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Význam laktátu v diagnostice mitochondriálních onemocnění u dětí

Role of lactate in diagnostics of mitochondrial disorders in children

Disertační práce

PhD Thesis

Školitel: Prof. MUDr. Jiří Zeman, DrSc.

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ABBREVIATIONS

A	acetoacetate
ADP	adenosine diphosphate
ATP	adenosine triphosphate
ATP6	ATP synthase 6
ATP12	ATP synthase mitochondrial F_1 complex assembly factor 2
B	3-hydroxybutyrate
BCS1	Saccharomyces cerevisiae bcs1 protein
COX I, II	cytochrome *c* oxidase subunit I, II
COX 10, 15	cytochrome *c* oxidase assembly protein 10, 15
CPEO	chronic progressive external ophtalmoplegia
CSF	cerebrospinal fluid
CT	computer tomography
cyt	cytochrome
DGUOK	deoxyguanosine kinase
DNA	deoxyribonucleic acid
DIDMOAD	diabetes insipidus, diabetes mellitus, optic atrophy, deafness
FAD	flavin adenine dinucleotide
IMM	inner mitochondrial membrane
IF	impact factor
KSS	Kearns-Sayre syndrom
L	lactate
L/P	lactate/pyruvate ratio
LDH	lactate dehydrogenase
LHON	Leber hereditary optic neuropathy
LRPPRC	leucine-rich PPR motif-containing protein
MELAS	mitochondrial myopathy, encephalopathy, lactic acidosis and stroke-like episodes
MERRF	myoclonic epilepsy associated with ragged-red fibers
MRI	magnetic resonance imaging
MTCYB	cytochrome *b* of complex III
mtDNA	mitochondrial deoxyribonucleic acid
NAD$^+$	nicotinamide adenine dinucleotide
NARP	neuropathy, ataxia, and retinitis pigmentosa
ND 3, 5, 6	complex I, subunit 3, 5, 6
nDNA	nuclear deoxyribonucleic acid
OGTT	oral glucose tolerance test
OMM	outer mitochondrial membrane
OXPHOS	oxidative phosphorylation
P	pyruvate
PC	pyruvate carboxylase
PDHA1	pyruvate dehydrogenase complex, E1-alpha polypeptide 1
PDHB	pyruvate dehydrogenase complex, beta polypeptide
PDHc	pyruvate dehydrogenase complex
PDHE3	dihydrolipoamide dehydrogenase (DLD), pyruvate dehydrogenase component E3
PEO	progressive external ophtalmoplegia
POLG1	DNA polymerase gamma
ROS	reactive oxygen species

SDHA	succinate dehydrogenase, subunit A
SDHC	succinate dehydrogenase, subunit C
SDHD	succinate dehydrogenase, subunit D
SANDO	sensory ataxic neuropathy, dysarthria, ophthalmoplegia
SUCLA2	succinate-coenzyme-A ligase
SURF1	Surfeit 1
TIM	translocase of the inner mitochondrial membrane
TK2	mitochondrial thimidine kinase
TMEM70	transmembrane protein 70
TOM	translocase of the outer mitochondria membrane
tRNA	transfer RNA
UCP	uncoupling protein
UQCRB	ubiquinone-binding protein
UV	ultraviolet light

CONTENTS

1. INTRODUCTION

Mitochondria are double-membrane embedded semiautonomous organells of eucaryotic cells. Main task of mitochondria is to oxidize substrates to produce energy and to sustain amphibolic pathways, such as the tricarboxylic acid cycle essential for survival. According to endosymbiotic theory, the origin of mitochondria lies in the engulfment of aerobic bacterial cell by primitive eucaryotic cell, approx. 2,300 million years ago. This hypothesis would explain the presence of mitochondrial DNA and proteosynthetic apparatus (Feng et al. 1997).

For different disciplines, mitochondria represent distinct object of interest. For the molecular anthropologists they represent a key to the beginnings of mankind, "mitochondrial Eve" and the differentiation of ethnic groups. For the physiologists and molecular biologist, they afford an excelent subject of studying structure-function relationship, active molecular transport, dual genome controlled mechanism, and metabolic control on a subcellular level. For the physicians, they represent the possible cause of several well-defined diseases and syndromes, but also on the other hand illnesses with extremely variable combinations of clinical signs, great diagnostic challenges and narrow therapeutic possibilities (Chinnery 2010).

A significant progress has been made in the large and dubious field of mitochondrial science. However, even more and more questions arised which remain to be answered. Main focus of the Thesis work is the assessment of significance of lactate analysis in the diagnostics of mitochondrial diseases. The literary overview of the metabolism of lactate with clinical consequences is given in the second part of the theroetical section. In the first part, I try to outline a very brief introduction to the mitochondrial medicine.

1.1. Mitochondrial overview

1.1.1. The beginnings of mitochondrial research

Mitochondrial research has started about 170 years ago. The earliest records on intracellular structures that probably represent mitochondria go back to the 1840s. Mitochondria were probably first recognized by A. Kölliker in the middle of the 19th century. He described them as granules in striated muscle (Kölliker 1856). In 1890, R. Altmann described primitive, ubiquitously occuring self-replicating bodies that he stained specifically and referred to as „elementary organisms" living inside cells and carrying out vital functions. He called them

"bioblasts". In 1898, the cytologist C. Benda introduced the name mitochondria in his studies on the thread-like granules that he observed during spermatogenesis. The name originates from the Greek "mitos" ($\mu i\tau o\varsigma$, thread) and „chondros" ($\chi o\nu\delta\rho i\nu$, granule) (Ernster and Schatz 1981).

A German-born American biochemist and physician Leonor Michaelis famous for his work with Maud Menten in enzyme kinetics found that the redox dye Janus Green B serves as a specific supravital stain of mitochondria in 1900. He such demonstrated that these organelles had oxidoreduction activities, however he did not related his observations with mitochondria role in cellular oxidation. This feature became the „official portrait" of mitochondria until 1952, when Romanian cell biologist and Nobel Prize holder George Palade used transmission electron microscope to show the first images of the folds of the mitochondria membrane (Masters 2009). In 1912, B.F. Kingsbury (Kingsbury 1912) postulated that mitochondria serve as „a structural expression of the reducing substances concerned in cellular respiration". Unfortunately, there was no direct evidence for his conclusion, until development of mitochondria isolation protocol and determination of their chemical constitution. In 1934, Bensley and Hoerr (Bensley 1934) described the isolation of a fraction from guinea-pig liver that at least partly consisted of mitochondria. This method opened the way to the identification of mitochondria role in cell respiration.

Early studies on cell respiration and oxidative phosphorylation were performed from the early 1910s. In guinea-pig liver extracts, Warburg reported in 1913 that cell oxidative reactions are associated with insoluble cellular structures. In 1925, Keilin described the cytochromes. This discovery led to the definition of the respiratory chain as a sequence of catalysts comprising the dehydrogenases on one end and "respiratory enzyme" (*Atmungsferment* according to Warburg) on the other (Keilin 1925).

Considerable progress was made in elucidating the reaction pathways and energetics of aerobic metabolism during the second half of the 1930s. Nobel Prize holder, Albert Szent-Györgyi, identified fumaric acid and other steps of the citric cycle in his work on cellular respiration. The citric acid cycle (Szent-Györgyi-Krebs cycle, Krebs cycle) was formulated in 1937 by Hans Krebs, who already identified the urea cycle in 1932. For the discovery of fundamental importance he was awarded Nobel Prize for physiology in 1953 (Krebs and Johnson 1937).

In kidney homogenates, Kalckar presented his first observations leading to the demonstration of aerobic phosphorylation. In 1941, Lipmann (Lipmann 1941) suggested a scheme of „phosphate-bond energy" as a general form of energy conservation in cellular metabolism. In
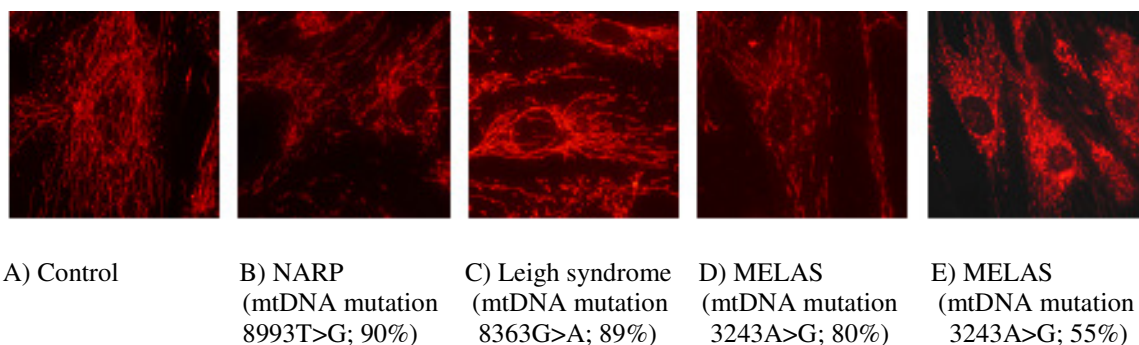
1943, Ochoa published his results indicated that the aerobic oxidation of pyruvate raises 3 moles of organically bound phosphate for each oxygen atom consumed. In 1948-1949, Friedkin and Lehninger proved the occurrence of respiratory chain-linked phosphorylation. In rat liver mitochondria, Kennedy and Lehninger (Kennedy 1949) also demonstrated the aerobic oxidation of citric acid cycle metabolites and fatty acids. Furthermore, they described accompanying formation of ATP from inorganic phosphate and ADP.

In the early 1950s, the isolation of structurally well-preserved mitochondria was possible, thus allowing the discovery of certain organized features of the mitochondrial enzyme system. In 1951-1952, several laboratories (Niemeyer 1951; Rabinovitz 1951; Lardy 1952) demonstrated „respiratory control" with isolated mitochondria, which required a certain degree of structural intactness. This effect consisted of a control of the respiratory rate by the availability of inorganic phosphate and the phosphate acceptor ADP. Kielley and Kielley (Kielley 1951) discovered another organized feature of intact mitochondria in 1951: the „latency" of the ATPase activity, which was stimulated by agents that damage the mitochondrial structure. In 1957, Chance and Hollunger showed that intact mitochondria catalyze an energy-dependent reduction of endogenous NAD^+ by succinate (Chance 1957). Summarised, these as well as others observations indicated that the coupling of respiration to phosphorylation requires a structural feature of the mitochondrion in addition to a set of functional enzymes. However, the understanding of the nature of this structural feature had to await better knowledge of mitochondrial ultrastructure and in particular, of the role of membranes in energy transduction. This topic will be further discussed in the particular chapter.

In short, mitochondrial research was initially focused on studying of fundamental metabolic and bioenergetic functions (oxidative phosphorylation, OXPHOS), Krebs cycle, heme biosynthesis, β-oxidation of fatty acids, and the metabolism of certain amino acids (Ernster and Schatz 1981). Several other research areas have grown important: e.g. targeting and import of proteins into mitochondria (Neupert and Herrmann 2007), biogenesis of Fe/S clusters (Lill and Kispal 2000), or the role of mitochondria in controlling apoptosis in higher eukaryotes (Jiang and Wang 2004). Mutations in mitochondrial DNA and other mitochondrial defects have been to higher and higher extent linked to a wide range of human diseases: numerous neuropathies and myopathies (Wallace 2005), premature ageing of mice (Trifunovic et al. 2004) and many others. The endosymbiont hypothesis, which was adumbrated by Altman in 1890, was fueled after the discovery of mitochondrial DNA in the 1960s (Chance 1961). It was reincarnated in its modern formulation by Margulis (Margulis 1970), and disputed by the serial endosymbiosis theory by Taylor (Taylor 1987).

1.1.2. Structure of mitochondria

Mitochondria are traditionally depicted as elongated cylindres with diameter of 0.5-1 micrometer. But as early as 1914-1915 Lewis and Lewis (Lewis 1914) described extensive changes in the position and shape of mitochondria in animal tissue cultures. These observations were later extended by several investigators with the time-lapse records (Frederic 1952). Nowadays we know that mitochondria are highly dynamic and remarkably plastic structures. They have a great ability to change their shape, fuse with one another and then separate again (Soubannier and McBride 2009). They are not present in the form of many individual organells in the cell but they constitute dynamic mitochondrial networks (Bereiter-Hahn and Voth 1994; Soubannier and McBride 2009). The structure and function of mitochondria also differ in particular tissues depending on the energetic demands of such tissue (Benard et al. 2006).

| A) Control | B) NARP (mtDNA mutation 8993T>G; 90%) | C) Leigh syndrome (mtDNA mutation 8363G>A; 89%) | D) MELAS (mtDNA mutation 3243A>G; 80%) | E) MELAS (mtDNA mutation 3243A>G; 55%) |

Fig. 1. Mitochondrial network in cultured human fibroblasts. Imunostaining was performed with primary antibodies against voltage-dependent anionic channel (VDAC, mitochondrial porin) conjugated with AlexaFluor 594. Magnification 600×. % represent heteroplasmy level. Courtesy of Mgr. Olina Kostková, Ph.D.
MELAS - mitochondrial myopathy, encephalopathy, lactic acidosis and stroke-like episodes; NARP - neuropathy, ataxia, and retinitis pigmentosa

The number of mitochondria in a cell varies broad among different species. It also depends on tissue type. Cells can contain several hundreds to thousands mitochondria (Alberts 2002). These variations are related to the specific function of the tissue. The mitochondrial proteome is dynamically regulated and a number of mitochondrial proteins also vary widely. In human cardiac mitochondria, 615 distinct types of proteins have been identified (Taylor et al. 2003). On the other hand, in *Murinae*, 940 proteins encoded by distinct genes have been reported

(Zhang. 2008). However, the number of mammalian mitochondrial proteins is estimated to 1,130-1,500 (Mootha et al. 2003).

A fact that mitochondria are surrounded by a membrane was suggested on the basis of early observations made with the light microscope. The first electron micrographs of mitochondria, published by Albert Claude, Keith Porter and Ernest Fullam in 1945, confirmed this conclusion (Masters 2009).
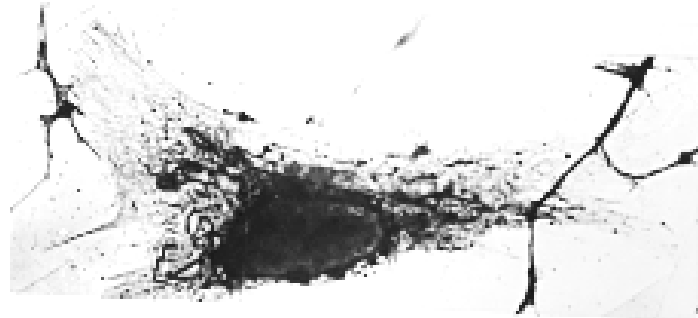
Fig. 2. The famous first electron micrograph of a cell (cultured fibroblast originating from a chick embryo) published by Porter, Claude and Fullam. It reveals mitochondria, the Golgi apparatus and a "lace-like reticulum" which Porter later named the "endoplasmic reticulum". Magnification 1600×.
(from http://www.rockefeller.edu/rucal/journey/journey.html)

Detailed studies of the mitochondrial ultrastructure became possible after the development of the thin sectioning techniques in the early 1950s. The first high-resolution electron micrographs were published in 1952-1953 by Palade and Sjöstrand. Several hypothetical models of mitochondria structure were proposed. Palade found that the mitochondrion is surrounded by a membrane, which is folded in ridges inside the mitochondrion; he named these ridges *cristae mitochondriales*. Another interpretation of these structures was proposed by Sjöstrand as "septa model". Sjöstrand also outlined existence of a double limiting membrane surrounding the mitochondrion, which was soon confirmed by Palade (Masters 2009).

Mitochondrial topography is highly specialised and comprises several compartments that carry out specialized functions. Each mitochondrion is bounded by outer and inner mitochondrial membrane. These two membranes define two separate compartments: the inner matrix and narrow intermembrane space. The inner membrane is highly convoluted and forms infoldings knowns as *cristae*. As mentioned above, ultrastructure of mitochondria depends not even on the tissue type, but on the physiological state and the devolopmental stage (Fawcett 1981).
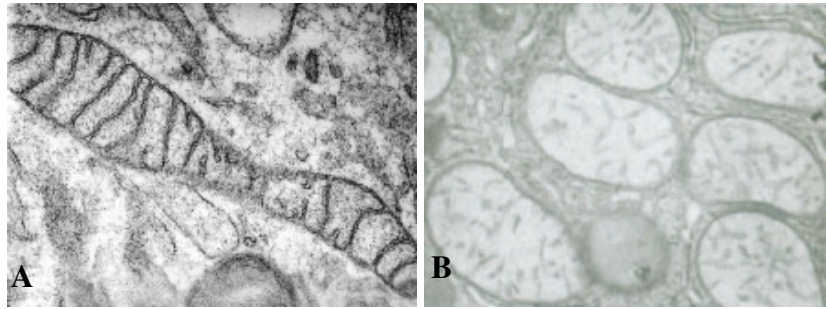
Fig. 3. Electronmicroscopic image of mitochondria in cultured human fibroblasts (A, magnification 44000×) and hepatocytes (B, magnification 15000×). Notice elongated shape of fibroblasts mitochondria, double membrane, well-developed cristae and spheric shape of hepatocytal mitochondria. (Lea, Temkin et al. 1994)

Outer mitochondrial membrane (OMM) encloses the entire organelle and carries many copies of porin. Porin, a transport protein forming aqueous channels, underlies permeability of OMM for molecules up to of 5,000 Da of their molecular mass, including small proteins (Alberts 2002).

Intermembrane space is chemically equivalent to the cytosol. It is the space between the outer membrane and the inner membrane. Proteins destined for the transport through the intermembrane space are translocated by special transport proteins. These are known as translocase of the outer mitochondria membrane (TOM) and translocase of the inner mitochondrial membrane (TIM) (Neupert and Herrmann 2007).

Inner mitochondrial membrane (IMM) is highly specialised and highly impermeable to all molecules. It contains more than 151 different polypeptides. A typical hallmark for IMM is a very high protein-to-phospholipid ratio (more than 3:1 by weight). The number of proteins of IMM represents about 20% of the total protein content of a mitochondrion (Alberts 2002). IMM contains a high proportion of an unusual phospholipid, cardiolipin, which may help to make the inner membrane impermeable. The IMM does not contain porins and is subcompartmentalized into numerous cristae.

Proteins of the IMM can be divided into groups according to their functions (Alberts 2002):

1. proteins performing the redox reactions of oxidative phosphorylation
2. ATP synthase
3. transport proteins regulating molecules passage into and out of the matrix

4. protein import machinery and

5. mitochondria fusion and fission protein

Cristae are IMM infoldings projecting into the matrix. They expand the surface area of the IMM and increase its ability to produce ATP. In typical liver mitochondria, cristae constitute about 30% of the total cell membrane (Alberts 2002), with the area of the inner membrane about five times greater than the outer membrane in the cells with high-energy demands. Cristae are studded with F_1 particles (oxysomes). Cristae membranes of different shapes have been observed: tubular, lamellar and ever triangular (Fawcett 1981).

The inner matrix is a space surrounded by the IMM. It is viscous in comparison with cytosol (Soboll 1976). Matrix contains highly concentrated mixture of hundreds of enzymes, different number of mitochondrial genome copies, mitochondrial ribosomes and tRNAs. In the matrix, oxidation of pyruvate and fatty acid, part of the urea cycle and haem biosynthesis, as well as the citric acid cycle, take place.

1.1.3 Oxidative phosphorylation

The system of oxidative phosphorylation (OXPHOS) serves as a major energy-supplying process, which utilises redox equivalents (NADH and $FADH_2$) raised by substrates oxidation to ATP. Coupling between substrates oxidation and phosphorylation of ADP was discovered sixty years ago, but the precise mechanism of this energy converting mechanism remained long unknown. Finally the chemiosmotic theory based on substrate oxidation and ADP phosphorylation using electrochemical potential of transmembranous proton gradient μ_{H+} was proposed by Mitchell (Mitchell 1961). According to this theory, the oxidation of the substrates is coupled to synthesis of ATP by the function of separate proton pumps in respiratory chain complexes, and ATPase. The electrochemical potential produced by proton pumping in respiratory chain is utilized by ATP synthase, which at the expense of this potential synthesizes ATP. The principles of chemiosmotic theory became widely accepted and the British biochemist Peter D. Mitchell was awarded Nobel Prize in biochemistry in 1978.

Fig. 4. Prof. Peter Mitchell.
http://nobelprize.org/nobel_prizes/chemistry/laureates/1978/mitchell-bio.html

OXPHOS consists of 5 enzymatic complexes, which are made of approximately 90 structural proteins. Only 13 proteins of them are encoded by mtDNA. Nuclear DNA codes the rest of structural subunits and assembly proteins, which take part in biogenesis of particular complexes and assembly of whole OXPHOS system. More than 200 such proteins are known at present (Valente et al. 2007). OXPHOS complexes occur either individually or as supramolecular structures named „supercomplexes". The organisation of supercomplexes probably influences the electron transport effectivity, OXPHOS complexes stability and inner mitochondrial membrane structure (Schafer et al. 2006; Boekema and Braun 2007).

OXPHOS consists of following 5 complexes:

Complex I, NADH: coenzyme Q oxidoreductase (EC 1.6.5.3) is an L-shaped molecule with larger integral membrane part and smaller arm sticking out into mitochondrial matrix. It contains several electron transporters, flavin mononucleotide (FMN), multiple Fe-S centres and probably also bound coenzyme Q. It is the largest complex in the electron transport chain. In mammalian mitochondria it is composed of at least 43 subunits and its approximate molecular mass is 800 kDa. Seven of these subunits are encoded by mitochondrial DNA (Capaldi et al. 1977; Chomyn et al. 1985).

Complex II, succinate: coenzyme Q oxidoreductase (EC 1.3.5.1) has an approximate molecular mass of 120 kDa and it consists of four subunits: hydrophobic large and small cytochrome *b* binding proteins containing one heme (SDH-C and SDH-D), hydrophilic flavoprotein SDH-A, and hydrophilic subunit SDH-B, which contains three Fe-S clusters. The

two latters are located on the matrix side and have the enzymatic activity (Hanstein et al. 1971). All four subunits of complex II are encoded by nuclear DNA (Capaldi et al. 1977). Complex II catalyses the oxidation of succinate to fumarate; it passes electrons directly to the quinone pool. It serves as the only direct link between the citric acid cycle and the electron transport chain in the mitochondrial membrane (Davis et al. 1972).

Complex III, ubiquinone: cytochrom c oxidoreductase, (EC 1.10.2.2) is composed of 11 subunits. It contains Fe-S cluster, two b type hemes and the heme of cytochrome c_1. Complex III is located asymmetrically across the inner mitochondrial membrane. It is functional as a dimer with molecular mass of 480 kDa. Only the cytochrome b subunit is encoded by mtDNA, the other subunits are encoded by nuclear DNA (Rieske et al. 1967).

Complex IV, cytochrome c oxidase, (EC 1.9.3.1) is the terminal enzyme of the respiratory chain, which transfers electrons to molecule of oxygen. It contains 13 subunits with four redox centers: heme a_3 and heme a, and copper ions Cu_A and Cu_B. Only the largest three proteins COX-1, COX-2 and COX-3, which build the catalytic core of the enzyme, are encoded by mtDNA. The molecular mass of complex IV is 205 kDa (Capaldi 1990).

Complex V, mitochondrial F_1F_0 – ATP synthase (EC 3.6.3.14) has a molecular mass of 600 kDa. It consists of two subcomplexes: the extramembranous F_1 and membrane intrinsic F_O subcomplex. These two subcomplexes are attached by the central and peripheral stalks (Weber 2006). The F_1 complex consists of five subunits in $\alpha_3\beta_3\gamma\delta\epsilon$ stoichiometric ratio, with a ring of α and β subunits alternating around a single γ unit. The inhibitor protein (IF_1) is believed to act as a bridge among several subunits of F_1. F_O complex is composed of 10 subunits, which are arranged into two domains – c-ring to located in the axis of the central stalk and the attached second domain, which creates the peripheral stalk. MtDNA encodes only two hydrophobic proteins: subunit a and A6L.

Fig.5. System of oxidative phosphorylation – OXPHOS.
http://ccforum.com/content/10/5/228/figure/f1?highres=y
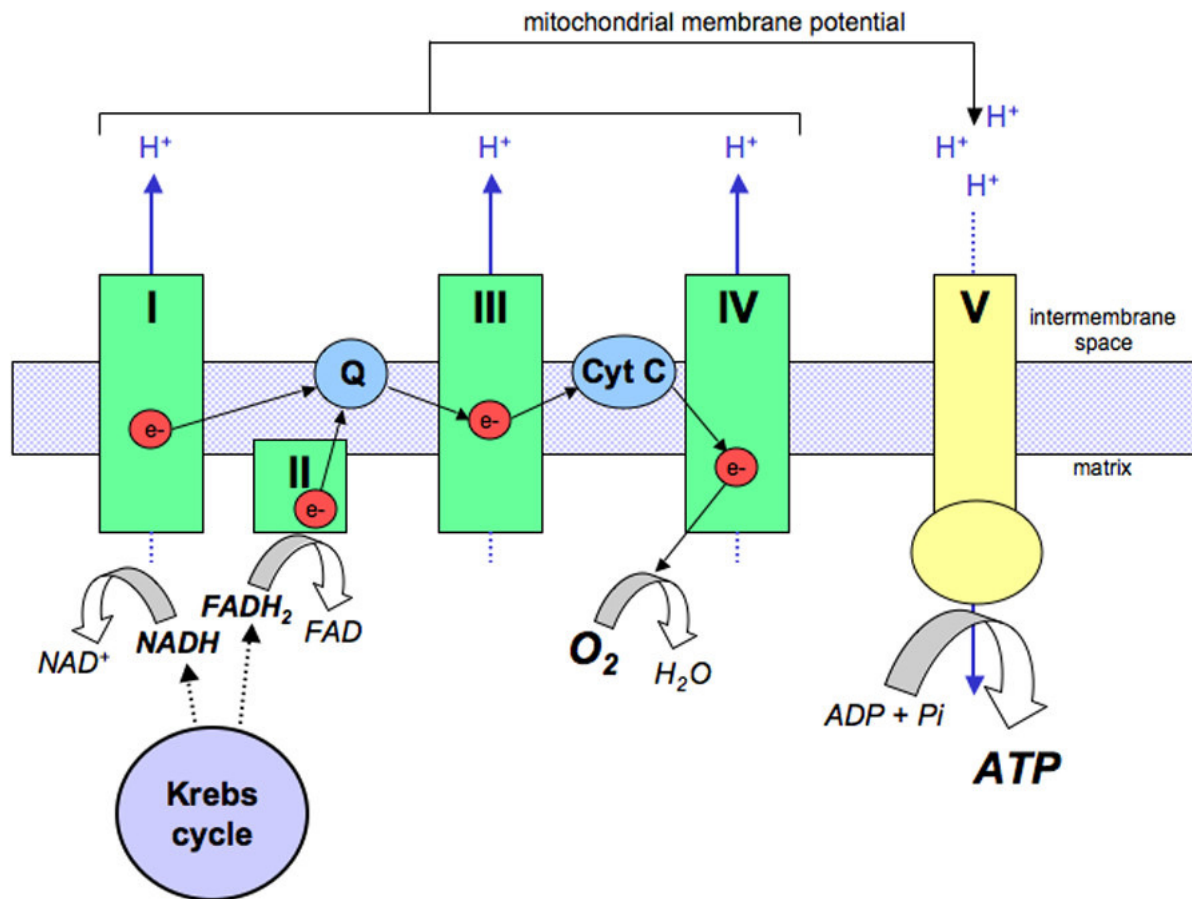
Reducing equivalents enter the OXPHOS system in the form of NADH and $FADH_2$. NADH is produced in the cytoplasm by the glyceraldehyde-3-phosphate dehydrogenase reaction, and in the mitochondria in the tricarboxylic acid cycle. Flavin adenine dinucleotide ($FADH_2$) is produced by the PDHc reaction and the tricarboxylic acid cycle. Electrons donated by reducing equivalents enter the complex I or complex II and initiate series of iron- or copper-mediated oxidation-reduction reactions that culminate in the reduction of molecular oxygen to water. Complex I, complex III, and IV are believed to act as proton pumps that create an electrochemical gradient across the mitochondrial inner membrane. This gradient represents the driving force for ATP synthesis by membrane associated complex V, the mitochondrial F_1F_O ATP synthase (Boyer 2000). F_1F_O ATP synthase operates as a reversible rotary motor. The F_O converts the electrochemical gradient of protons into torque to force the F_1 motor for anticlockwise rotation. Different interactions of the three β subunits with the central single γ subunit induce asymmetry at the three catalytic sites. The catalytic sites on each of the three β subunits cycle between three binding states for substrates and products in so-called

"alternating sites" mechanism. The sequentially changing conformation of the F_1 β subunit affects the varying affinity for ADP, P_i and ATP. ADP and P_i are bound to the catalytic site of the F_1 β subunit. The macroenergetic bound formation and ATP release occur after further turn of the domain. During one twist of the central stalk three ATP molecules are released (Weber 2006). It is appropriate to mention that F_1F_O ATP synthase may work also in the reverse mode as an ATP hydrolase. The ATP is synthesized intramitochondrially and then translocated to cytosole in exchange for ADP by ADP/ATP translocator (Weber 2006).

The membrane gradient of protons can be also used to generate heat instead of ATP. The heat production is based on presence of the uncoupling proteins (UCP) in mitochondia of mammals and other higher eukaryotes. Genes for UCP represent an important example of developmentally regulated genes participating in the control of mitochondrial energy conversion (Brauner et al. 2003).
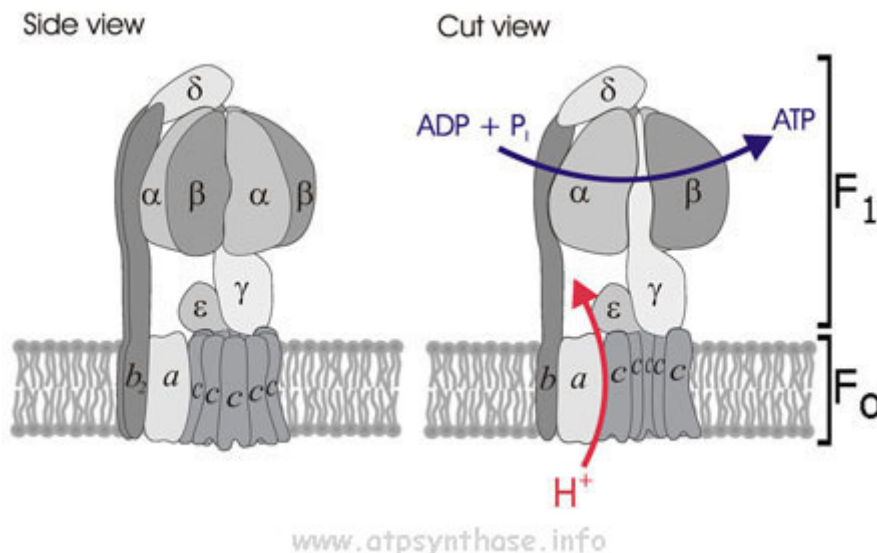
Fig. 6. Function of ATP-synthase. F_1F_O ATP synthase operates as a reversible rotary motor. The F_O converts the electrochemical gradient of protons into torque to force the F_1 motor for anticlockwise rotation. The sequentially changing conformation of the F_1 β subunit affects the varying affinity for ADP, P_i and ATP. ADP and P_i are bound to the catalytic site of the F_1 β subunit. The macroenergetic bound formation and ATP release occurs after further twist of the domain. From: http://www.atpsynthase.info/Basics.html

The OXPHOS enzyme complexes also functionally interact with other parts of the mitochondrial metabolism: citrate cycle *via* complex II and complex I (Schagger and Pfeiffer 2001), with glycerolphosphate shuttle *via* mitochondrial glycerophospate dehydrogenase (mGPDH) (Werner and Neupert 1972), and with fatty acid oxidation enzymes (they transfer electrons to Coenzyme Q) (Sumegi et al. 1991).

1.1.4. Reactive oxygen species

Oxygen used by mitochondria is reduced to water by series of reactions that produce potentially highly reactive oxygen species (ROS). These are generated, when unpaired electrons interact with molecular oxygen (O_2) and thus generate highly reactive superoxide ions (O_2^-), hydroxyl radicals (OH·), and hydrogen peroxide (H_2O_2). ROS are thus a natural byproduct of the normal oxidative phosphorylation. They have important roles in cell signalling. However, during conditions of environmental stress (e.g. UV or heat exposure), ROS levels can increase dramatically. This may result in significant damage of cell structures: damage of both mitochondrial and nuclear DNA, oxidations of polydesaturated fatty acids in lipids (lipid peroxidation), oxidations of amino acids in proteins and oxidative inactivation of specific enzymes by oxidation of co-factors. This cumulates into a situation known as oxidative stress.

Cells posses several mechanisms to defend themselves from ROS: with enzymes (e.g. manganese superoxide dismutases, catalases, lactoperoxidases, glutathione peroxidases and peroxiredoxins) but also small molecules (e.g. ascorbic acid, tocopherol, uric acid, and reduced glutathione) (Peng and Jou 2010). It has been shown that disorders of OXPHOS may lead to increased generation of ROS. The pathogenic mechanism thus includes not only the energy deprivation but also the enhanced oxidative stress (Houstek et al. 2006). The increased ROS production has also been connected to the apoptosis, neurodegeneration, Alzheimer disease, and aging (Liu et al. 2002; Muller et al. 2007).

1.1.5. Genetics of mitochondria

Mitochondria and OXPHOS are functionally controlled by two genomes: nuclear DNA (nDNA) and mitochondrial DNA (mtDNA) as the only exception in the biology of man. Mitochondrial DNA is inherited exclusivelly maternally (DiMauro and Schon 2003). However, it seems to be very likely that some exceptions do exist. In 2002, Marianne Schwartz and John Vissing reported a patient with mitochondrial myopathy and a microdeletion in ND2 who had inherited most of his muscle mtDNA from his father (Schwartz and Vissing 2002).

Both Meves and Regaud suggested a role of mitochondria as „bearers of genes" already in 1904. However, the existence of mitochondrial DNA was confirmed only in 1963 (Nass and

Nass 1963). The first pathogenic mutation was described by the team of Anita Harding, who published a case report of patient with mitochondrial myopathy with large deletion of mtDNA in the year 1988 (Holt et al. 1988).

After establishing a symbiotic relationship, the loss of redundant genes and the transfer of genes from the bacterium to the nucleus lead to the currently observed distribution of genes between the two genomes (Gray 1999). Human mtDNA is a circular doublestrand molecule with length of 16,569 base pairs. Beside the genes for 12S and 16S rRNAs and 22 tRNAs, it encodes only 13 from approximately 1130 – 1500 mitochondrial proteins. Only the D-loop is a noncoding stretch, containing the promoters for L and H strand transcription. All coding sequences are contiguous with each other without introns (Anderson et al. 1981; Meisinger et al. 2008).

Each mammalian mitochondrion contains up to 10 mtDNA copies per organelle. Each human cell, except mature erythrocytes, contains hundreds to thousands of mitochondria and, thus, 10^3-10^5 copies of mtDNA. During cellular division, mitochondria are randomly distributed to the daughter cells. The stochastic partitioning of mitochondria during cell division, a process called 'replicative segregation', therefore provides a mechanism for unequal distribution of mutated mtDNA. Mixture of mutant and wild-type mtDNA within each cell is called heteroplasmy (Holt et al. 1988; Holt et al. 1990).

The percentage level of mutant mtDNA may vary among organs and tissues within the same family, and also organs and tissues within the same individual. It may also vary with age. The mutant load of affected tissue is directly related to the severity of the phenotype in some diseases. Usually, the pathogenic treshold is high in most tissues. The level of heteroplasmy, at which the pathogenic mtDNA mutation manifests biochemically and clinically, is called the threshold effect (Shoffner 1995). Heteroplasmy and the threshold effect are crucial concepts by which to understand the extraordinary clinical heterogeneity of mtDNA-related diseases (DiMauro 2011). However, for most mitochondrial disorders caused by mutation in mtDNA the situation is more complicated, with very important role of the genetic backround. A good example was given by Hudson et al. (Hudson et al. 2007), who described a greater risk of visual loss in LHON, when patients harbouring each of three major mutations belonged to specific haplogroups.

The rate of mutations in mtDNA is 10 to 17 times higher than in nuclear genes with a similar function (Wallace et al. 1987). This fact is usually explained by absent histones, higher reactive oxygen species (ROS) products of OXPHOS, and the lack of effective DNA repair mechanism (Kang and Hamasaki 2002). All of deletions and duplications (Holt et al. 1988;

Zeviani et al. 1988), point mutations for structural units (Shoubridge 2001), tRNA (Shoffner et al. 1990), and rRNA genes (Lu et al. 2010) have been described.

The mutations in mtDNA are an important cause of mitochondrial diseases. However, the majority of mitochondrial diseases are caused by mutations of genes in nuclear DNA. In children, the percentage of mitochondrial disorders due to mutations in nuclear DNA is estimated to be approximately 80% (Zeviani and Di Donato 2004). All of mutations in genes for structural subunits, genes for assembly factors, translation factors, and mutations associated with multiple mtDNA deletions or mtDNA depletion have been described. The first nucleary encoded mitochondrial defect was published by Bourgeron in 1995, who described two girls with Leigh syndrome, in whom complex II deficiency were diagnosed. (Bourgeron et al. 1995). Structural defects have been described also in all other complexes of oxidative phosphorylation: in complex I (Smeitink and van den Heuvel 1999), in complex III (Haut et al. 2003), in complex IV (Massa, et al. 2008), and very recently by cooperation of Salzburg and Prague groups in complex V (Mayr et al. 2010). More and more genes for ancillary proteins have been described. In the region of the central Europe, mutations affecting genes for assembly factors of complex IV are more frequent (Bohm, et al. 2006). Our group has also described the group of patients with the first known ancillary protein for complex V (Cizkova et al. 2008, Honzik et al. 2010).

The right coordination of nuclear and mitochondrial genoms expression is necessary for proper OXPHOS function. To date, nine nuclear genes have been associated with mtDNA depletion syndromes, *TK2*, *DGUOK*, *POLG1*, *SUCLA2*, *SUCLG*, *PEO1*, *RRM2B*, *TYMP* and *MPV15* (DiMauro 2011).

Tab. 1. Genetic classification of human mitochondrial disorders.
According to Chinnery 2010.

Nuclear DNA mutations
Nuclear genetic disorders of the mitochondrial respiratory chain, mutations in structural subunits
Leigh syndrom with complex I deficiency (*NDUFS1, NDUFS4, NDUFS7, NDUFS8, NDUFV1*)   Leigh syndrom with complex II deficiency (*SDHA*)   Leukodystrophy with complex II deficiency (*SDHAF1*)   Cardiomyopathy and encephalopathy (complex I deficiency) (*NDUFS2*)   Optic atrophy and ataxia (complex II deficiency) (*SDHA*)   Hypokalaemia and lactic acidosis (complex III deficiency) (*UQCRB*)
Nuclear genetic disorders of the oxidative phosphorylation system, mutations in assembly factors

Leigh syndrome (*SURF1, LRPPRC*) Hepatopathy and cardiomyopathy (*SCO1*) Cardiomyopathy and encephalopathy (*SCO2*) Leukodystrophy and renal tubulopathy (*COX10*) Hypertrophic cardiomyopathy (*COX15*) Encephalopathy, liver failure, renal tubulopathy (with complex III deficiency) (*BCS1L*) Encephalopathy, myopathy and hypertrophic cardiomyopathy (*TMEM70*)
Nuclear genetic disorders of the mitochondrial respiratory chain, mutations in translation factors
Leigh syndrome, liver failure, and lactic acidosis (*GFM1*) Lactic acidosis, developmental failure, and dysmorphism (*MRPS16*) Myopathy and sideroblastic anemia (*PUS1*) Leukodystrophy and polymicrogyria (*TUFM*) Leigh syndrome and optic atrophy with COX deficiency (*TACO1*)
Nuclear genetic disorders associated with multiple mtDNA deletions or mtDNA depletion
Autosomal progressive external ophtalmoplegia (*POLG1, POLG2, C10orf2, SLC25A4*) Mitochondrial neurogastrointestinal encephalomyopathy (thymidine phosphorylase deficiency) (*TYMP*) Alpers-Huttenlocher syndrome (*POLG1*) Infantile myopathy / spinal muscular atrophy (*TK2*) Encephalopathy and liver failure (*DGUOK*) Hypotonia, movement disorder, and/or Leigh syndrome with methylmalonic aciduria (*SUCLA2*) Hypotonia, encephalopathy, renal tubulopathy, lactic acidosis (*RRM2B*) Mitochondrial encefaphalopathy with combined respiratory chain deficiency (*AIF1*) Reversible hepatopathy (*TRMU*) Myopathy with cataract and combined respiratory chain deficiency (*GFER*)
Other
Coenzyme Q10 deficiency (*COQ2, COQ9, CABC1, ETFDH*) Barth syndrome (*TAZ*) Cardiomyopathy and lactic acidosis (mitochondrial phosphate carrier deficiency) (*SLC25A3*)
Mitochondrial DNA mutations and rearragements
mtDNA rearragements (deletions and duplications)
Chronic progressive external ophtalmoplegia Kearns-Sayre syndrome Diabetes and deafness
mtDNA point mutations
Protein-enconding genes
Leber hereditary optic neuroapthy (LHON) (m.3460G>A, m.11778G>A, m.14484T>C) Neurogenic weakness with ataxia and retinitis pigmentosa/Leigh syndrome (m.8993T>G, m.8993T>C)
tRNA genes
MELAS (m.3243A>G, m.3271T>C, m.3251A>G) MERRF (m.8344A>G, m.8356T>C) Chronic progressive external ophtalmoplegia (m.3243A>G, m.4274T>C) Myopathy (m.14709T>C, m.12320A>G) Cardiomyopathy (m.3243A>G, m.4269A>G) Diabetes and deafness (m.3243A>G, m.12258C>A) Encephalomyopathy (m.1606G>A, m.10010T>C)
rRNA genes

Nonsyndromic sensorineural deafness (m.7445A>G)
Aminoglycoside-induced nonsyndromic deafness (m.1555A>G)

1.1.6. Clinical aspects of mitochondrial disorders

Mitochondrial disorders represent a clinically, biochemically and genetically heterogeneous group of diseases associated with dysfunction of the oxidative phosphorylation system (OXPHOS). In these disorders, organs with the highest energy demand (brain, heart and skeletal muscles) are predominantly affected (Wallace 1999).

The first mitochondrial disorder was recognized in 1962, when Swedish endocrinologist Rolf Luft described a young woman with severe hypermetabolism not due to thyroid dysfunction (Luft et al. 1962).

The diagnosis of Luft disease was confirmed by an excellent correlation between biochemical abnormalities (loose coupling) and clinical features (uncontrolled muscle metabolism). Notably, the first described mitochondrial disease is probably also the most rare one. Only two patients suffering from this disease have been described. The molecular diagnosis has never been found, although many candidate genes from cultured fibroblasts were examined. Luft disease introduced also the concept of "organellar medicine" because the classical paper by Henry-Géry Hers on inborn lysosomal diseases was not published until 3 years later (Hers 1965; DiMauro 2011).

Fig. 7. Professor Rolf Luft, 1914–2007.
From http://www.ncbi.nlm.nih.gov/pmc/articles/PMC2292419/

Since then, mitochondrial disorders have emerged as major clinical entities. More than 350 mutations, structural changes and deletions in mtDNA are known at present. These are responsible for more than 30 defined mitochondrial syndromes and diseases (Tuppen et al. 2010). Mutations in nuclear DNA have been found in other more than 80 mitochondrial

diseases (Spinazzola and Zeviani 2007). More than 119 defined diseases of mitochondrial energetic metabolism are thus known. (OMIM; www.ncbi.nlm.nih.gov/omim)

1.1.6.1. Incidency

Elliot and colleagues (Elliott et al. 2008) showed that the prevalence of pathogenic mtDNA mutations is at least 1 to 200. However, the prevalence of mitochondrial disorders, which includes both of mtDNA and nDNA disorders, is estimated to 1 to 5,000 (Skladal et al. 2003A; Thorburn 2004; Taylor and Turnbull 2005). The mitochondrial etiology can be found in 2-3% patients with non-insulin dependent diabetes mellitus, in 2-3% patients with aquired blindness, or in 1% of patients with brain stroke event (stroke-like episode) (Kokotas et al. 2007). Increased incidency of mitochondrial diseases mirrors the improvement in diagnostics as well as better knowledge of this group of inherited metabolic disorders.

1.1.6.2. Clinical manifestation

The clinical manifestation of mitochondrial diseases is very heterogenous. The presentation usually depends on generalised or tissue-specific decrease in ATP production. Some mitochondrial disorders affect a single organ (e.g., the eye in Leber hereditary optic neuropathy), but many involve multiple organ systems. Virtually any organs may be impaired, but the organs with highest energetic demands are typically involved, including brain, muscle, heart and liver (Scaglia et al. 2004; Bohm et al. 2006).

The clinical presentation and course in patients with mitochondrial syndromes are extremely diverse, even among patients with identical enzymatic or genetic defects. Many mitochondrial disorders may manifest as a characteristic cluster of clinical features (DiMauro and Schon 2001; Munnich and Rustin 2001). These include e.g. Kearns-Sayre syndrome (KSS), chronic progressive external ophtalmoplegia (CPEO) (Moraes et al. 1989), mitochondrial encephalomyopathy with lactic acidosis and stroke-like episodes (MELAS) (Hirano et al. 1992), myoclonic epilepsy with ragged-red fibres (MERRF) (Hammans et al. 1993), neurogenic weakness with ataxia and retinitis pigmentosa (NARP) (Holt et al. 1990), or Leigh syndrome (Ciafaloni et al. 1993). However, there is often considerable clinical variability and many affected individuals do not fit into one particular category. Mutations in *POLG1* gene, which have emerged as a major cause of mitochondrial disease, illustrate this well. They have been associated with severe multi-system disease, including Parkinson´s syndrome (tremor,

hypokinesia, rigidity), autosomal dominant or recessive CPEO, a MERRF-like phenotype, Alpers-Huttenlocher syndrome, ataxia, or premature menopause (Chinnery 2010).

Mitochondrial diseases may manifest at any age (Leonard and Schapira 2000). Clinical spectrum covers prenatal complications, acute neonatal metabolic disroder, manifestation in childhood but also in adult age. Until recently it was generally thought that the mitochondrial disorders due to mutations in nuclear genes manifest in childhood and disorders due to mtDNA mutations demonstrate in late childhood or adult life (Skladal et al. 2003B; Bohm et al. 2006). However, recent advances have shown that many mtDNA disorders present in childhood, and many nuclear genetic mitochondrial disorders manifest in adult life (Finsterer 2006A).

Tab. 2. List of the most frequent mitochondrial syndromes
According to Finsterer 2006A; Honzik et al. 2010A; Chinnery 2010.

Disorder	Primary features	Additional features	Genetic defect
Alpers-Huttenlocher syndrome	hypotonia, farmacoresistant epilepsy, liver failure, progressive neurologic deterioration	renal tubulopathy	*POLG1* gene
Barth syndrome	cardiomyopathy, myopathy, cyclic neutropenia	short stature	*Taffazin* gene
Costeff syndrome	optic atrophy, extrapyramidal symptoms, spasticity		*OPA3* gene
Friedreich´s ataxia	gait disturbance, speech problems, vision and hearing impairment hypertrophic cardiomyopathy	diabetes mellitus, scoliosis	*FRAXA* gene
Mohr-Tranebjaerg syndrome	deafness, dystonia, cortical blindness, spasticity	dysphagia	*TIMM8A gene*
Sensory ataxic neuropathy, dysarthria, ophthalmoplegia (SANDO)	migraine, seizures, dysarthria, severe sensory ataxic neuropathy, PEO	hyporeflexia, dysphagia, nystagmus	*POLG1 gene*
TMEM70 protein deficiency	encephalopathy, myopathy, hypertrophic cardiomyopathy, psychomotor delay	IUGR, short growth, failure to thrive, hypospadia in boys	*TMEM70* gene
Wolfram syndrome (DIDMOAD)	diabetes insipidus, diabetes mellitus, optic atrophy, deafness		*WFS-1* gene

Chronic progressive external ophtalmoplegia (CPEO)	external ophtalmoplegia, bilateral ptosis	mild proximal myopathy	mtDNA deletions
Kearns-Sayre syndrome (KSS)	PEO, pigmentary retinopathy, heart block, cerebellar ataxia	bilateral deafness, myopathy, dysphagia, diabetes mellitus, hypoparathyroidism, dementia, CSF protein >1g/l	mtDNA deletions or rearrangements
Pearson syndrome	sideroblastic anemia of childhood, pancytopenia, exocrine pancreatic failure	renal tubular defects	mtDNA deletions or rearrangements
Neurogenic weakness with ataxia and retinitis pigmentosa (NARP)	neuropathy, ataxia, pigmentary retinopathy	basal ganglia lucencies, abnormal electroretinogram, sensorimotor neuropathy	mtDNA mutations, e.g. 8993T>G
Leber´s hereditary optic atrophy (LHON)	acute or subacute onset of visual loss, optic atrophy, decrease of visual acuity	onset in young adulthood	MtDNA mutations, e.g. 3460G>A, G11778G>A
Mitochondrial encephalopathy with lactic acidosis and stroke-like episodes (MELAS)	stroke-like episodes at age <40 years, seizures and/or dementia, ragged-red fibers and/or lactic acidosis	diabetes mellitus, cardiomyopathy, deafness, pigmentary retinopathy, cerebellar ataxia	mtDNA mutations, e.g. 3243A>G ($tRNA^{Leu}$ gene)
Myoclonic epilepsy with ragged red fibres (MERRF)	myoclonic epilepsy, ragged red fibres	exercise intolerance, poor night vision, hearing loss	MtDNA mutations, e.g. 8344A>G ($tRNA^{Lys}$ gene)

IUGR – intrauterinne growth retardation, PEO - progressive external ophtalmoplegia, CSF – cerebrospinal fluid

1.1.6.3 Clinical symptoms and laboratory data

Nervous system

The range of neurological manifestations of mitochondrial disorders is broad and includes almost all CNS functions. The cognitive involvement in the means of psychomotor delay or regression of the psychomotor development is the prominent clinical feature of the mitochondrial disorders in the childhood. In the older children in whom the psychological assessment is possible, all of abstract reasoning, verbal memory, visual memory, language

(naming, fluency), executive or constructive functions, calculation, attention (attention deficit disorder, decreased attention span), or visuo-spatial functions may be impaired (Finsterer 2006A).

Seizures are common in mitochondrial disorders. 50% from 113 children with mitochondrial disorders manifested seizures in the study of Scaglia (Scaglia et al. 2004). Epilepsy may start at infancy as infantile spasms, West syndrome, myoclonic jerks, astatic seizures, or myoclonic epilepsy (Finsterer 2006A).

Stroke-like episodes usually affect the occipital and parietal lobe, occur at young age, and manifest clinically as focal weakness, parestesia, speech disturbance, homonymous hemianopsia, hemihypesthesia, or seizure. Stroke-like episodes are accompanied by difficultly treatable migrainous phenomena. Both are typical for MELAS syndrome, however, they may occur also in other mitochondrial disorders (Kaufmann et al. 2004).

Mitochondrial movement disorders are often associated with basal ganglia degeneration (Shoffner et al. 1995). Pyramidal signs, spasticity, or hyperreflexia are common CNS manifestations of mitochondrial disorders. Ataxia is particularly prevalent in MELAS, MERRF, Leigh syndrome, KSS, NARP, and Friedreich ataxia. Dysarthria is a common feature, dysphagia and nystagmus belong to rarer signs in patients with cerebellar atrophy or brainstem involvement (Finsterer 2006A).

Psychiatric or neuropsychological abnormalities may be also present in mitochondrial disorders, although they are more frequent in adults. However, it is worth to mention that children with mitochondrial disorders may manifest autistic features or depression (Finsterer 2006A).

CT may show basal ganglia calcification and/or diffuse atrophy. MRI may show focal atrophy of the cortex or cerebellum, leukoencephalopathy, cerebellar atrophy and symmetrical spongiform lesions in basal ganglia and brain stem (Leigh syndrome) (Barragan-Campos et al. 2005; Scaglia et al. 2005). EEG may document the generalized slow wave activity or the specific epileptiform discharges. EMG may show myopathic features, or neuronal involvement, typically axonal sensorimotor polyneuropathy.

Tab. 3. CNS manifestations of syndromic mitochondrial disorders.
From Finsterer 2006A.

Syndrome	psychiatric abnormalities	neuropsychological deficits	stroke-like episodes	Migraine	epilepsy	extrapyramidal manifestation	spastictiy	ataxia	hypotonia	Hypopituitarsim	dysarthria	Dysphagia	nystamus
MELAS	+	+	+	+	+	-	-	+	-	+	-	-	-
MERRF	+	+	+	-	+	-	+	+	-	+	+	-	-
MNGIE	-	+	-	-	-	-	-	-	-	-	+	-	-
LS	+	+	-	+	+	+	+	+	+	-	-	-	+
LHON	+	-	-	-	-	+	-	-	-	-	-	-	-
KSS	+	+	+	-	-	-	-	+	-	+	-	+	-
NARP	+	+	-	-	+	-	-	+	-	-	-	-	-
FA	-	-	-	+	-	+	-	+	-	-	+	+	-
MSL	-	-	-	-	-	-	+	+	-	-	-	+	-
MDS	+	+	-	-	-	-	-	-	+	-	+	-	+

CNS, central nervous system; MELAS, mitochondrial encephalomyopathy, lactic acidosis, stroke-like episodes; MERFF, myoclonic epilepsy and ragged red fibers; MNGIE, mitochondrial neurogastro-intestinal encephalomyopathy; LS, Leigh syndrome, maternally inherited Leigh syndrome; LHON, Leber's hereditary optic neuropathy; KSS, Kearns-Sayre syndrome; NARP, neurogenic muscle, weakness, ataxia, and retinitis pigmentosa; FA, Friedreich's ataxia; MSL, multiple systemic lipomatosis (Mandelung's disease); MDS, mitochondrial depletion syndrome.

Vision and hearing

In the group of 113 children with mitochondrial disorders, 45% patients had strabism, 35% had optical atrophy, 27% ptosis, 25% nystagmus and 15% pigmental retinitis (Scaglia et al. 2004). Progressive external ophtalmoplegia (PEO), which is typical mainly for mtDNA depletion syndromes (Finsterer 2001), is not often in children. Sensorineural hearing loss was detected in 21% of the same group (Scaglia et al. 2004). Deafness may be present in patients with various syndromes: MELAS, DIDMOAD, MERRF, Mohr-Tranebjaerg or *SUCLA2* gene mutations (Carrozzo et al. 2007).

Heart

Cardiomyopathy is present in 22 - 40% of children with mitochondrial disorder (Holmgren et al. 2003; Scaglia et al. 2004; Bohm et al. 2006). However, its prevalence differs in particular syndromes and it can also change its severity with the course of the disease (Honzik et al. 2010). The hypertrophic cardiomyopathy is more common, although the dilated cardiomyopathy is also not rare (Scaglia et al. 2004, Barth et al. 1983). Frequent electrocardiographic findings are atrioventricular (AV) block, Wolff-Parkinson-White

(WPW) syndrome, bundle branch block, QT prolongation, ST and T-wave abnormalities and atrial fibrillation (Finsterer 2006B).

Skeletal muscle

Skeletal muscle affection can manifest as a hypotonia and muscle weakness, myopathy (CK elevations are not always present), exercise intolerance, resting muscle pain (van de Glind et al. 2007). It can mimic facioscapulohumeral muscle dystrophy (Filosto and Mancuso 2007), or spinal muscular atrophy (SMA-like) (Magner et al. 2010).

Gastrointestinal tract

The severe gastrointestinal manifestation is present in patients with myoneurogastrointestinal encephalopathy (MNGIE) syndrome. The symptomatology includes the attacks of abdominal pain and discomfort, diarrhoea, malabsorption syndrome, vomiting and severe dystrophy. The combination of encephalopathy and hepatopathy is typical for Alpers-Huttenlocher syndrome. The syndromes with mtDNA depletion may present the malabsorption due to exocrinne pancreatic insufficiency. Dysphagia is also a frequent sign of mitochondrial disorders (Gillis and Sokol 2003).

Kidneys

Kidneys as organs with high energetic demands may be also affected in mitochondrial disorders, although the kidney disease are rarely in the limelight of general clinical picture. Renal manifestations include polycystic kidneys, nonspecific nephritis, focal, segmental glomerulosclerosis, or tubular dysfunction, which may turn into chronic renal failure, event. requiring haemodialysis. Fanconi´s syndrome may occur in an isolated form or as part of a multisystem disease (Kuwertz-Broking et al. 2000). In our group of hildren with cytochrome *c* oxidase deficiency, renal involvement in children were quite rare, in less than 5% of patients (Bohm et al. 2006).

Endocrine glands

Children with mitochondrial disorders may present with short stature, hypothyroidism, and hypogonadism. As already mentioned, 2-3% of non-insulin dependent diabetes mellitus is

given on account of OXPHOS dysfunction (Kokotas et al. 2007). Endocrinological symptoms are in the limelight of maternally inherited diabetes and deafness (MIDD), and diabetes insipidus, diabetes mellitus, optic atrophy, deafness (DIDMOAD or Wolfram) syndrome. Non-sense and missense germline mutations in complex II genes have been found in patients with familiar pheochromocytoma, with or without paraganglioma, and in sporadic pheochromocytoma (Astuti et al. 2001).

Haematological manifestation

Cyclic neutropenia is a typical finding in Barth syndrome. Sideroblastic macrocytic anemia as well as thrombocytopenia may be found in mitochondrial depletion syndrome as KSS, CPEO and Pearson syndrome. In single cases either permanent or recurrent eosinophilia can be observed, not attributable to any of the established causes. All these manifestations are partially resistant to adequate therapy (Finsterer 2007).

Tab.4. Non-neurological manifestations of mitochondriopathies.
Adapted from Finsterer 2006B.

Endocrinological system involvement
hypopituitarism, short stature, diabetes insipidus, hypothyroidism, hyperthyroidism, hypoparathyroidism, hyperparathyroidism, polyphagia, polydipsia, hyperhidrosis, insufficiency of the suprarenal gland, amenorrhoea, hypogonadism, gynaecomastia
Heart involvement
cardiomyopathy, rhythm abnormalities, left ventricular hypertrabeculation
Gastrointestinal symptoms
dysphagia, gastrointestinal dysmotility, pseudoobstruction, recurrent vomiting, hepatopathy, recurrent pancreatitis, exogene pancreas insufficiency, villous atrophy, malabsorption, diarrhoea, weight loss, anorexia
Kidneys
renal cysts, tubulopathy, focal-segmental glomerulosclerosis, Toni-Debre-Fanconi syndrome
Haematological abnormalities
anemia, leucopenia, thrombocytopenia

1.1.6.4. Diagnostics of mitochondrial diseases

The diagnostics of OXPHOS syndromes and diseases is enormously demanding. This is mainly because of various clinical manifestation and broad genetic heterogeneity. The multidisciplinary approach is necessary (Thorburn 2000).

For a diagnostics of mitochondrial disorders it is necessary to take in account the particular family and personal history, the course of the disease, the comprehensive clinical examination, results of specialized examinations (especially cardiology, visual fundus examination, brain imaging, EMG), the basic but also laboratory analyses in body fluids, and examination of bioptic samples of muscle, skin, and eventually liver. The definitive diagnosis is confirmed by the molecular-genetic analysis.

Biochemical examination

The most prominent biochemical marker of mitochondrial disorders is elevated lactate level in body fluids. The review of diagnostic value of elevated lactate and its differential diagnosis is in detail compiled in the second part of the theoretic part of the Thesis. The biochemical markers include the creatine kinase activity, which may be elevated as a sign of muscle involvement, elevated liver enzymes present e.g. in the Alpers-Huttenlocher syndrome, and hyperammonaemia which may be linked to TMEM70 protein deficiency and NARP syndrome (Honzík et al. 2010A). The amino acid profile in blood may show the hyperalaninaemia as an indirect sign of lactic acidosis, increased glutamine level as a marker of hyperammonaemia, elevated branched aminoacids in the case of PDH E3 deficiency. Increased excretion of lactate and the citric acid cycle intermediates, such as malate, succinate, 2-oxoglutarate, and fumarate can also be found. An extremely high excretion of fumaric acid and 2-hydroxyglutarate are found in fumarase deficiency and 2-oxoglutarate dehydrogenase deficiency, respectively (Rubio-Gozalbo et al. 2000). Methylmalonic aciduria may be present in deficit of SUCLA2 (Carrozzo et al. 2007). 3-methylglutaconic aciduria is a marker of growing importance pointing to mitochondrial disorders. All of Barth syndrome, TMEM70 protein deficiency, Costeff syndrome, *POLG1*, *SUCLA2*, MEGDEL and others not yet specified on molecular level show these findings (Wortmann et al. 2010). Typical biochemical findings in patients with MNGIE syndrome (thymidine phosphorylase deficit) are increased plasma thymidine and deoxyuridine levels. The generalized unspecified aminoaciduria may be present in mitochondrial disorders as a result of tubulopathy.

Tab. 5. Main biochemical markers used in the diagnostics of mitochondrial disorders.
From Suomalainen 2011.

Examination	Finding in RC deficiency	Differential diagnostics	Source of artefact
lactate in blood/plasma/CSF	increased or normal	neuroinfection, malignancy, seizures, stroke	struggling child, sampling error (stasis)
pyruvate in blood/ CSF	increased or normal lactate/pyruvate ratio	pyruvate dehydrogenase or carboxylase deficiencies, biotinidase deficiency	poor sample handling
amino acids in plasma/CSF/urine	elevated alanine	aminoacidopathies	recent diet, fasting
organic acids in urine	increased excretion of tricarboxylic acid cycle intermediates, methylmalonic aciduria, methylglutaconic aciduria, dicarboxylic aciduria	amino- and organic acidopathies, fatty acid disorders, renal immaturity in children <1 years; artefacts from diet, drugs, fasting	recent diet, drugs, fasting
acylcarnitine profiling/ carnitine analysis in blood	low free carnitine, elevated acyl/free carnitine ratio	fatty acid oxidation defects, aminoacidemias, organic acidemias	
creatine kinase in blood	increased	other metabolic myopathies, muscle dystrophies	

CSF – cerebrospinal fluid

Enzymatic studies

Only in a minority of mitochondrial disorders with well-recognized phenotype, it is directly possible to indicate the molecular genetic analysis (Chinnery 2010). Although some algorhytms for direct genetic diagnostics of mitochondrial disorders have been already proposed (e.g. Honzik et al. 2010B), muscle biopsy remains the gold standard for the diagnosis of mitochondrial disorders. It is important to evaluate the chance for succesful diagnosis prior to deciding on muscle biopsy, which is, especially when performed under general anesthesia, an invasive procedure, with an additional risk for patients with a dysfunction in oxidative phosphorylation, severe muscle hypotonia, cardiac symptoms, and CNS abnormalities (Morava et al. 2006).

A biopsy from skeletal muscle is usually the first examined. The reason for this is the affection of skeletal muscle in majority of patients. The muscle biopsy is in most cases accompanied by the skin biopsy with further fibroblast cultivation (Thorburn et al. 2004).

Assays to quantify OXPHOS enzyme activities are usually based on spectrophotometry, although polarography and substrate oxidation studies are also potent diagnostic tools (Rodenburg et al. 2010). The interpretation of enzymatic measurements is complicated.

The evaluation with respect to age-matched controls is especially necessary in infants and there is only a small margin between patient ranges and control ranges (Rustin et al. 1994).

The important part of the muscle biopsy examination is also the particular histological analysis. Ragged red fibres and COX negative fibres together with abnormal shape of mitochondria may be present (Morava et al. 2006).

Molecular genetic analyses

A significant progress has been made in the diagnostics of mitochondrial disorders with the development of new methods in the molecular genetic analysis. Whole mtDNA sequenation is provided with the suspicion on mitochondrial disorder in several laboratories, the preparation of various diagnostic mitochips is in progress. New methods can be illustrated by identifying of TMEM70 gene as cause for complex V deficiency by our group. In cooperation with the group of Ing. S. Kmoch, PhD. (Institute for Inherited Metabolic Disorders, General Teaching Hospital and First medical faculty of Charles University) and dr.Houštěk, DrSc. (Department of Bioenergetics, Physiological Institute of the Academy of Science of the Czech Republic) the expression of 1632 genes necessary for cell cycle, signalisation and mitochondrial biology was analysed. Gained express profiles were compared to the results of two patients with ATP synthase defiency due to mt902ΔTA microdeletion and results of genetic heterogenous group of 11 children, in which the molecular-genetic diagnosis had not been known yet. The new oligonucleotide chip (h-MitoArray) was constructed for the purposes of the study (Cizkova et al. 2008). The comparison of express profile found in fibroblasts of patients and of controls led to the classification of the three groups of patients: M group corresponded to patients with already known mtDNA mutation, while groups N1 and N2 coresponded to patients with the unclear defect in nuclear DNA. The synchronnic supression of mitochondrial biogenesis and the cell cycle arrest was found in the M group. The increased expression of genes for complex I and decreased expression for complexes III and V, and significantly reduced expression of genes responsible for cell signalisation dependent on phosphorylation and the marks of activated apoptosis. The apoptosis activation was also found in the N2 group. Further significant changes have not been found. Several nuclear DNA candidate genes with possible causal relationship were thus selected by the thoroughgoing analysis of the expression profile of the N1 group (Cizkova et al. 2008).

In eight selected children with isolated ATP synthase deficit and 3-methylglutaconic aciduria from six families the linkage analysis was done (whole genomic mapping). The *TMEM70*

gene (transmembrane protein 70) at 8th chromosome was the most probable candidate. The mutation (c.317-2A>G) was found in this gene, subsequently. In the further course of our work, this mutation was confirmed in 24 children in homozygote state. One child was identified as a compound heterozygote for the previous mutation and for the c.[118_119insGT] mutation, his phenotype did not differ from the others. The causality of the mutations in *TMEM70* gene was then proved on cultured fibroblasts. The results were published in the Nature Genetics (Cizkova et al. 2008).

As there is no effective disease modifying treatment for patients with primary disorders of the mitochondrial respiratory chain, and despite advances in the supportive care, the disease course can be relentlessly progressive leading to severe disability and early death (Leonard and Schapira 2000; DiMauro and Schon 2001). These factors make the demand for exact genetic diagnosis of the disease more important in the view of further possibility of accurate genetic counselling (Thorburn and Dahl 2001).

1.1.6.5 Treatment

Current treatment of mitochondrial disorders is largely supportive and the disorders may cause significant morbidity and premature death. Dietary modifications, pharmacological agents and exercise therapy have been tried in individual cases and small cohorts. However, the recent Cochrane review has not showed any efficacy of these interventions. There is currently no clear evidence supporting the use of any intervention in mitochondrial disorders (Chinnery 2006). As a body of genetic and pathophysiological knowledge of mitochondrial disorders grows, some new and possibly hopeful approaches emerge. Most research in this field is still at the level of single cell research.

These new treatment strategies include (1) gene therapy (replacement or repair), controlled regulation of specific transcriptional regulators, and altering the balance between wild-type and mutated mtDNA in the case of mitochondrial disorders due to mutation in mtDNA, and (2) metabolic manipulation.

Ad 1. Prospect of gene therapy approaches can roughly be divided into three groups: (1) import of wild-type copies or relevant sections of DNA or RNA into mitochondria, (2) manipulation of mitochondrial genetic content, and (3) rescue of a defect by expression of an engineered gene

product from the nucleus (allotopic or xenotropic expression). A promising progress has been made in the last decade in this field (Cwerman-Thibault et al. 2010). A few examples will be given to illustrate this problematics. In 2004, Tarassov's group showed that the respiratory defect in cybrid cells bearing the MERRF mutation was partially rescued when the yeast homologue was expressed in the nucleus, as imported tRNA was correctly aminoacylated and able to participate in mitochondrial translation (Kolesnikova et al. 2004). Tanaka demonstrated the gene therapy based on mitochondrial delivery of the restriction endonuclease, SmaI in human cell lines. The *SMAI* gene fused to mitochondrial targeting signal (MTS) was transiently expressed in hybrids carrying mtDNA with 8993 T>G mutation. This mutation was specifically eliminated from mitochondrially targeted SmaI, followed by repopulation with wild-type mtDNA, which resulted in restoration of both intracellular ATP levels and mitochondrial membrane potential (Tanaka et al. 2002).

An example of possible transcription regulation interference may be given on the peroxisome proliferator-activated receptor γ co-activator 1 (PPAR-γ/PGC-1α). It is a transcriptional factor regulator that stimulates transcription of genes involved in cellular energy metabolism (Wenz et al. 2008). PGC-1α stimulation through exercise is known to increase mitochondrial mass and oxidative capacity (Taivassalo and Haller 2005). A level of PGC-1α is further physiologically regulated by calorie intake but also pharmacologically, e.g. by benzafibrate or resveratrol (Koene and Smeitink 2011). A mouse model for cytochrome *c* oxidase (COX) deficiency with transgenic expression of PGC-1α was used to evaluate to verify the role in mitochondrial disorders. PGC-1α expressing mice with COX deficiency had a delayed onset of myopathy, increased mitochondrial biogenesis, increased ATP levels and increased health and lifespan compared to controls (Wenz et al. 2008).

Ad 2. A several mechanisms have been so far considered to be influenced by „metabolic manipulators": (1) prevention of oxidative damage by reactive oxygen species, (2) amelioration of lipid peroxidation, (3) correction of altered membrane potential, and (4) restoration of calcium homeostasis (Koene and Smeitink 2011). Administration of antioxidant, Co-enzyme Q10 (CoQ10; ubiquinone) belongs to this group. Its main role its in therapy of CoQ10 biosynthesis defects, where high doses of CoQ10 lead to clinical improvement (Quinzii et al. 2007), but it is often administred in other mitochondrial disorders (Wang et al. 2008). The most extensively studied of mitochondria-targeted antioxidants is MitoQ, which contains the antioxidant quinone moiety covalently attached to a lipophilic triphenylphosphonium cation.

MitoQ has now been used in a range of *in vivo* studies in rats and mice and in two phase II human trials (Smith and Murphy 2010). From other substances is worth-mention the CGP37157 at least, a benzothiazepine drug, which was shown to restore the important calcium metabolism and ATP production in cybrid cells expressing the m.8993T>G (NARP) mutation (Brini et al. 1999).

1.2. Lactate and its metabolism

Lactate (2-hydroxypropanoic acid) is the end product of anaerobic glucose metabolism. It is further utilized either *via* oxidation within the citric acid cycle, depending on the presence of oxygen and/or well functional oxidative phosphorylation, or *via* gluconeogenesis within the Cori cycle. Lactate thus represents the link between aerobic and anaerobic metabolism (Thomas 1998).
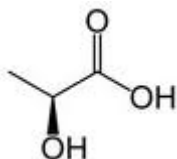
Fig. 8. Basic chemical characteristics of lactic acid. IUPAC name: 2-hydroxypropanoic acid. Molecular formula $C_3H_6O_3$. Molar mass 90.08 g.mol^{-1}. Melting point of L-isomer 53 °C. Boiling point 122 °C at 12 mmHg; pK_a 3.86.

1.2.1. Discovery and isolation

The lactic acid was first discovered in 1780 by the Swedish chemist Carl Wilhelm Scheele, who found lactic acid in samples of sour milk. The milk origin of the first discovery of lactic acid led to the origin of the trivial name for this molecule ("lactic," or relating to milk). In 1810 the presence of lactic acid was verified in other organismal tissues and products, such as fresh milk, ox meat, and blood. In 1833, pure samples of lactic acid had been prepared and the chemical formula for lactic acid was determined. The finding that lactic acid exists in two optical isomers (D- and L-isomers) was made in 1869, with the L-isomer having biological metabolic activity. Due to the prevalence of lactic acid formation from fermentation reactions, fermentation was the main direction of early scientific inquiry into the biochemistry of lactic acid production (Robergs et al. 2004).

1.2.2. Lactate level regulation

Lactate metabolism is regulated very precisely; therefore, in healthy children, the lactate concentration in body fluids only rarely exceeds the reference range (Benoist et al. 2003; Robinson 2006). The plasma lactate level reflects the equilibrium between its production and

its comsuption by different tissues. Elevated blood lactate concentrations can be found in cases of inappropriately high accumulation or disturbed utilization (Thomas 1998).

Lactate is generated by the reduction of pyruvate. Lactate level thus depends on 1) pyruvate level and 2) shift of concentration equilibrium of lactate and pyruvate.

1. Pyruvate:

A) Origin of pyruvate

Pyruvate arises *via* three pathways: 1) Glycolysis as part of the Embden-Meyerhof pathway, the major portion of pyruvate is synthesized via this pathway. 2) During proteolysis by dehydrogenation and aminotransferase reactions. Substrates for pyruvate synthesis are predominantly the amino acids alanine, glycine, serine, cysteine, threonine, and tryptophan. Under basal conditions, proteolysis accounts for an approximately 15% of lactate synthesis. 3) Oxidation of lactate by lactate dehydrogenase (LD) (Thomas 1998).

B) Pyruvate utilization

Pyruvate undergoes the carboxylation catalyzed by pyruvate carboxylase, or it enters the citric cycle *via* pyruvate dehydrogenase complex. Pyruvate carboxylase and pyruvate dehydrogenase complex are the key regulation sites. Lactate excess in the circulation is encountered by the liver, pyruvate carboxylase becomes more active. Likewise, when muscle encounters increased lactate under aerobic conditions, the resulting increase in pyruvate activates the pyruvate dehydrogenase complex by inhibition of pyruvate dehydrogenase kinase. It is worth to mention that the shutdown of pyruvate metabolism by defects in the mitochondrial respiratory chain will use the same regulation points (Stacpoole 1993).

2. Lactate/Pyruvate equilibrium

The blood lactate-to-pyruvate (L/P) molar ratio reflects the equilibrium between product and substrate of the reaction catalyzed by lactate dehydrogenase. The L/P ratio is correlated with the cytoplasmic $NADH/NAD^+$ ratio and is used as a surrogate measure of the cytosolic oxido-reduction state (Robinson 2006). All circumstances leading to intracellular increase of the

pyruvate concentration, the formation of H^+, and the decrease of NAD^+, will raise the concentration of lactate (Stacpoole 1993).

1.2.3. Lactate flux

The cumulative total of lactate produced per day glycolytically is between 70 and 110 g for the human body. From red cells and skin come 33.5 g each with another approximately 20 g from skeletal muscle, brain and a small amount from intestinal mucosa (Fig. 9) (Robinson 2006). The capacity for homeostatic maintenance of lactate is dependent mainly on rates of gluconeogesis in gluconeogenic tissues with some oxidative removal by muscle tissue (Ahlborg and Felig 1982).
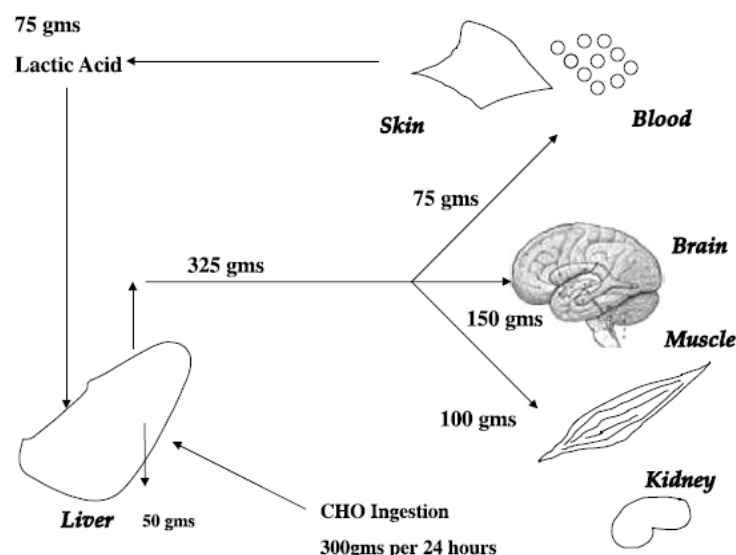
Fig. 9. The daily turnover of glucose and lactic acid in the human body.
The average 70 kg man ingests 300 g of carbohydrate per 24 h. After processing by the gastrointestinal tract and liver, 250 g of this is released as glucose plus another 75 g generated by the Cori cycle for a total liver output of 325 g. Approximately 150g of glucose is utilized by the brain and 100 g used by other tissues including heart, skeletal muscle and kidney. Other tissues, particularly skin and erythrocytes, metabolise glucose to lactic acid accounting for a net production of 75 g lactic acid per day. Small amounts of lactate are produced by brain and skeletal muscle (25–30 g) but an equivalent amount is re-used oxidatively by heart and kidney cortex. Thus, 75 g of lactic acid is returned to the liver for processing *via* gluconeogenesis to glucose.
According to Robinson 2006. gms - grams

1.2.4. Lactate level and acid-base status

From a biochemical perspective, the cellular production of lactate has several benefits for the cell. First, the lactate dehydrogenase (LDH) reaction also produces cytosolic NAD^+. Producing of NAD^+ helps to maintain the cytosolic redox potential (NAD^+/NADH) and it also provides a substrate necessary for the glyceraldehyde-3-phosphate dehydrogenase reaction. By keeping this reaction in run, it is possible to continue ATP regeneration from glycolysis. It is also necessary to emphasize that as a proton is consumed for every pyruvate molecule catalyzed to lactate and NAD^+, LDH reaction also serves as an imporant intracellular buffer. The LDH reaction is thus alkalinizing the cell, not acidifying (Robergs et al. 2004).

The lactate is removed from the cell by the monocarboxylate transporter. It gets into the circulation, where it can be taken up and used as a substrate for metabolism in other tissues (Hagberg et al. 1985; Juel et al. 1996). This monocarboxylate transporter serves also as a symport for proton removal from the cell. Lactate and a proton leave the cell stoichiometrically *via* this transporter mechanism. To conclude, lactate production helps to maintain intracellular pH by consuming one proton at its production by LDH reaction and excreting one proton from the cell, when a molecule of lactate leaves the cell as well. This intracellular buffer mechanism consequently retards the emergence of lactic acidosis also in blood (Robergs et al. 2004).

However, accumulation of lactate in blood to levels higher than 5 mmol/l (Thomas 1998), or more than 7 mmol/l (Vassault 2008) leads to lactic acidosis. The hyperlactataemia and lactic acidosis should be thus distinguished, although due to some simplification this is not always done in clinical praxis. The hyperlactataemia should then represent a condition characterized by an increased lactate concentration and a blood pH$\geq$7.33-7.34 in conjunction with normal (physiological) acid-base status or alkalosis. On the other hand, lactic acidosis occurs with the blood pH$\leq$7.33-7.34, when H^+ accumulation together with increased lactate synthesis cannot be compensated (Thomas 1998, Maasová 1998, Boron 2004).

The different and even more precise terminology is used according to some authors. Hulin discriminates between acidosis, where pH in blood is in normal range, and acidaemia, with acidic pH. According to this statement, theoretically three possible states of increased lactate could be classified: 1) hyperlactataemia with a mild increase of lactate with no change of blood pH, 2) lactic acidosis as a condition with increased lactate level, which would lead to pH shift, but this is compensated by blood buffer mechanisms, and 3) lactic acidaemia with increased lactate level leading to acidic pH (Maasová 1998).

1.2.4. Etiology of increased lactate level

Increased lactate level is a very common biochemical finding in paediatrics. Lactic acidosis is found both in patients with inherited metabolic diseases and in those with various acquired conditions. It was hypothesized that the majority of acquired cases of lactic acidosis reflects an abnormality in the mitochondrial metabolism of pyruvate and ATP production (Stacpoole 1997A). A good example of such situation is shock. The tissue hypoperfusion, hypoxia, and severe dehydration lead to accelerated glycolysis and lactate production and to impaired pyruvate oxidation. This may be possibly through inhibition of pyruvate dehydrogenase activity (Vary et al. 1986). Many acquired conditions can give rise to persistent hyperlactacidaemias - chronic diarrhoea, persistent infection (mainly of urinary tract), hyperventilation, hepatic failure, and others. Many drugs and toxins such as sedatives-hypnotics, anesthetics, carbon monoxide, and cyanide can inhibit respiratory chain enzyme activity with the emergence of lactic acidosis (Stacpoole 1993). From rarer causes, the HIV infection may result in marked and persistent hyperlactataemia (Mhiri et al. 1991). Possible secondary causes should be ruled out before considering inborn errors of metabolism. On the other hand, it must be emphasized that these circumstances can also sometimes trigger an acute decompensation in a patient with an inborn error of metabolism that has not been diagnosed so far. In severely ill patients many non-specific abnormalities (such as respiratory acidosis, severe anoxic hyperlactacidaemia and secondary hyperammonaemia) can disturb the primary findings. Poggi-Travert used the presence of ketosis as a helpful discriminating factor between secondary and primary hyperlactacidaemia. Ketosis was absent in most hyperlactacidaemias secondary to tissue hypoxia, whereas it was a frequent finding in most inborn errors of metabolism (Poggi-Travert et al. 1996). This fact reflects also our observations in general, however many exceptions out of this rule may be found (e.g. beta-oxidation disorders).

Tab. 6. Underlying causes and diseases resulting in hyperlactataemia and more rarely, in lactic acidosis. According to Thomas 1998; Zeman et al. 1998.

Cause/disease	Assessment
Strenous muscular activity, e.g. as in - **sport activities** - **grand mal seizures**	The increased release of lactate from the muscle during strenous physical activity is usually compensated for by a rise in lactate oxidation. Despite an increase in lactate turnover, the resulting plasma lactate elevation is only about three times that of the resting value, i.e. hyperlactataemia. During sports activities, e.g. during a one-hour period of jogging, a rise in lactate to about 6 mmol/l is observed after approximately 10 min with a subsequent decrease after 30 min to a concentration of approx. 2 mmol/l. This concentration is then maintained for the rest of the jogging period due to the availability of aerobic energy in the meantime. In intense physical activity involving the arms seems to result in a higher increase of lactate than that involving the legs. Following intense sport activities, lactic acidosis normalizes faster given low-level physical activity then at rest.
Infusions	Sodium bicarbonate administration and carbohydrate (glucose, fructose, sorbitol, xylitol) infusions may result in hyperlactataemia without lactic acidosis.
High insulin dosage during glucose intolerance	High insulin dosage in diabetics can result in hyperlactataemia with lactate concentrations up to 8 mmol/l and a transient decline of pH caused by increased peripheral lactate production and inhibition of gluconeogenesis.
Hyperventilation	Hyperventilation, e.g. as observed in patients undergoing intensive care treatment or in patients with diseases of the central nervous system, leads to respiratory alkalosis. Lactate synthesis is increased.
Postoperative state	Hyperlactataemia with lactate concentrations of up to 5 mmol/l usually occurs postoperatively within a period of 48 h and is compensated by the body´s own regulatory mechanisms without the development of lactic acidosis.
Catecholamine infusion	Infusion of catecholamines, or other sympathomimetics, as well as the intake or administration of substances which release catecholamines, such as theophylline, cocaine, and ether, can cause hyperlactataemia which is the result of vascular constriction followed by a decrease in the perfusion of skeletal muscles and the liver. Hyperlactataemia is due to release from the muscles as well as a decreased hepatic uptake of lactate

Tab.7. Hyperlactataemias frequently progressing to lactic acidosis
According to Thomas 1998; Zeman et al. 1998.

Cause/Disease	Assesment
Shock, sepsis, ileus, myocardial infarction, cardiovascular collapse, congenital heart malformation, acute liver failure, hypoperfusion, meningitis	The systemic drop in arterial blood pressure and the compensatory adrenal and sympathetic nervous system-induced vasoconstriction result in reduced perfusion and hypoxia of skin and visceral organs, especially the liver and kidneys. Under these circumstances, mitochondrial ATP synthesis is disturbed while glycolysis is activated. Lactate which is then produced at a higher rate, cannot be utilized by the liver and kidneys.

Hyperlactataemia represents an independent prognostic parameter for assessing the chance of survival. Blood lactate concentrations >5 mmol/l in patients with sepsis admitted to intensive care units are associated with a mortality rate of 59% after 3 days and of 83% after 30 days. Following resuscitation of patients with acute circulatory disorders, mortality is >90% if the lactate exceeds 8 mmol/l. The concentration of lactate is a better indicator of tissue hypoxia than the anion gap and the pH of the blood because diseases such as acute liver failure or septic shock have a tendency to be associated with alkalosis. In severe shock, the measured rise in lactate amounts is up to 0.8 mmol/l per 10 min. After normal circulatory function is reestablished, lactate will be rapidly consumed with lactate concentrations declining at a rate of 4-10 mmol/l over a period of 10 min. |
Biguanide-associated lactic acidosis	While undergoing treatment with biguanides, lactic acidosis may develop in patients with type II diabetes mellitus. In the case of phenformin, the incidence is 1:4,000, in the case of buformin and metformin 1:40,000 to 1:80,000. Biguanides are said to cause a decline of the pH within the hepatocyte, thereby leading to inhibition of both lactate uptake as well as gluconeogenesis from lactate. Lactic acidosis ensues. Reduced renal function has been described in almost all such patients.
Postoperative detection of acute intraabdominal vascular occlusion	The assessment of postoperative lactate trends may allow recognition of intestinal ischaemia (aortic and intestinal arterial reconstructive surgery).
Fetal distress during labor and delivery	During labor and delivery, the blood lactate concentration and pH obtained from the fetal scalp are important parameters for assessing fetal distress. pH > 7.25 and lactate concentrations < 2 mmol/l suggest that underlying fetal distress is not present.
Intoxication with methanol, ethylene glycol, acetaminophen, salicylates	Intoxication with these substances leads to an anion gap frequently of > 20 mmol/l and lactic acidosis. Clinically, patients present with a variety of neurological symptoms including coma. Methanol is metabolized to formaldehyde and formic acid, ethylene glycol to glycolic acid and oxalic acid. These metabolites or the original substances which, as non-measurable anions, all greatly increase the anion gap and inhibit the mitochondrial oxidation of pyruvate; development of lactic acidosis ensues. Salicylate intoxication is associated with salicylate concentrations of > 300mg/l. In children, metabolic acidosis predominates. Adults show a mixed picture consisting of respiratory alkalosis and metabolic acidosis.

1.2.5. Increased lactate level in inherited metabolic disorders

Lactic acidosis is known to be a presenting feature of many inborn errors of metabolism: organic acidurias, urea-cycle defects (mainly citrulinaemia), fatty acid oxidation defects, disorders of liver glycogen metabolism, disorders of liver gluconeogenesis, the PDHc deficiency, or Krebs-cycle defects; and dysfunction of the oxidative phosphorylation. The latest are considered to be the most common causes of congenital forms of lactic acidosis (Stacpoole et al. 1997A).

The differential diagnosis of congenital lactic acidosis was presented by Zeman et al. (1998). They published a retrospective study of 230 children with suspected congenital lactic acidosis. Younger children manifested mainly the CNS involvement, the older children manifested the myopathic symptoms. The inherited metabolic disorder was diagnosed in 49 children, i.e. 21% of the group. The mitochondrial disorder was diagnosed in 23 children. 10 children had an isolated complex IV deficiency, two children had the combined complex I and IV deficiency, three children had the PDHc deficit, and one child had a point mutation in mtDNA with ATP synthase deficit. Seven children had the beta-oxidation disorder (4x MCAD a 3x LCHAD). The liver glycogen storage metabolism was proved in 13 children. Three had organic aciduria, two of them urea cycle deficit (OTC, citrulinaemia) and fructose metabolism disorder was found in three children. The low free and total carnitine level was found in five children as a result of valproate treatment (Zeman et al. 1998).

Tab. 8. Causes of congenital lactic acidosis.
According to Thomas 1998; Zeman et al. 1998; Vassault 2008.

Group of diseases/ disorders	Metabolic defect
OXPHOS defect	isolated/combined respiratory chain complex deficiencies, ATP synthase deficiency
citric cycle disorders	2-oxolutarate dehydrogenase complex deficiency, fumarase deficiency
pyruvate dehydrogenase complex deficiency	pyruvate decarboxylase (E1) deficiency, dihydrolipoic transacetylase (E2), dihydrolipoyl dehydrogenase (E3) deficiency
pentose phosphate pathway and gluconeogenesis disorders	glucose-6-phosphatase deficiency, fructose-1,6-bisphosphatase deficiency, phosphoenol pyruvate carboxykinase deficiency
glycogen storage disorders	acid maltase deficiency (Pompe disease), glycogen debrancher deficiency (Cori-Forbes disease), muscle glycogen phosphorylase

	deficiency (McArdle disease), muscle phosphofructokinase deficiency (Tauri disease)
ketolysis disorders	succinyl coenzyme A: 3-oxo acid transferase deficiency
carboxylases deficiencies	biotidinase deficiency, biotin deficiency
organic acidurias	methylmalonic aciduria, priopionic aciduria

1.2.6. Differences in lactate levels in particular disesases with congenital lactic acidosis

The degree of lactataemia is in itself not discriminatory for the particular disorder (Poggi-Travert et al. 1996). In disorders of gluconeogenesis (fructose bisphosphatase), the level of lactic acid can reach 15 mmol/l. The level of lactic acid may negatively correspond with the level of glycaemia in these disorders. Combination of lactic acidosis and severe hypoglycaemia can become life-threatening. Such setting is even more pronounced in glycogen storage disease type I. Both glycogenolysis and gluconeogenesis are effectively compromised by deficiency of glucose-6-phosphatase (GSD type 1a), glucose-6-phosphate translocase (GSD type 1b), or the endoplasmic reticulum phosphate translocase (GSD type 1c). The elevated lactate level is present constantly (Moses and Parvari 2002). In contrast, in glycogen storage disease types III and VI and in glycogen synthase deficiency, hyperlactataemia is only observed in the post-prandial period when the patient is fed with a carbohydrate-rich diet, whereas lactate concentrations are normal when the patient is fasted or hypoglycaemic. In these disorders, the level of lactate rarely if ever reaches 7 mmol/l (van den Berghe 1996). In case of pyruvate carboxylase deficiency, the literature is not consistent. Poggi-Travert describes permanent hyperlactataemia, observed both in the fed and fasted states, which tends to decrease within a short fast (Poggi-Travert et al. 1996). On the other hand, Robinson (Robinson 2006) describes increase in lactate level in fasting with a tendency towards hypoglycaemia, however, with attenuated effect. In pyruvate dehydrogenase, 2-oxo-glutarate dehydrogenase and respiratory-chain disorders, maximum lactate levels are observed in the fed state (Poggi-Travert et al. 1996).

When samples are not collected at the time of acute metabolic decompensation, it must be emphasized that metabolic abnormalities are quite variable and may not be apparent at the particular time of testing. The practical solution to these variations is to obtain several samples of blood and urine at several times in the day and night and eventually under different dietary conditions (e.g. during an acute illness, after fasting, after a highcarbohydrate and/or high-protein diet, or after physical exercise) or to provide the glucose tolerance test (s.

below). Differences in lactate levels in PDHc and disorders of OXPHOS are discussed in a following chapter.

1.2.6. Lactate origin in OXPHOS disorders

Patients with primary disorders of the citric cycle, and oxidative phosphorylation may develop abnormal lactate accumulation. Oxidation of NADH and $FADH_2$ produced by the PDHc reaction and the tricarboxylic acid cycle is hampered by impaired electron transfer through the respiratory chain. The consequent rise in the intramitochondrial $NADH/NAD^+$ ratio inhibits PDHc activity. Pyruvate oxidation decreases, and pyruvate is converted to lactate, resulting in an increased lactate/pyruvate ratio. Another important consequence is the decrease of the ATP production (Stacpoole et al. 1997A).

1.2.7 Lactate/pyruvate ratio is lower in PDHc deficiency than in OXPHOS disorders

The rise in the intramitochondrial $NADH/NAD^+$ ratio in case of impaired cellular respiration results in increased lactate/pyruvate (L/P) ratio. In pyruvate dehydrogenase (PDHc) deficiency, the metabolic block is upstream to the respiratory chain. The level of pyruvate rises as it is not metabolized at the sufficient amount. On the other hand, reducing equivalents raised in other reactions, e.g. citric cycle, may be metabolized by functional respiratory chain. Theoretically, the cytoplasmic oxido-reduction state is predicted to be less altered and L/P ratios are normal or lower than in disorders of OXPHOS (Poggi-Travert et al. 1996). An L/P molar ratio >25 in case of elevated lactate level is considered increased and suggestive of a primary respiratory chain dysfunction, whereas a ratio <25 is thought to be consistent with PDHc deficiency. Moreover, other thresholds have been suggested: Poggi-Travert, DeBray, Robinson (Poggi-Travert et al. 1996; Robinson 2006; Debray et al. 2007). Some authors consider values under 15 as significant (Barnerias et al. 2010). The usefulness of the cut point to correctly classify patients into primary respiratory chain dysfunction and PDHc deficiency was evaluated only in 2007 by Debray et al (Debray et al. 2007). He found a strong positive association between L/P ratio and blood lactate in patients with non-PDH congenital lactic acidosis, whereas this association was weak in children with PDHc deficiency. Consequently, the diagnostic accuracy of the L/P ratio for differentiating between non-PDH and PDHc deficiency forms of congenital lactic acidosis increased at higher lactate concentrations.
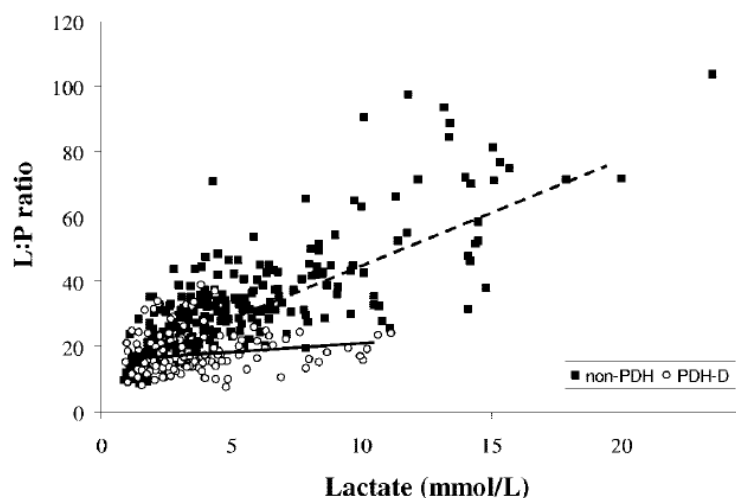
Fig. 9. Scatter plot of L/P ratio vs lactate concentration in patients with congenital lactic acidosis. According to Debray et al. 2007.
■, non-PDH group; ○, PDH-D group. The *solid line* represents the regression line for the PDH-D group, and the *dashed line* represents the regression line for the non-PDH group.

1.2.8 The role of lactate in the diagnostics of mitochondrial disorders

The majority of proven mitochondrial oxidative disorders present with a raised blood or CSF lactate. This is often accompanied by a raised lactate to pyruvate ratio signifying a change in cellular redox state. It is a common presenting feature in some mitochondrial disorders (MTDs), intermittent in others and for some of MTDs, it is not a presenting feature at all (Robinson 1993; Scaglia et al. 2004; Bohm et al. 2006; Robinson 2006). The lactate level analysis thus plays a major role in the diagnostics of mitochondrial disorders on the biochemical level (Morava et al. 2006).

In general, the more severe the defect is, the more likely it is to display an increased lactate in body fluids (Robinson et al. 1990). Increased blood lactate was found in 85% and increased blood alanine in 65% of 180 children with COX deficiency. The CSF lactate level was elevated in 81% of examined cases (Bohm et al. 2006). Only 60% of 113 patients with mitochondrial disorders had elevated blood lactate sampled on >1 occasion in paper published by Scaglia and colleagues (Scaglia et al. 2004). Increased lactate level may be only intermittent. A normal level of blood lactate in a child with clinical suspicion of mitochondrial disorder thus does not exclude mitochondrial etiology of his disease (Stern 1994; Magner et al. 2011A). Repeated blood lactate assessment is thus important in children for whom clinical

suspicion of mitochondrial disorder exists but who have presented normal lactate levels in previous analyses.

There are some studies indicating that lactic acidosis occurs predominantly in patients with mitochondrial diseased based rather on mtDNA than nuclear DNA defects. This is illustrated by study of Böhm et al. who found elevated lactate levels regularly in patients with combined respiratory chain defects and known mtDNA mutations (79 patients) (Bohm et al. 2006). On the other hand, there are mitochondrial diseases, such as NARP and its infantile variant maternally inherited Leigh syndrome, in which blood lactate is often normal or mildly increased at best (Dimauro et al. 2004). There are also literary evidences, that lactic acidosis can be present also in syndromes caused by specific nuclear genes mutations. For example in the study of Cizkova et al. (2008), lactic acidosis was present in all 27 patients with mutation in the nuclear gene *TMEM70* coding an assembly protein for ATP synthase. Anyway, presence of lactic acidosis/elevated lactate concentrations is relative more constant in some syndromes than others. For example, severe infantile lactic acidosis (>5 mmol/l) is typical for patients with severe manifestation of PDHA1 and DLD deficiencies, severe nDNA or mtDNA defects of complex I, severe type B pyruvate carboxylase deficiency and *SURF1* defects accompanied by COX deficiency (Robinson 2006). Degree of lactate level elevation is also dependent on severity of syndrome manifestation in every single patient.

Tab. 10. The occurence of lactic acidosis in severity assorted by particular mitochondrial defect. According to Robinson 2006.

Lactate level	Mitochondrial disorders - deficiency or mutations in								
	complex I	complex II	complex III	complex IV	complex V	PC	PDH	tRNA	MtDNA depletion
normal <2.0 mmol/l	some nuclear mtDNA LHON mutations	*SDHC SDHD*	some cyt *b*	some *LRPPRC*	*ATP6*	type C	mild *PDHA1 PDHE3*	low heteroplasmy *tRNALeu tRNALys*	
mildy increased 2–5 mmol/l	nuclear defects mtDNA *ND5,ND6*	*SDHA*	*MTCYTB UQCRB*	*LRPPRC COX 10 COX 15 COX I COX II*	*ATP 6* Leigh *ATP12*	type A	*PDHA1 PDHB DLAT DLD PDP1*	high heteroplasmy *tRNALeu tRNALys*	*DGUOK TK2 SUCLA2 POLG1*
high >5 mmol/l	nuclear defects mtDNA *ND3,ND5*		*BCS1*	*SURF1*		type B	severe *PDHA1*		

ATP6 - ATP synthase 6; ATP12 - ATP synthase, mitochondrial F1 complex, assembly factor 2; BCS1 - S. cerevisiae bcs1 protein; COX I, II cytochrome c oxidase subunit I, II; COX 10, 15 -cytochrome *c* oxidase assembly protein 10, 15; DGUOK - deoxyguanosine kinase; LRPPRC - Leucine-rich PPR motif-containing protein; MTCYB- cytochrome *b* of complex III; ND 3, 5, 6 - complex I, subunit 3, 5, 6; PC - pyruvate carboxylase; PDHA1 - pyruvate dehydrogenase complex, E1-alpha polypeptide 1; PDHB - pyruvate

dehydrogenase complex, beta polypeptide; PDHE3 - dihydrolipoamide dehydrogenase (DLD), pyruvate dehydrogenase component E3; POLG1 - DNA polymerase gamma; SDHA - succinate dehydrogenase, subunit A; SDHC - succinate dehydrogenase, subunit C; SDHD - succinate dehydrogenase, subunit D; SUCLA2 - Succinate-coenzyme-A ligase; SURF1 - Surfeit 1; TK2 - mitochondrial thimidine kinase; UQCRB - ubiquinone-binding protein

1.2.8.1. Mitochondrial disorders in childhood and cerebrospinal fluid lactate

No strict correlation exists between lactate levels in blood and cerebrospinal fluid. The rate of lactate transport across the blood-brain-barrier is low under normal circumstances (LaManna et al. 1993) and lactate concentration in cerebrospinal fluid behaves largely independently of plasma levels, even in samples obtained simultaneously (Hutchesson et al. 1997). The lactate level in children with mitochondrial disorders is usually increased to a greater degree in cerebrospinal fluid than in plasma (Hutchesson et al. 1997; Benoist et al. 2003).

1.2.8.2. The role of alanine level examination in the diagnostics of the mitochondrial disorders

As alanine is formed from pyruvate by alanine transaminase at a rate proportional to intracellular lactate levels, and thus serves as an indirect marker of increased lactate level, an assignment of alanine levels is also a part of the diagnostic work up in the children with suspicion of mitochondrial disorder.

Morava et al. described significantly higher blood alanine levels in neonates with mitochondrial dysfuncion, with good capability of increased alanine levels to distinguish patients with respiratory chain disease from those with transient lactic acidosis (Morava et al. 2006). According to the third diagnostic protocol of mitochondrial disorders assessment published by Wolf and Smeitink (2002), an absolute elevation in alanine above 450 µmol/l is a factor used to determine the likelihood of mitochondrial disease. The increased alanine level in CSF and/or blood is also an important biochemical criterion in the study of Morava et al. (2006A).

1.2.9. Functional tests used in the diagnostics of patients with primary lactic acidosis

1.2.9.1. The forearm ischaemic exercise test (McArdle test)

Ischemic exercise testing is used in evaluation of patients with suspected McArdle disease, also known as glycogen storage disease type V. During ischaemia and physical activity involving the forearm in healthy individuals, the maximal rise in lactate (5-25 times that of normal range) occurs after 1.5 minutes distal to the compression area, with normalization not until 20-30 minutes later (Thomas 1998). Lack of an increase in the blood lactate concentration during exercise is indicative of a defect in conversion of glycogen (or glucose) to lactate, consistent with the deficiency of skeletal muscle phosphorylase in this disease. Other glycogen storage diseases, such as deficiencies of phosphofructokinase and debranching enzyme, would also yield an abnormal ischemic exercise response (Zaman and De Raedt 2000). The ischemic exercise test is not a frequently performed test and its interpretation has its limits (Zaman and De Raedt 2000). This test cannot be provided in small children because of absent compliance. The test is also not free or risk, as a late-onset rhabdomyolysis with massive myoglobinuria, which some patients with McArdle disease could develope subsequently to this test (Meinck et al. 1982).

1.2.9.2. Glucose tolerance test and its interpretation

A glucose tolerance test is a medical test in which glucose is given and blood samples taken afterward to determine how quickly it is cleared from the blood. The test is usually used to test for diabetes mellitus, insulin resistance, and sometimes reactive hypoglycaemia or rarer disorders of carbohydrate metabolism. In the most commonly performed version of the test, an *oral glucose tolerance test* (OGTT), a standard dose of glucose is ingested by mouth and blood levels are checked two hours later. Many variations of the GTT have been devised over the years for various purposes, with different standard doses of glucose, different routes of administration, different intervals and durations of sampling, and various substances measured in addition to blood glucose. One of these variations is used in the diagnostics of mitochondrial disorders (Craig et al. 2009).

The test is based by glucose administration (1.75g/kg body weight; maximum 75g) as a 10% solution in water orally or by nasal tube in 5-10 min. A blood sample, in which the levels of glucose, lactate (L), pyruvate (P), 3-hydroxybutyrate (B) and acetoacetate (A) are examined,

has to be taken before the glucose administration. We take the control sample after one hour at our department, which seems to be the most informative. However, some authors take blood samples *á* 30 minutes for 3 hours (Poggi-Travert et al. 1996).

A marked decrease of lactate from an elevated level at zero time is observed in disorders of gluconeogenesis and in glucose-6-phosphatase deficiency; an exaggerated increase occurs in other glycogen storage diseases, glycogen synthase deficiency, and PDHc deficiency. In respiratory-chain disorders, both L/P and B/A ratios are elevated and can be associated with a significant post-prandial hyperketonaemia (Rotig et al. 1990; Munnich et al. 1992). In pyruvate carboxylase deficiency (isolated or secondary to biotinidase or holocarboxylase synthetase deficiency), the L/P ratio is very high and is associated with post-prandial hyperketonaemia and a normal or low B/A ratio (Saudubray et al. 1976; Robinson 1993). Ketone bodies decrease in defects of gluconeogenesis, glycogen synthase deficiency and glycogen storage disease types III and VI. They can paradoxically increase in pyruvate carboxylase deficiency (with a low 3-hydroxybutyrate/acetoacetate ratio) and in respiratory-chain disorders (with a high 3-hydroxybutyrate/acetoacetate ratio) (Poggi-Travert et al. 1996). In pyruvate dehydrogenase deficiency, the L/P ratio is normal or low and there is no ketosis (Poggi-Travert et al. 1996).

1.2.10. Therapy of increased lactate with dichloroacetate

Regulatory site of pyruvate dehydrogenase complex is a target of possible therapeutical approach (Stacpoole 2010). Dichloroacetate (DCA) enters mitochondria and stimulates the activity of the PDHc by inhibiting the phosphorylation (and inactivation) of the E1α subunit catalyzed by pyruvate dehydrogenase kinase (Stacpoole 1989). In some patients with E1α deficiency, DCA may also stabilize the enzyme and decrease its rate of turnover (Fouque et al. 2003; Morten, et al. 1999). By maintaining PDHc in its active, unphosphorylated form, DCA accelerates the aerobic oxidation of glucose, pyruvate, and molecules such as alanine and lactate, that are in equilibrium with pyruvate, to acetyl coenzyme A that enters the citric cycle. Electrons of reducing equivalents NADH and $FADH_2$ generated by the PDHc and by the dehydrogenases of the citric cycle, enter the respiratory chain. Administration of DCA should thus theoretically stimulate both the oxidative removal of lactate (and accompanying protons) and the synthesis of adenosine triphosphate in mitochondria with at least residual activity of PDHc, citric cycle and respiratory chain. (Stacpoole et al. 1997B).

Stacpoole et al. 2006 published the double-blind study in 43 children patients with persistent or intermittent lactic acidosis. In this heterogeneous population of children with congenital lactic acidosis (including PDHc defect, citric cycle defects, respiratory chain defects), oral administration of DCA for 6 months the postprandial increase in circulating lactate in these children was blunted. However, the DCA administration did not improve the clinical outcome (Stacpoole et al. 2006). Patients in previous study tolerated the treatment well, but some toxicity of DCA had been described on the nervous system and liver in human in other studies (Kaufmann et al. 2006). New epidemiological research on DCA are planned more selectively on populations that may be predisposed to DCA-associated health complications because of age or genotype (Stacpoole 2010).

2. AIMS OF THE THESIS

The heterogeneity of mitochondrial disorders is a challenge for routine diagnosis. The diagnostic work-up starts with a careful clinical examination, searching for typical sets of symptoms. Because of the highly variable phenotypes, the clinical suspicion of a mitochondrial disorder is often delayed, or the disease remains undiagnosed. No sufficiently specific and sensitive laboratory investigations or biochemical markers exist. The golden diagnostic examination standard upon suspicion of mitochondrial disorder remains the skeletal muscle biopsy with histological and biochemical analysis. However, muscle biopsy represents an invasive procedure, with an additional risk for patients with a dysfunction in oxidative phosphorylation, severe muscle hypotonia, cardiac symptoms, and CNS abnormalities. Therefore it is very important to evaluate the chance for succesful diagnosis prior to deciding on muscle biopsy.

To fulfil this statement it is necessary to characterise the natural course of the mitochondrial disorders in childhood. The aim of this PhD Thesis is a brief contribution to this effort with special focus on importance of lactate analyses in body fluids.

Three specific aims have been stated in this work:

A. To analyse the role of lactate examination in the differential diagnosis between children with mitochondrial disorders and children with other diseases.

The mitochondrial disorders and some neurological or inherited metabolic disorders may present a very similar clinical manifestation. Especially the discrimination from children with epilepsy is very important as a substantial part of mitochondrial children manifests seizures. Lactate as a possible biomarker thus could help to indicate the muscle biopsy in proper cases and prevent its unnecessary misuse.

B. To study the lactate level differences in various mitochondrial syndromes.

As lactate level may vary from one mitochondrial syndrome to the other, the aim was to analyse the lactate in children with various mitochondrial diseases.

C. To characterise the clinical and laboratory data of neonates with mitochondrial disorders and to suggest new diagnostic algorhytms.

The diagnostics of neonates with metabolic disorders is especially demanding. The symptoms as hypotonia, refusal to eat, failure to thrive, cardiorespiratory failure, are often unspecific. The aim was to prepare new diagnostic algorhytms of mitochondrial disorders in neonatal period.

3. METHODS AND MATERIAL

3.1. Patients

During my 5-year stay at the Department of Pediatrics of Charles University I had a possibility to see app. 1000 children with various metabolic disorders. A significant part of them was admitted with a suspicion on mitochondrial disorder. 107 children ranging in the age from 1 month to 13 years were included in the crucial study of the PhD thesis characterising the role of lactate in differential diagnosis of mitochondrial disorders. In this study patients were divided into five groups according to their primary diagnosis.

Group I consisted of 24 children with mitochondrial encephalopathy resulting from isolated or combined deficiency of respiratory chain complexes or ATP synthase; the ages of the children in this group ranged from 1 month to 13 years (M/F 16/8). Mutations in nuclear or mitochondrial genes were identified in half of the patients in this group. All children in group I, but none from any other groups, fulfilled the diagnostic criteria for mitochondrial diseases published by Walker and colleagues (Walker et al. 1996). Six children had complex I deficiency (NADH:ubiquinone oxidoreductase); eight had complex IV deficiency (cytochrome *c* oxidase), including two with mutations in the *SCO2* gene and three with mutations in the *SURF1* gene; four children had NARP syndrome (Neurogenic muscle weakness, Ataxia, Retinitis Pigmentosa) with complex V deficiency (ATP synthase); six children had combined respiratory chain complex deficiencies including one patient with MELAS syndrome (Mitochondrial myopathy, Encephalopathy, Lactic Acidosis and Stroke-like episodes), one with MERRF syndrome (Myoclonic Epilepsy, Ragged-Red Fibers), and one with Alpers-Huttenlocher syndrome due to mutations in *POLG1* gene. In seven children, the samples for biochemical analysis were obtained after an attack of brief seizures. The duration of seizures was less than 3 min and lumbar puncture was performed between 30 min and 72 h after the seizures. No seizures were observed in the other 17 children.

Group II consisted of 32 children with epilepsy (1 month to 6 years, M/F 23/9). Patients with epilepsy underwent lumbar puncture for purposes of the broad-differential diagnosis mostly due to poorly compensated seizures. No metabolic disorders were recognized. In 15 children, the samples for biochemical analysis were obtained 3.0±0.6 h after seizures (range between 1 and 8 h). The duration of seizures in the study group was relatively short, ranging from 0.5 min to 2 min. Seizures persisted for more than 30 min, fulfilling status

epilepticus criteria, in only one patient. The other 17 children in Group II were without recent history of seizures at the time of analysis.

Group III consisted of 23 children with psychomotor retardation (5 months to 6 years, M/F 15/8) with developmental quotient below 50 points. For children up to 1 year, a delay in achievement of psychomotor milestones greater than three months was considered as a criterion of psychomotor retardation. These patients without history of seizures were ascertained for the suspicion of various hereditary metabolic disorders, none of which were confirmed.

Group IV consisted of 12 children with bacterial meningitis (1 month to 2 years, M/F 4/8) due to *Streptococcus pneumoniae* (5x), *Streptococcus agalactiae* (2x), *Neisseria meningitidis* B (2x), *Neisseria meningitidis* C (1x), and *Escherichia coli* (2x), respectively.

Control group V was represented by 16 children with acute febrile illnesses or meningism without any laboratory findings of neuroinfection (3 months to 2 years, M/F 9/7).

3.2. Biochemical studies

Lactate

Blood samples for lactate analyses were obtained from patients in groups after overnight fasting without venostasis. For lactate measurement, samples were deproteinized immediately by the addition of two volumes of 8% (v/v) perchloric acid. CSF specimen were obtained by lumbar puncture. The CSF specimen were obtained simultaneously with the blood samples; all lumbar punctures were indicated for diagnostic purposes by consultants. All specimen were transported on the wet ice to the laboratory, where the analyses were performed immediately. Lactate levels were measured at the Institute of Inherited Metabolic Disorders, First Faculty of Medicine, Charles University in Prague and General University Hospital in Prague (IIMD). A spectrophotometric method based on the lactate dehydrogenase-catalyzed oxidation of lactate to pyruvate, in which NADH is formed, was used. In this method, the intensity of NADH absorbance, measured at 340 nm, is proportional to the lactate concentration in the sample. The analytical variation coefficients at 1.5 and 5.2 mmol/l were 2.4%, and 2.2%, respectively (Noll 1984). Pathological concentrations of metabolites were defined as values higher than: blood lactate >2.3 mmol/l, CSF lactate >2.1 mmol/l. CSF samples with blood contamination were removed from the study with exclusion criterion of more than 50 erythrocytes/3 μl.

Amino acids and organic acids

Amino acids in serum and CSF were analyzed by ion exchange chromatography with ninhydrin detection on an automatic amino acid analyzer (AAA 400, Ingos, Czech Republic) at IIMD. Aliquots of serum (300 μl) were diluted with lithic buffer (composition: citrate, lithium citrate and lithium chloride, pH 4.8), 4-chlorphenylalanine as internal standard and deproteinized by adding sulfosalicylic acid (final volume 500 μl). The analytical CVs at 250 and 500 μmol/l were 6.1%, and 6.0%, respectively (Hyánek 1991).

The profile of organic acids in urine was analyzed by gas chromatography and mass spectrometry (Chalmers 1982).

3.3. Enzymatic and protein studies

The muscle mitochondria gained by biopsy or autopsy were isolated according to Makinen and Lee without use of protease (Makinen et al. 1968) and stored in the liquid nitrogen.

The respiratory chain enzymes activities, NADH-coenzym Q10-oxidoreductase (NQR, complex I), succinyl-coenzyme Q10-oxidoreductase (SQR, complex II), coenzym Q10-cytochrome *c* oxidoreduktase (QCCR, complex III), and cytochrome *c* oxidase (COX, complex IV) were measured spectrophotometrically (Pelley et al. 1976; Rustin et al. 1994).

Specific oligomycin-sensitive F_1F_O-ATP synthase hydrolytic activity was measured in ATP generating systems as described by Baracca et al (1987). The steady-state levels of F_1F_O-ATP synthase were determined by blue-native polyacrylamide gel electrophoresis (BN-PAGE, Schagger 1991) followed by Western blot using monoclonal mouse antibodies against ATP synthase alpha subunit (Mitosciences) (Fornůsková et al 2008).

PDH complex activity and E1 subunit activity were assayed as the release of $^{14}CO_2$ from [^{14}C]pyruvate. The activity of PDH complex in the lymphocytes was measured according to Sheu et al. (1981), and in isolated muscle mitochondria according to Constantin-Teodosiu et al. (1991). The activity of the E1 subunit of the PDH complexes was measured according to Van Laack et al. (1988). The amounts of pyruvate dehydrogenase complex (PDHc) were estimated by Western blot.

The activity of control enzyme citrate synthase was determined spectrophotometrically (Srere 1969). Protein content was determined by the method of Lowry et al. (1951).

3.4. Molecular-genetic analyses

DNA was isolated from muscle and/or blood by method of phenol extraction. The presence of prevalent mutations was analyzed by PCR-RFLP, mitochondrial DNA and selected nuclear genes were studied by method of direct sequenation at the ABI 3100 Avant analysator (Applied Biosystems).

3.5. Statistics

Data was analyzed with Kruskal-Wallis one-way analysis of variance on ranks with Dunn's method used for pair-wise multiple comparison. To compare subgroups, Student's t-test was used. If not specified, $P<0.05$ was considered as significant. Statistical analysis was performed using the SigmaStat 3.5 program (Systat Software, Inc.).

3.6. Ethics

All studies with children were approved by the Committees of Medical Ethics at all collaborating institutions. Informed consent was obtained from parents.

4. RESULTS AND DISCUSSION

4.1. The role of lactate analysis in the differential diagnosis between children with mitochondrial disorders and children with other diseases.

Elevated CSF-lactate is a reliable marker of mitochondrial disorders in children even after brief seizures

Martin Magner, Karol Szentiványi, Ivana Švandová, Pavel Ješina, Markéta Tesařová, Tomáš Honzík and Jiří Zeman

The aim of our study was to ascertain the diagnostic value of lactate and alanine analyses in blood (B) and cerebrospinal fluid (CSF) in children with mitochondrial disorders in comparison to children with epilepsy, psychomotor retardation, meningitis and meningism. To address this problem, we ascertained the diagnostic value of lactate and alanine in B and CSF in children with mitochondrial disorders (n=24), epilepsy (n=32), psychomotor retardation (n=23), meningitis (n=12) and meningism (n=16).

- The most important fact showed in our study is that relatively brief seizures lasting less than 2 minutes did not increase lactate concentration in CSF. Increased CSF lactate levels were found in 83% of children with mitochondrial disorders but in only 3% of children with epilepsy and 9% of children with psychomotor retardation. Our results thus suggest that CSF-lactate level may serve as a reliable marker discriminating mitochondrial patients with seizures from patients with epilepsy if the sample of cerebrospinal fluid is not obtained within a short time after an attack of prolonged seizures. This fact is important to consider, because a substantial proportion of patients with mitochondrial disorders may manifest seizures.

- 21% of the children with mitochondrial disorders showed only mildly increased CSF lactate levels (between 2.2 and 3.0 mmol/L). This finding may be of special importance, because, in mild elevation of CSF-lactate may be wrongly considered to be a result of seizures or even overlooked.

- Two-thirds of children with mitochondrial disorders had higher lactate concentrations in CSF than in blood. This confirmed the previously known fact, that CSF lactate examination is of higher sensitivity than that of blood lactate levels examination.

- The last important result of our study was the confirmation of the role of alanine assessment. Although no correlation was found between lactate and alanine levels in CSF, alanine concentration in blood was increased in 54% of mitochondrial patients but in only 3% of children with epilepsy and 4% of children with psychomotor retardation. Our results were thus in accord with the third diagnostic protocol of mitochondrial disorder assessment published by Wolf and Smeitink (Wolf and Smeitink 2002), in which an absolute elevation in alanine above 450 µmol/l is a factor utilized to determine the likelihood of mitochondrial disease.

4.2. The lactate level differences in various mitochondrial syndromes

SURF1 missense mutations promote a mild Leigh phenotype

Piekutowska-Abramczuk D, Magner M, Popowska W, Pronicki M, Karczmarewicz E, Sykut-Cegielska J, Krmiec T, Jurkiewicz E, Szymanska-Debinska T, Bielecka L, Krajewska-Walasek M, Vesela K, Zeman J and Pronicka E

Clin Genetics **2009;76:195-204. IF 3.206**

SURF1 gene mutations are the most common cause of Leigh syndrome (LS), a rare progressive neurodegenerative disorder of infancy, characterized by symmetric necrotizing lesions and hypervascularity in the brainstem and basal ganglia, leading to death before the age of 4 years. Most of reported mutations create premature termination codons, whereas missense mutations are rare. The aim of this study was to characterize the natural history of Leigh syndrome patients carrying at least one missense mutation in the *SURF1* gene. Nineteen such patients (8 own cases and 11 reported in literature were compared with a reference group of 20 own c.845_846delCT homozygous patients, and with other LS SURF cases described in the literature. Disease onset in the studied group was delayed. Acute failure to thrive and hyperventilation were rare, respiratory faillure did not appear before the age of 4 years. Dystonia, motor regression and eye movement dissociation developed slowly. The number of patients who survived 7 years of life totaled 9 of 15 (60%) in the ´missense group´ and 1 out of 26 (4%) patients with mutation s leading to truncated proteins.

The study showed that:

- The presence of a missence mutation in the *SURF1* gene may correlate with a milder course of the disease and longer survival of Leigh patients
- Normal magnetic resonance imaging (MRI) findings, normal blood lactate value, and only mild decrease of cytochrome *c* oxidase (COX) activity are not sufficient reasons to forego *SURF1* mutation analysis in differential diagnosis.

Two patients with clinically distinct manifestation of pyruvate dehydrogenase deficiency due to mutations in PDHA1 gene

Magner M., Vinšová K., Tesařová M., Hájková Z., Hansíková H., Wenchich L., Ješina P., Smolka V., Adam T., Vaněčková M., Zeman J. and Honzík T.

In this study we presented first two patients with PDHc deficit due to mutations in *PDHA1* gene in the Czech Republic. We documented the broad variability of clinical symptoms of this disease. Although the Leigh syndrome was diagnosed in MRI examination in both patients, the clinical course differed significantly. In patient 1, the initial hypotonia with psychomotor retardation was observed since early infancy. The child gradually showed symptoms of spasticity and arrest of psychomotor development. In patient 2, the disease manifested by seizures and hyporeflexia in toddler age. The diagnosis was confirmed at the age of seven years after attacks of dystonia and clinical manifestation of myopathy with normal mental development.

- The increased blood and cerebrospinal fluid lactate levels were present in both our patients. In concordance with other studies, biochemical investigations did not help to predict the phenotype of patients, as the correlation between biochemical findings and clinical manifestation is poor and even impossible in the PDH deficiency (Robinson et al. 1987). The patients with various severity of clinical impairment may show mild elevation of lactate and alanine levels in blood or very severe lactic acidosis. Decreased lactate/pyruvate ratio in case of lactic acidosis (normal range 10-20) supports the clinical suspicion on PDHc deficit. Some authors consider already values under 15 significant (Barnerias et al. 2010). Lactate/pyruvate ratios indicate a suspicion on PDHc deficit in both our patients using Barnerias´s criteria.

- Enzymatic analyses revealed PDHc deficiency in isolated lymphocytes in the first but not in the second patient. The direct measurement of PDH E1 subunit revealed deficiency in this individual. We thus proved that normal PDHc activity may not exclude the disease. The literary data on correlation among the PDHc activity, E1αβ protein level and clinical phenotype exists in PDHc deficit were reviewed in the discussion. No clear correlation could be found.

- In patient 1, a novel hemizigous mutation c.857C>T (Pro250Leu) was detected in the X-linked *PDHA1* gene. Mutation c.367C>T (Arg88Cys) was found in patient 2.

Mitochondrial disorders of respiratory chain complexes in children

J. Zeman, M. Magner

in ***Praktische Aspekte bei Diagnostik und Therapie von Mitochondriopathien und ketogener Diät. Eds. W. Sperl, J. Mayr. Heilborn: SPS Verlagsgesellschaft, 2007. ISBN 978-3-936145-38-0***

The text reviews current knowledge of prevalence, clinical spectrum, diagnostic approach and possibilities in the field of mitochondrial medicine in children. In association with biochemical diagnosis, it is emphasized that OXPHOS disorders are often accompanied by excessive production of lactic acid and development of metabolic acidosis. According to our experiences, increased lactate and alanine levels in blood and cerebrospinal fluid are present in more than 80% children, usually with increased lactate/pyruvate ratio.

4.3. The characterisation of clinically and laboratory data of neonates with confirmed mitochondrial disorders. The proposal of new diagnostic algorhytms.

Mitochondrial encephalocardio-myopathy with early neonatal onset due to TMEM70 mutation

Tomáš Honzík, Markéta Tesařová, Johannes A Mayr, Hana Hansíková, Pavel Ješina, Olaf Bodamer, Johannes Koch, Martin Magner, Peter Freisinger, Martina Huemer, Olga Kostková, Rudy van Coster, Stanislav Kmoch, Josef Houštěk, Wolfgang Sperl, Jiří Zeman

The aim of this multi-site survey was to characterise the natural course of a novel mitochondrial disease with ATP synthase deficiency and mutation in the *TMEM70* gene. Retrospective clinical data and metabolic profiles were collected and evaluated in 25 patients (14 boys, 11 girls) from seven European countries with a c.317-2A→G mutation in the *TMEM70* gene.

In the majority of patients (92%), muscular hypotonia, apnoic spells and acute metabolic distress characterised by lactic acidosis and hyperammonaemia (86%) were present from birth. Artificial ventilation was necessary in 19 neonates. Only in 2 children was the onset of disease delayed until 1–3 months of age. Ten patients died during the first episode of metabolic disturbance within the first 6 weeks of life. Six others died later between 14 months and 4.5 years of age following metabolic deterioration as a result of acute respiratory infection or gastroenteritis. Interestingly, one of a pair of monochorionic–monoamniotic twins died during the neonatal period whereas the other twin was alive at the age of 8 years. Nine patients were alive, the oldest one being 13 years old. Failure to thrive and growth retardation (below the third percentile) were present in all patients surviving the neonatal period. Microcephaly was documented in 59% of all patients. Mild cranio-facial dysmorphy with low set ears, a prominent nasal bridge and retrognathia were apparent in 16/24 patients. In P23 (compound heterozygote), apart from the early neonatal onset similar to the other patients, the course of the disease was much milder allowing almost normal psychomotor development with attendance at a regular school.

Clinical and laboratory data in 75 children with neonatal manifestation of mitochondrial disease: proposal for diagnostic algorhytms

Honzík T., Tesařová M., Hansíková H., Wenchich L., Veselá K., Ješina P., <u>Magner M.</u>, Zeman J.

The diagnosis of mitochondrial diseases on biochemical and/or molecular level has been confirmed in more than 350 patients during last 18 years at our department. 75 of them manifested symptoms at the neonatal age. Aim of our work was to characterise the clinical and laboratory presentations of mitochondrial diseases at the neonatal age and to propose the algorythyms for the diagnosis of mitochondrial diseases at this age.

- The complex I deficiency was found in 5 children, complex IV deficiency in 40 children, ATP-synthase deficiency in 26 children, the PDHc deficiency in 2 children and 2 children had a combined respiratory chain deficiency.

- ATP-synthase deficiency due to mutations in *TMEM70* gene was found in 18 children, the 8993A>G mutation (NARP syndrome) was confirmed in 7 children. The mutation analysis confirmed the diagnosis of Barth syndrome in one child. We found the mutations in *SCO2* and *SCO1* genes, which encode the assembly proteins for complex IV, in 5 patients with complex IV deficiency. Pearson and Alpers-Huttenlocher syndrome were found in 2 children with the combined respiratory chain deficiency. The mutations in E3 subunit of PDHc were found in 2 children.

- A substantial lactic acidosis was present in 93 % neonates immediately after delivery. Hyperammonaemia was documented in 22% of children, mostly in children with Tmem70 protein deficit, in some children with isolated complex IV deficiency and in one child with the NARP syndrome. The increased creatin kinase activity was found in 28 % of the neonates. The selective metabolic screening showed the increased alanine concentration in blood and increased excretion of citric cycle intermediates (malate, fumarate, citrate, succinate, akonitate, 2-oxoglutarate). All neonates with Tmem70 protein deficit had the in creased elevation of 3-methylglutaconic acid.

- The diagnostic algorhytms for the diagnostics of neonates with suspicion of mitochondrial age were proposed.

Fig. 10. Diagnostic algorhytm for a neonate with a suspicion on mitochondrial disorder. According to Honzik et al. 2010.

IEM – inborn errors of metabolism, CMP – cardiomyopathy, U-3-MGA – 3-methylglutaconic acid in urine, S-BCAA – branched-chain amino acids in serum

4.4. DISCUSSION

The role of lactate analysis in the differential diagnosis between children with mitochondrial disorders and children with other disease

We showed that elevated cerebrospinal fluid lactate is a more reliable marker of mitochondrial etiology of disease in children than increased blood lactate. Also the discriminative value of the CSF-lactate to other diseases is high – this is valid even after short-lasting seizures. The main reason for this conclusion is that brief seizures lasting less than 2 minutes did not increase lactate concentration in the CSF in our study. This fact is of great importance in the differential diagnosis, as many of children with mitochondrial disorders manifest seizures and are mistreated under the diagnosis of epilepsy. We also showed that some children with mitochondrial disorders manifest only mild increase in lactate levels, and that even small increases in lactate require some explanation. Repeated lactate analyses are sometimes necessary, because lactate may be elevated only intermittently in children with mitochondrial disorders.

The lactate level differences in various mitochondrial syndromes

There are some mitochondrial diseases, for which the characteristic level of lactate can be found. The good example is the LHON syndrome, in which the increased lactate level is very uncommon. Robinson (Robinson 2006) even classified several mitochondrial disorders according to the severity of lactic acidosis (s.Table 10). However, as the clinical spectrum is very variable in many mitochondrial diseases, we believe in contradiction with Robinson, that the severity of particular phenotype is more deciding for the severity of the lactic acidosis than the particular syndrome.

In many mitochondrial diseases both of the severe forms with early manifestation and significant lactic acidosis, intermediate and mild forms with far milder course were described. As a good example may serve the example of SURF1 deficit. Robinson classified this disorder as the one with typical lactate level over 5 mmol/l (Robinson 2006). We demonstrated the group of the patients with milder clinical course and corresponding much lower levels of lactate (Piekutowska-Abramczuk et al. 2009). A PDHc deficit may be also taken as an example. We described two boys with severe and intermediate phenotypes (Magner et al. 2011B), but the clinical variability is much wider (Barnerias et al. 2010).

However, many exceptions may be found in this correlation. This is documented by case reports of two boys with PDHc deficit with quite different course of the disease, but similar levels of lactic acidosis (Magner et al. 2011B). The next very important exception from this rule are mitochondrial syndromes manifesting usually at higher age, in which the typical episodes of metabolic disturbance with severe lactic acidosis are observed, as MELAS (Connolly et al. 2010).

The characterisation of clinically and laboratory data of neonates with confirmed mitochondrial disorders. The proposal of new diagnostic algorhytms

The early presentation at the neonatal age (and thus very serious manifestation in the most of cases) is in concordance with severe lactic acidosis. This is documented by our works describing patients with mitochondrial disorders, which manifested at the neonatal age (Honzík et al. 2010A; Honzik et al. 2010B). On contrary, in the milder forms of the diseases with presentation at higher age, the incidence of lactic acidosis was far lower. Our work with SURF1 patients with missensse mutations illustrate this possibility (Piekutowska-Abramczuk et al. 2009). We may roughly conclude, that the severity of lactic acidosis correlates with the age of the disease onset and thus also with the severity of its clinical course.

We described the group of patients with mostly severe form of TMEM70 protein deficit in patients with manifestation in neonatal age (Honzik et al. 2010A). The milder course of the disease has been described, recently (Shchelochkov et al. 2010). The severity of single mutation can be wide spread on the scale of clinical manifestations of the disease. In our study with TMEM70 protein deficit patients with the same mutations had a variable clinical course of the disease. Interestingly, one of a pair of monochorionic-monoamniotic twins died during the neonatal period whereas the other twin was alive at the age of 8 years. General outcome is with no doubts affected also by allelic confuguration of the genome and/or many epigenetic factors.

The practical impact of the work is the proposal of the diagnostic algorhytms for the newborns with supicion on mitochondrial disorder.

5. CONCLUSION AND PRACTICAL IMPACT OF PHD THESIS

Lactate is an important biochemical marker of children with mitochondrial disorder. The main aim of this PhD Thesis was to evaluate some aspects of the role of lactate in mitochondrial disorders in order to help to better indicate and interpret its values. The most important results could be summarized as follows:

- Elevated cerebrospinal fluid lactate is a more reliable marker of mitochondrial etiology of disease in children than increased blood lactate. Also the discriminative value of the CSF-lactate to other diseases except neuroinfection is high – this is valid even after short-lasting seizures. The main reason for this conclusion is that brief seizures lasting less than 2 minutes did not increase lactate concentration in the CSF in our study. This fact is of great importance in the differential diagnosis, as many of children with mitochondrial disorders manifest seizures and are wrongly treated / mistreated under the diagnosis of epilepsy.

- There are some mitochondrial diseases, for which the characteristic level of lactate can be found. However, as the clinical spectrum is very variable in many mitochondrial diseases, the severity of particular phenotype is more deciding for the severity of the lactic acidosis than the particular syndrome.

- The early presentation of mitochondrial disease in the neonatal age was usually connected with severe lactic acidosis. On contrary, in the milder forms of the diseases with later onset, the incidence of lactic acidosis was far lower.

- The valuable clinical and laboratory data of 75 neonates diagnosed with mitochondrial disorders were evaluated. The special focus was given to the group of children with novel disease - TMEM70 protein deficit. Based on our experiences the diagnostic algorhytms for the newborns with supicion on mitochondrial disorder were proposed.

6. LIST OF PUBLICATIONS

6.1. Key publications for PhD Thesis

1. <u>Magner M</u>, Szentiványi K, Svandová I, Ješina P, Tesařová M, Honzík T, Zeman J.Elevated CSF-lactate is a reliable marker of mitochondrial disorders in children even after brief seizures. **Eur J Paediatr Neurol.** 2011;15:101-8. **IF 2.007**

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