory chain.

1.2 Mitochondrial biogenesis
Biogenesis of mitochondria results both in an increase in the number of mitochondria per cell (i.e., organelle proliferation) and in increase in the functional capabilities of mitochondria (i.e., organelle differentiation) [1]. Efficient mitochondrial biogenesis is essential through whole life, but of special importance is its involvement in proper embryonic and foetal development.

1.2.1 Embryonic and foetal metabolism
Mammalian adaptation to the aerobic extrauterine environment involves profound hormonal, physiological and metabolic changes which need to be finely tuned to avoid neonatal morbidity and mortality [2]. In the pre-compaction stage, respiratory rates are low and the main source of energy comes from an exocoelomic pyruvate and endogenous amino acids. With the proceeding compaction and blastocyst development, biosynthetic rates demand glucose as a predominant source of energy with increasing respiratory capacity. However, only recently it was proven that β-oxidation is also active during foetal life [3]. At the end of the first trimester, a threefold intraplaclental rise in oxygen has been measured [4, 5] which is accompanied with progressive ultrastructural changes of human mitochondria [6]. Full switch from glycolysis to oxidative phosphorylation at birth is of utmost importance for successful adaptation to extrauterine life in all neonates. The cellular competence for ATP provision relies on the adequate biosynthesis of OXPHOS. It is Gestational Week (GW) 22 to 23, that seems to be a limit of foetal viability [7]. Important regulatory changes of mitochondrial function on a genomic level, therefore, need to occur during this period. A profound change of transcription pattern was observed for several OXPHOS subunits within the foetal development [8]. On the other hand, it was hypothesized that OXPHOS protein expression in liver is exerted mainly after birth [9]. Based on these obser-
vations, it is probable that prenatal preparation may lean mainly on a transcriptional level spearheading for more flexible post-transcriptional regulation early after birth [9].

1.3 Mitochondrial disorders (MD)
MD are a group of clinically, biochemically and genetically heterogeneous diseases with the minimal prevalence estimated as 1 to 4,500. They may be associated with either OXPHOS dysfunction (due to mutated subunits or their assembly factors) or dysfunction of other mitochondrial processes, including mtDNA integrity maintenance, expression, transport machinery, mitochondrial biogenesis, apoptosis and cofactor biosynthesis. MD may manifest at any age and affect any organ of the body. The clinical presentation is, however, the most severe in high energy demanding tissues. Due to their extreme variability, diagnostics of MD is very demanding and requires an integrated approach: clinical, biochemical, histological and genetic. Population-based studies suggest that m.3243A>G mutation, responsible for Mitochondrial myopathy, Encephalopathy, Lactic Acidosis, and Stroke-like episodes (MELAS, OMIM 540000) and Leber Hereditary Optic Neuropathy (LHON) prevalent m.11778G>A, are the most common diseases—causing mtDNA mutations.

1.3.1 Mitochondrial Optic Neuropathies (MON)
The term optic neuropathy refers to damage to the optic nerve anywhere between the Retinal Ganglion Cells (RGC) and the lateral geniculate body. As the genetic basis of inherited optic neuropathies were uncovered, it became apparent that mitochondrial dysfunction is a recurrent theme underlying the loss of RGC in these disorders [10] which is believed to be due to their high–energy dependence. MON lead to chronic visual impairment or even registrable blindness with a significant detrimental impact on the overall quality of life. The two major prototypes of MON are LHON and Dominant Optic Atrophy (DOA). Although MELAS is usually associated with retrochiasmal visual loss due to Stroke-Like Episodes (SLE) in occipital or parietal lobes [11], it may less commonly present with optic neuropathy.

1.3.1.1 LHON
Leber hereditary optic neuropathy (LHON, OMIM 535000) is the most frequent MD affecting about 1/10,000 of the population [10]. Three pathogenic homoplasmonic point mutations of mitochondrial DNA at positions 11778/MT-ND4, 3460/MT-ND1 and 14484/MT-ND6 in Complex I are agreed to be causative for the majority (>90 %) of cases [12]. The disease is typical with its age– and gender–related penetrance, although neither of those may fully predict the individuals
in risk of the disease manifestation. Development of visual failure is nowadays believed to be a result of several risk factors. LHON is characterized by progressive painless visual deterioration that usually becomes binocular. Within its course, LHON is associated with changes in Retinal Nerve Fibre Layer (RNFL) and microangiopathy. These can be objectified by Spectral-Domain Optical Coherence Tomography (SD–OCT) and by a novel technique – SD–OCT–Angiography (SD–OCT–A), respectively. It is of importance that structural changes usually do not correlate with clinical phenotype and predictive value of these findings still remains unknown. Generally, eye is the only affected organ in LHON but a small subgroup of patients will develop additional extraocular features [13-15]. Patients with LHON plus (i.e. LHON in addition to other severe neurological abnormalities) are usually associated only with rarer pathogenic Complex I mutations in isolated pedigrees [14, 16-19]. Moreover, several recently described point mutations with pronounced inhibitory effect on Complex I activity have also been identified in patients with overlapping symptoms of LHON and MELAS [20-22], LHON/Leigh syndrome [23] or MELAS/Leigh syndrome [24] and it was further proposed the mechanism of microangiopathy contributes to the development of vision loss in these mitochondrial disorders [12].

1.3.1.2 DOA
DOA (OMIM 165500) is the most prevalent hereditary optic neuropathy with an estimated prevalence ranging from 1: 12,000 to 1: 50,000 [25, 26]. OPA1 is the major gene responsible for DOA and up to now, more than 200 mutations have been identified. DOA usually manifests within the first two decades of life with painless bilateral visual deterioration and tritanopia/generalized dyschromatopsia. Compared to LHON, the progression in DOA is slow, insidious with variable visual outcome [27].

1.3.1.3 Therapeutic management of MON
Recently, it was proven that early initiation of therapy with idebenone in LHON and DOA patients ameliorates visual functions and clinical trials testing several other promising therapies are underway. Incomplete penetrance and similarities with other disorders affecting the optic nerve cause considerable diagnostic difficulties and delay in therapy initiation. Establishing a multidisciplinary medical care centre dedicated to patients with MON in order to develop effective diagnostic and treatment algorithms and to research the underlying pathogenetic mechanisms is of crucial importance in the Czech Republic.
2 AIMS OF THE THESIS

Inborn errors of metabolism represent a heterogenous group of rare conditions, most having an incidence of less than 1 in 100,000 births. Present Thesis particularly focuses on disturbances in mitochondrial energy metabolism. Studying clinical, biochemical and molecular aspects of MD is the only way to improve quality of life of affected patients. Better understanding of mitochondrial functioning in various developmental spans may provide theoretical basis for better care of very premature neonates with early manifestation of MD. Moreover, with recent therapeutic advances we may allow for improvement or stabilization of visual deterioration in a group of MON. The creation of multidisciplinary centers would bring a better care management as well as harmonization of (pre--)clinical research. In addition, Department of Paediatrics and Adolescent Medicine is focused on diagnostics and treatment of other mitochondrial and non-mitochondrial rare diseases. The accurate diagnosis is important for prenatal testing and genetic counselling in the affected families, as well as for well-timed therapy initiation.

Specific aims of the Thesis were:

A) to characterize mitochondrial biogenesis in an organ– and gender–specific manner by examining human liver and muscle tissue;

B) to establish a National medical care Center for Patients with Mitochondrial Optic Neuropathies; to define clinical, biochemical and genetic aspects in large cohort of patients with optic neuropathies based on a mitochondrial dysfunction;

C) to describe pathobiochemical and molecular aspects of selected rare inherited errors of metabolism
3 RESULTS AND DISCUSSION

3.1 Relation to the aim

A) Mitochondrial biogenesis

3.1.1 Developmental changes of Cytochrome c Oxidase (COX), Citrate Synthase (CS), Succinate Coenzyme Q Reductase (SQR) and mtDNA in human foetal tissues

Due to the great advances in neonatal medicine the mortality of extremely premature newborns born in ≥24 GW is decreasing and their prognosis becomes significantly better than it was only a few years ago. As follows, guidelines on resuscitation are shifting back to earlier GW. This led us to study the energetic metabolism around this critical developmental point. Evaluation of the expression profile in a unique sample set of 25 foetal liver and 18 muscles collected between 14th and 29th GW revealed significant increase in COX4 and MT-ATP6 transcription levels in liver after the 22nd GW. We further studied the activity of mtDNA content and several mitochondrial enzymes. MtDNA amount positively correlated with the age of gestation only in liver (p=0.011). Interestingly, following the pattern of increasing gene expression, we could observe a marked drop in COX, CS and SQR activity after the GW 22 in both tissues. Based on our results, we suggest, that sufficient level of mtDNA and mitochondrially-encoded transcripts accumulated throughout the gestation, but mainly after 22nd GW, are crucial for adequate onset of mitochondrial energetic functions after birth, and, contrarily, that its inadequate capacity leads to high mortality of children born before this specific developmental point. Additionally, foetal Liver Haematopoiesis (LH) was surveyed by light microscopy. We observed a continuous decline of LH from 14th to 24th GW, not correlating with OXPHOS-specific activities, indicating their exclusive reflection of liver tissue processes. Our study supports the GW 22 is the edge of viability.


3.1.2 Developmental changes of COX, CS, SQR and mtDNA in human foetal tissues

In order to document the effect of both age and gender on the interpretation of mitochondrial functional state the enzymatic activities (CS, COX and SQR) and mtDNA content were assessed in the set of 205 human muscle samples. A significant difference in mtDNA content was found among boys up to 10 years of age and the older male groups (0-10 vs. 11-20 y: p=0.001; vs. 21-40 y: p=0.005 and vs. 41-78 y: p=0.005). The female mtDNA content per cell did not vary with age group (p>0.05). In both groups, no significant age group dependency was detec-
ted in any of enzymatic activities. Nevertheless, the enzyme activities normalized to mtDNA showed a downward trend through lifetime only in the male group. This, rather unexpected finding, could indicate that skeletal muscle loses its efficiency to produce enzyme specific activity per mtDNA unit with age. This may represent the effect of sex hormones, or gender specificity in mitochondriogenesis. Due to the progressive decline of testosterone levels after 20 years of age, the increase in mtDNA content with age could be a compensatory effect in men when mitochondrial transcription declines and oxidative stress is increasing. The sex differences in the normalized enzyme activities could reflect a distinct compensatory mechanism for age–related impairment of muscle mitochondria.


3.2 Relation to the aim B) Creation of specialized center for MON – optimization of diagnosis and management strategy; characterization of large cohort of patients with MON

3.2.1 *Extraocular features in LHON*

In our group of 54 (20 symptomatic) individuals with LHON, the average age at onset was 24 years. All but 2 symptomatic patients were men, manifesting with progressive, painless, binocular vision loss with the involvement of the fellow eye either sequentially (n=18) or simultaneously (n=2). Altogether, 16 patients were clinically asymptomatic, but detailed neuroophthalmic examination revealed discrete alterations (i.e. subclinical carriers). The remaining 18 patients were completely asymptomatic. Interestingly, extraocular symptoms were expressed in 11 carriers (hearing loss, peripheral neuropathy, myopathy, tremor). All but two (symptomatic woman and man, both carrying m.3460G>A with tremor, peripheral neuropathy and myopathy, and isolated tremor, respectively) had m.11778G>A. The most remarkable feature is the dominance of extraocular symptoms over only slightly affected visual functions in a family with m.11778G>A mutation. In 4 patients with myopathy and hearing loss or peripheral neuropathy, visual impairment is completely absent. In all four patients, available muscle specimen showed focal subsarcolemmal accumulation of SDH product and COX negative fibres in up to 5 % and 15 % of muscle fibres, respectively. Enzymology of the muscle biopsy revealed a severe Complex I defect in two of the patients (Complex I-III: 19-47 % of controls; Complex II-III: 39 % of controls). Both mtDNA and whole exome sequencing were performed and none of the variants found could explain the development of extraocular symptoms which supports the
exclusive role of m.11778G>A. Due to their high incidence, especially in Czech female pa-
tients, we emphasize the need for their search and management even in subclinical or asympto-
matic cases. We report that plus symptoms without the concomitant visual failure may be the
only presentation in LHON.

Kolarova H. et al. Extraocular features may be the essential presentation in m.11778G>A
LHON carriers. (in preparation).

3.2.2 Peripapillary microcirculation in LHON

In this first prospective observational comparative case series, we aimed to study the peripa-
pillary capillary network with SD–OCT–A in LHON. Herein, total of 12 eyes of six individuals
with LHON were imaged and compared to 6 eyes of three healthy controls. All three affected
individuals were males (two carriers of m.11778G>A and one carrier of m.14484T>C). In five
affected eyes SD–OCT–A showed the reduction of radial peripapillary microvascular network
that correlated with RNFL thinning and visual field loss. The same finding was observed in one
patient treated with Idebenone for 14 months that underwent almost full recovery of his left eye
with Best Corrected Visual Acuity (BCVA) of 0.87. Interestingly, the other eye showed normal
ocular findings 14 months after onset, which is an extremely rare finding, described in the lite-
rature in very few adult cases [28-31]. We have not observed any differences between unaffec-
ted mutation carriers and control eyes. Although subjective examination (BCVA) showed an
improvement of visual functions in our patients treated with Idebenone, radial peripapillary mi-
crovascular network dropout on SD-OCT-A remained stable. This observation may suggest that
improvement of RGC function relies more on functional changes of mitochondria, than on im-
provement in RNFL and/or microvascular network. Our findings confirm that the peripapillary
microvascular network is highly abnormal in eyes manifesting LHON but not in mtDNA
mutation carriers. They also support the hypothesis that microangiopathy contributes to the
development of mitochondrial disorders. Results of SD–OCT–A imaging in LHON patients in
this study raise the expectation that the method could be used in diagnosing and monitoring
function and apoptosis of RGC.

(submitted)

3.2.3 Sequence of symptoms in 50 Czech m.3243A>G carriers

The clinical and laboratory data of 33/50 symptomatic individuals with MELAS based on
m.3243A>G were analysed in a retrospective study. Fully expressed phenotype (i.e. with SLE)
was observed in 42% patients, whereas 58% were oligosymptomatic. The mean age of onset was 17.4±12.9 years. Remarkably, 3 patients presented very early (1 month, 6 months and 2 years, resp.). Similar to [32], all of them had a positive history of early developmental delay, which may represent a risk factor for earlier and more severe presentation of MELAS-specific symptoms. Overall, 58% manifested ≥4 symptoms; 4 of our patients remained monosymptomatic up to 12 years of follow-up. The most common presentations were sensorineural hearing loss (76%), myopathy (56%) – which was also the most frequent symptom at onset (18%) – and Chronic Progressive External Ophthalmoplegia (CPEO, 45%). The median time interval between the 1st and 2nd and the 2nd and 3rd symptoms was 5.0±8.3 (0-28) and 2±6.0 years (0-21), respectively. Ophthalmic involvement was observed in 22 patients and was mostly represented by ptosis and/or CPEO. Only in 2 patients, we verified the optic atrophy with retinitis pigmentosa. A difference in survival probability was noted between the juvenile (onset <16 years) and the adult groups (p=0.005). It seems that in MELAS any symptom may come first and stay isolated for a long-time interval. This finding highlights the importance of a lifelong follow-up.


3.2.4 Unique presentation of LHON/MELAS syndrome based on m.13046T>C

A 12-year-old girl with otherwise unremarkable medical history presented with abrupt, painless loss of vision. Ocular examination confirmed bilateral optic nerve atrophy with BCVA 0.125 in the right eye and 0.02 in the left eye. Standard automated perimetry revealed severe bilateral visual field loss and visual evoked potentials showed overall reduced amplitudes with prolonged P100 wave latencies. Despite the suggestive presentation, initial genetic analyses ruled out three LHON prevalent mutations. Over the next months, the girl developed moderate sensorineural hearing loss (30 dB loss in the right and 20 dB in the left ear), vertigo, migraines, anhedonia and thyroiditis, which are findings frequently associated with oligosymptomatic MELAS. Furthermore, metabolic workup documented elevated cerebrospinal fluid lactate of 4.25 mmol/l (controls <2.1) and enzyme histochemistry of muscle biopsy revealed signs of myopathy with focal subsarcolemmal accumulation of SDH product in up to 5% of muscle fibers and focal weakening of reaction in COX staining. There was a 50% reduction in the activity of Complex I of the respiratory chain in isolated mitochondria from muscle biopsies. MtDNA sequencing revealed a pathogenic heteroplasmic mutation m.13046T>C in the ND5 subunit of complex I (MT-ND5), previously described in a single case with a 2-way overlap syndrome.
MELAS/Leigh [33]. Quantification of heteroplasmy in examined tissue showed the following: urine 71 %, muscle 70 %, hair follicles 44 %, fibroblasts 40 %, buccal smear 34 % and blood 27 %. Surprisingly, the mutation was not detected in either of these tissues in any of the relatives. The present case emphasizes that in cases of vision loss suspected to be due to a mitochondrial disorder, mutations in other subunits of Complex I must be considered.


3.2.5 Molecular genetic analysis of OPA1 in 44 probands with optic atrophy

A total of 44 probands of British, Canadian and Czech (including 1 Czech Roma) origin referred with the diagnosis of bilateral optic atrophy to the University Hospital of Wales and General University Hospital in Prague were screened by direct sequencing of OPA1 coding regions and flanking intronic sequences in order to determine the molecular genetic cause of their condition. Detected rare variants were assessed for pathogenicity by in silico analysis. Prior to the start of the study, participating probands were tested negative for the 3 prevalent mutations associated with LHON. A total of 29 heterozygous pathogenic OPA1 mutations were identified, of these 7 were novel. The great majority (22; 76 %) of the detected pathogenic mutations were, based on in silico analysis, evaluated to lead to unstable transcripts resulting in haploinsufficiency [34-36]. One of the two mutations c.1148A>G in near proximity to intron–exon boundaries (2nd exonic 3 nucleotides), predicted to lead to an amino acid substitution, was found out to cause in–frame skipping of exon 11 (p.Val346_Phe383del) [37]. This case points out the fact that interpretation of mutations needs always to be put into context of nucleotide position within the open reading frame so that variants interfering with splicing process are not wrongly indicated as substitutions. Three probands with the following disease–causing mutations c. 1230+1G>A, c.1367G>A and c.2965dup were documented to suffer from hearing loss and/or neurological impairment. Our study expanded the spectrum of OPA1 mutations and supported the view that haploinsufficiency is the most common disease mechanism in DOA. In patients with optic atrophy after excluding three LHON prevalent mutations, OPA1 screening, including its flanking intronic regions, should be performed.


3.2.6 MON – optimization of centered care

Recent advances in therapeutic management of MON patients are very promising and highlight
the importance of early diagnosis. Nevertheless, incomplete penetrance, in addition to variable clinical manifestation, causes substantial differential diagnostic difficulties and often leads to therapeutic delay. The main focus of this Thesis was therefore the application of our results and experiences in clinical practice. In 2013, we succeeded in creating a **National Center for Patients with Mitochondrial Optic Neuropathies** in order to bring the best care to patients with rare mitochondrial optic diseases, improve diagnostic tools and harmonize clinical and preclinical research. In 2016, the Ophthalmology and Pediatrics Departments and the Institute of Inherited Metabolic disorders joined, as the only institutions of such specialization, the European Reference Network (ERN) on Eye and ERN for Hereditary Metabolic Diseases. In 2017, we become members of the National Board of LHON Experts. In our Laboratory for study of Mitochondrial Disorders there were 160 and 17 genetically confirmed LHON and DOA patients, respectively, but before the creation of our Center, only a handful of them benefitted from the regular follow-up care. The average diagnostic delay of MON was more than 12 months – a time when the irreversible tissue damage of the optic nerve already occurs. Since 2013, we managed to examine a group of 54 patients with LHON, 17 patients with DOA, 33 patients with MELAS and 10 patients with unknown cause of MON which provided the base for the above mentioned publications. Thanks to the early diagnosis in our Center, we were able to initiate therapy with coenzyme Q analogue (idebenone) in 8 symptomatic (5–LHON, 2–DOA, and 1–LHON/MELAS overlap syndrome) and 3 patients with subclinical signs of the disease in a period of 4 weeks - 9 months after the disease onset. This treatment has led to significant amelioration of visual impairment in 4 patients. Two probands were included in phase III clinical trial of gene therapy in Munich (see below).


### 3.2.7 Ocular gene therapies in patients with inherited retinal disorders

Because of relatively easy accessibility and visualization allowing monitoring efficacy, retina turns out to be a convenient compartment with tremendous treatment potential. At present, gene–based clinical trials have been registered for several monogenic retinal disorders: Leber Congenital Amaurosis 2 (LCA2), retinitis pigmentosa 38, Usher syndrome 1B, Stargardt disease, choroideremia, achromatopsia, X-linked retinoschisis and LHON. Apart from RPE65 gene therapy for LCA2 and *MT-ND4* for LHON which has reached phase III, all other trials are in investigation phase I and II, i.e. testing the efficacy and safety. Since the damage to the retina
sets in quickly, time window for therapeutic intervention is very short. Due to the absence of proper legislation in our country, none of the dozens of gene therapy studies are currently underway in the Czech Republic. However, due to the prompt diagnosis in our Center, two male patients with m.11778G>A LHON prevalent mutation, entered the phase III trial with gene therapy GS010 proceeding in Munich (RESCUE Study, GenSight Biologics, Paris; Study No: GS–LHON–CLIN–03A), and are, therefore, the first Czech patients approved for this kind of treatment. The first study conducted by the group of Wan showed promising results of GS010 with significant ameliorations in visual acuity and visual field [38]. With the development of novel therapeutic approaches, establishing not only clinical but also molecular genetic diagnosis is of crucial importance. Although very appealing, gene therapy still concerns an experimental therapeutic approach, yet only available by clinical trials. Nevertheless, recent rapid advances in this field have allowed us to hope that gene therapy will be a causal treatment not only for devastating retinal disorders within the next few years.


3.3 Relation to the aim C) Pathobiochemical and molecular aspects of selected rare inherited errors of metabolism

3.3.1 Characterization of three patients with Lipoprotein Lipase (LPL) Deficiency

First two Czech and one Russian patient with LPL deficiency are described. Patient 1 presented since the 1st week of life with recurrent abdominal pain; Patient 3 with abdominal distension and hepatosplenomegaly since the 2nd month of life. Patient 2, asymptomatic younger brother of Patient 1, was diagnosed in the 1st week of life. Lipaemia retinalis and splenomegaly were present in two children, hepatomegaly in Patient 3 and acute pancreatitis in Patient 1. Interestingly, in our patient siblings, enlargement of kidneys was also found, which, to our best knowledge, has not been described so far. All children had lactescent serum, profound hypertriacylglycerolaemia (124±25 mmol/l; controls < 2.2), hypercholesterolaemia (22.8±7.3 mmol/l, controls < 4.2) and low HDL-cholesterol level (0.42±0.02 mmol/l) that persisted even after long–term diet. Their serum LPL mass did not increase after heparin injection (6.3, 5.6 and 4.6 ng/l, respectively. Both siblings were homozygous for a novel c.476C>G (p.Ser159Th) and c.1421C>G (p.Ser474Term). Patient 3 was compound heterozygous for novel c.604G>A
(p.Asp202Asn) and c.698A>G (p.Tyr233Cys) in the LPL, respectively. A low–fat diet with 0.5-0.6 g of fat/day, introduced in all patients has led to normalization of lipid profile and clinical symptoms. We emphasize the need for considering LPL deficiency in neonates and young infants with abdominal pain and hypertriacylglycerolaemia as early treatment may prevent development of life–threatening pancreatitis.


3.3.2 Aberrant Apolipoprotein C-III (Apo C-III) glycosylation in Glycogen Storage Disorders (GSD)

It is increasingly evident that secondary glycosylaton abnormalities might occur in other, non–Congenital Disorders of Glycosylation (CDG) metabolic diseases. The aim of this study was to examine N-/O-glycosylation in our group of 24 patients with various types of Glycogen Storage Diseases (types 0, Ia, non-Ia, III and IX) in order to determine whether there is a broader biochemical overlap between the CDG and GSD syndromes. Transferrin N-glycosylation was found to be normal in the majority of the analyzed samples. While the sialylation of Apo C-III in GSD types 0, Ia and non-Ia was rather variable and overall borderline, in the patients with GSD types III and IX it was found to be consistently markedly reduced (p=0.0004). A notable elevation of the aglycosylated form of ApoC-III with absolutely unoccupied glycosylation site was found by subsequent analyses using sodium dodecyl sulfate polyacrylamide gel electrophoresis and MALDI-TOF mass spectrometry, predominantly in patients with GSD types III and IX. The ApoC-III glycosylation profile became more pathological in a period of non–compliance with frequent hypoglycaemias and the status of ApoC-III sialylation perhaps reflects metabolic (de)compensation in these patients. Considering the unavailability of enzymatic assay for GSD type III or certain unreliability of this investigation in GSD type IX, our finding could help clinicians better target and select appropriate genetic analysis for the diagnostic confirmation. We also hypothesize that ApoC-III hypoglycosylation in these disorders results from reduced availability of the nucleotide-monosaccharides, specifically UDP-GalNAc, in the corresponding glycosylation reactions.

4 CONCLUSION

The overall goal of the present Thesis was to study the mitochondrial biogenesis within foetal, neonatal and adult period and to characterize MON as well other two rare inherited errors of metabolism on clinical, biochemical and molecular level.

The contributions of this work include:

- Our study of mitochondrial biogenesis in early human development significantly supported the fact that GW 22 is the edge of viability, which has to be taken into account in upcoming discussions about guidelines on resuscitation of preterm neonates.
- We succeeded in creating a National Center for Patients with Mitochondrial Optic Neuropathies in order to in order to bring the best care to patients with rare mitochondrial optic diseases. Within the centre, we managed to examine large cohort of patients with various types of MON, bring their biochemical and molecular characterization, expand genotypes and phenotypes associated with the disorders and introduce novel diagnostic and therapeutic approaches.
- We described the first Czech patients with LPL deficiency and disease–related kidney enlargement in our patient siblings is suggested to be a novel presentation of this a rare disorder of lipid metabolism. Novel finding of markedly hypoglycosylated ApoC-III in patients with GSD types III and IX could be helpful for clinicians in cases of suspected patients, in whom the clinical picture strongly suggests a particular type of GSD, but the corresponding enzymatic assays are within the normal range.

In conclusion, the proposed aims of the thesis were accomplished. Present work contributed to better understanding of mitochondrial biogenesis within the foetal development as well as adult life. Secondly, we have managed to characterize the biochemical and molecular–genetic background in more than 200 patients, and both selected cases and cohort studies were published, bringing novel phenotype and genotype findings. Finally, the main contribution of this Thesis was the application of our results and experience in clinical practice. Our results bring evidence that centralized care management significantly improves quality of life in families affected by mitochondrial optic neuropathies.
5 REFERENCES


6 LIST OF PUBLICATIONS (Total IF 18.526)

Publications in extenso, that constitute the basis of the PhD thesis:

a) with IF


b) without IF


The first author of 9 chapters: Kolarova H., Honzik T., Zeman J.: Poruchy cykly močoviny (Urea Cycle Disorders); Fenylketonurie (Phenylketonuria); Glykogenóza typ Ia (Glycogenosis Type Ia); Glykogenóza typ non-Ia (Glycogenosis Type non-Ia); Glykogenóza typ III (Glycogenosis Type III); Leberova hereditární optická neuropatie (LHON syndrom) (Leber Hereditary Optic Neuropathy); Deficit lysosomální kyselé lipázy (Lysosomal Lipase Deficiency); Deficit lipoproteinové lipázy (Lipoprotein Lipase Deficiency); Hypofosfatázie (Hypophosphatasia); Syndrom MELAS (Mitochondrial myopathy, Encephalopathy, Lactic Acidosis, and Stroke–like episodes). (Syndrome MELAS).

Other publications in extenso

a) with IF

b) without IF