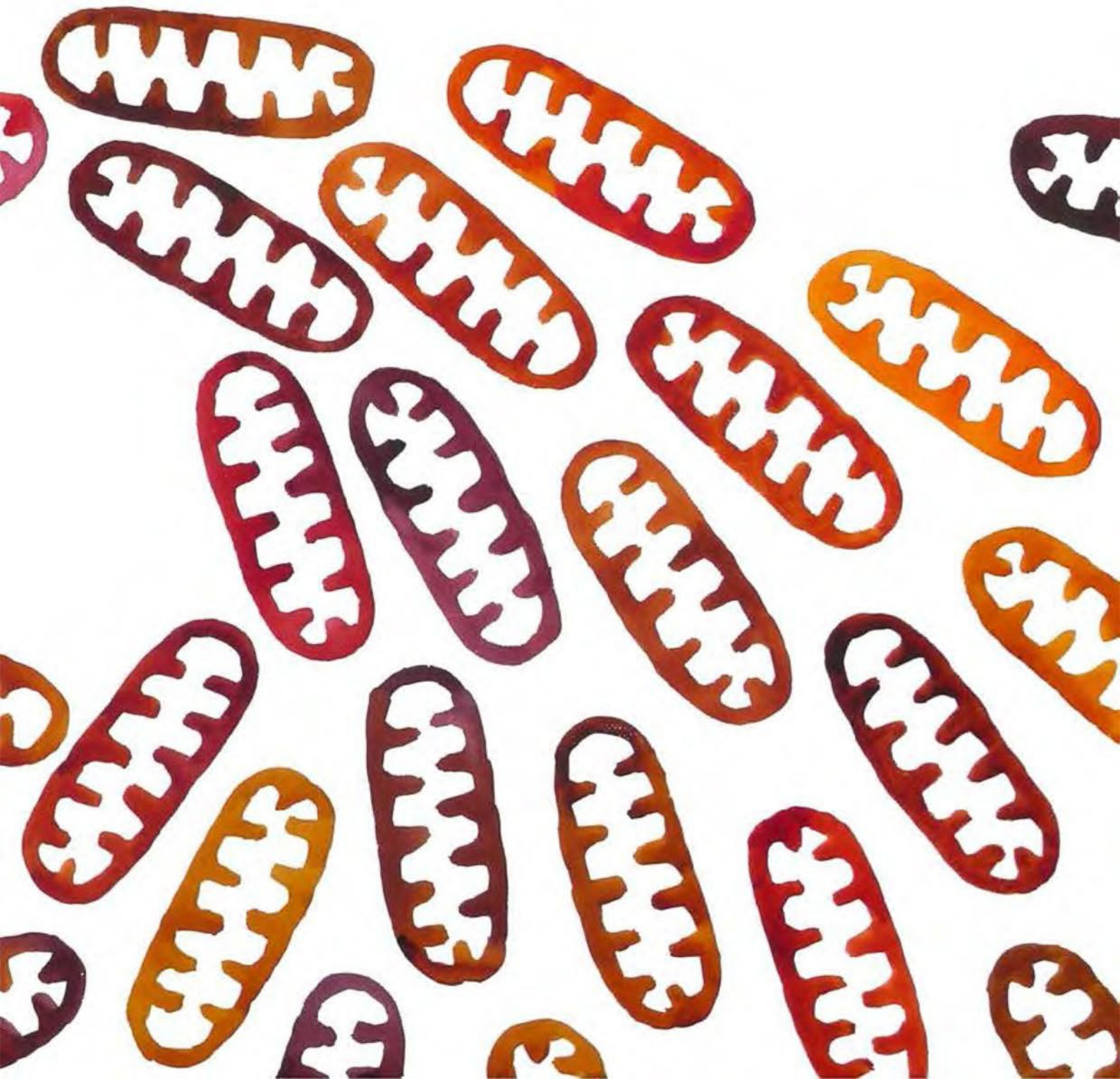
MiP 2015
11th Conference on Mitochondrial Physiology



2015 MiP*art by Alžběta Kumstátová*

MiP2015

11th Conference on

Mitochondrial Physiology

Luční Bouda, Giant Mountains National Park

Czech Republic

7 – 11 September 2015

Editors

Alena Pecinová

Tomáš Mráček

Petr Pecina

Location

The Krkonoše or Giant Mountains in English lie in the north of the Czech Republic at the border with Poland. They measure approximately 35 km in length, with their main ridges and valleys arranged in a direction from northwest to southeast. The highest peak - Sněžka is the Czech Republic's highest point with an elevation of 1,603 meters (5,259 ft.).
While the mountains are relatively compact and their altitude is at best moderate, the variety in the landscape's flora and fauna, far exceeds that of similarly sized mountains throughout Europe. This is due to Krkonoše's unique geographical location - in fact, the mountains form the northernmost montane border of Central Europe. This makes the mountains a natural barrier, inhibiting cold and wet winds blowing in from the west and the Atlantic Ocean.
In the past, recurrent periods of glaciation, have seen the Scandinavian glacier to move south and bring northern tundra into the Central Europe along its southern boundary. On the other side of Central European basin the Alpine glaciers expanded as well and thus the non-iced ridges of Krkonoše appeared at the natural crossroads, where northern and alpine habitats merged. When the glaciers retreated (approx. 20,000 years ago) the peak ridges of Krkonoše became an isolated place, separated by vast Central European woodlands from other mountain ranges. The same became true for many plant and animal species, trapped here as a glacial relicts.
Justifiably, this area is now subject of environmental protection. On both sides of the border, large areas of the mountains are designated national parks and together they constitute a cross-border biosphere reserve under the UNESCO Man and the Biosphere Program. The Czech Krkonoše National Park (KRNAP) was founded in 1963, making it the second national park on the territory of former Czechoslovakia (after High Tatra mountains of Slovakia), but also the oldest one in the present Czech Republic. Its area is approximately 370 square kilometers (140 sq mi) and it covers not only the subalpine zone but also large parts at the foot of the mountains.

Venue

Luční bouda (The Meadow Chalet) lies at the mountain plain at 1,410 m (4,626 ft.) above sea level. It is the oldest and also highest situated mountain hut in Krkonoše. Another important first is held by its own microbrewery, which is the highest one in Central Europe.

Lying in the Sudeten area of the Czech Republic, with mixed Czech and German population, history of the Chalet resembles history of the whole region in a miniature. The origins of Meadow Chalet date back to the second half of 16th century. It was an important farm taking care for surrounding grasslands and meadows which supported several dozens of cattle and goats. But it was often used by tourists as their base for trips to Sněžka. There even used to be a bell attached at the front wall, which was used to wake up the travelers, so that they wouldn't miss the sunrise on the top of Sněžka. Even scientists made their footprint in the history of Meadow chalet. In 1876 it was bought by a conservationist Christopher Haering, who had operated a meteorological station here. During his era it was also the meeting place of many naturalists and their observations were put together into a book published by a botanist Josefina Kablíková.

All this came to an end at the dawn of WW2, when during the retreat of the Czechoslovak army the Chalet burned down. However, due to its strategic location, Germans immediately started works on its restoration. The construction project was designed by the famous architect Ludwig Stigler, a graduate engineer from Berlin. The building was a pride of the German Empire and its completion at Easter of 1940 was celebrated throughout the region. Until the end of the war, it was used as a training center of the Wehrmacht Army, and for the German Air Force.

Post-war confiscation of German property passed the ownership of the chalet to various communist sport organizations until it ended up in the hands of the Czech Tourist Club at the beginning of the new democratic era. However, later on it was privatized, came into disrepair and even had to close completely for two years. However, its present owners have worked hard to bring it back to its former glory.

Mitochondrial Physiology Network 20.17: 86 pp (2015)

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11:00	R	Session 1A	Session 2	Session 3	P
12:30	R	Lunch	Lunch	Lunch	A
13:30	I	Walks & talks	Walks & talks	MiP Excursion	R
15:45	V	Session 1B	Session 2	Session 4	T
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17:00	L	Session 1B	Mitoeagle Session	MiP General Assembly	R
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19:00	Dinner	Posters 1	Posters 2	Social evening	
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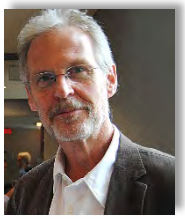
Section A1: Manifestation and etiology of mitochondrial diseases

A1-01 Posttranscriptional regulation of mitochondrial gene expression



Antonicka H and Shoubridge Eric A

Montreal Neurological Institute, Genetics, Montreal, Canada

Regulation of the translation and turnover of mRNAs is central to the control of gene expression. Eukaryotes have evolved mechanisms to sequester mRNAs and their associated RNA-binding proteins into non-membrane delimited bodies called RNA granules as a mechanism to control these processes and to respond to changing cellular demands and physiological stresses. We recently identified mitochondrial RNA granules, and showed that they contain newly synthesized mitochondrial RNA and the RNA-binding protein GRSF1, suggesting that compartmentalization of mitochondrial RNAs might also be important for mitochondrial gene expression. Silencing of GRSF1 resulted in dysregulation of mitochondrial transcription, aberrant loading of mRNAs and lncRNAs onto mitochondrial ribosomes, and compromised mitochondrial ribosome biogenesis. We have further characterized the mitochondrial RNA granule proteome, and find that it contains a large toolbox of proteins dedicated to RNA metabolism including proteins involved in transcript processing, poly(A) addition, rRNA and tRNA modification, mRNA degradation, and ribosome biogenesis. I will discuss recent developments in this area and the relevance to mechanisms of mitochondrial disease.

A1-02 The etiology of age-dependent disease: A story of two genomes

McManus Meagan J[1], Wen Chen H[1], Picard M[1], Potluri P[1], Morrow R[1], Angelin A[1], Narula J[2] and Wallace DC[1]

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If decreased mitochondrial vitality drives aging, then the hereditary anomalies of age-related disease may be explained by the complex interaction between the Mendelian and non-Mendelian mitochondrial genes, which together determine mitochondrial function. To investigate this hypothesis, we analyzed the relative importance of mtDNA and nDNA mutations in heart disease, the number one cause of mortality in the U.S. We first examined a 13-generation Mennonite pedigree with autosomal recessive cardiomyopathy due a mutation in the mitochondrial adenine nucleotide translocator-1 (ANT1). Substantial variability in the progression of heart disease segregated with maternal lineage, and the severity of cardiomyopathy correlated with the mtDNA haplogroups (Strauss, et al 2013). To determine the causative nature of this correlation, we examined the influence of

inherited mtDNA mutations on ANT1-cardiomyopathy in the mouse. We introduced homoplasmic mtDNA ND6 or COI missense mutations into the mouse female germ line, generating mice with complex I or IV deficiency, respectively, and analyzed Ant1-dependent cardiomyopathy on the different mtDNA backgrounds. On wt mtDNA background, the Ant1-/- mice developed a distinctive concentric dilated cardiomyopathy, characterized by substantial myocardial hypertrophy, ventricular dilation and shortened lifespan. Loss of ANT1 impaired F_oF_1ATPase assembly and prevented dimerization, leading to "kinky" cristae architecture. The mtDNA ND6 mutation accelerated Ant1-/- age-dependent cardiomyopathy, as evidenced by increased ultrastructrural abnormalities, bioenergetic defects, sensitized mitochondrial permeability transition, increased mtDNA damage, and heart failure that ultimately attenuated the lifespan. Our results are the first to prove the cause-and-effect relationship between mtDNA variation and the penetrance of age-related disease and mortality in mammals.

Sněžka (1,603 m), Giant Mountains National Park

A1-03 Unraveling the causes of the clinical heterogeneity of coenzyme Q10 deficiency due to different molecular defects in *Coq9* gene

Luna-Sánchez M[1,2], Díaz-Casado E[1,2], Barca E[3], Hidalgo-Gutiérrez A[1,2], Barriocanal-Casado E[1,2], Quinzii CM[3], López Luis C[1,2]

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Primary coenzyme Q10 (CoQ10) deficiency is due to mutations in genes involved in CoQ biosynthesis. The disease has been associated with five major phenotypes, but a genotype-phenotype correlation is unclear [1]. Here, we compare two mouse models with a genetic modification in *Coq9* gene (*Coq9*^{Q95X} and *Coq9*^{R239X}). The comparison was done by biochemical, molecular, genetics, histopathological and phenotypic analyses. *Coq9*^{R239X} mice manifest severe widespread CoQ deficiency associated with fatal encephalomyopathy [2]. In contrast, *Coq9*^{Q95X} mice exhibit mild CoQ deficiency manifesting with reduction in CI+III activity and mitochondrial respiration in skeletal muscle, and late-onset mild mitochondrial myopathy. We show that these differences are due to the levels of COQ biosynthetic proteins, suggesting that the presence of a truncated version of COQ9 protein in *Coq9*^{R239X} mice destabilizes the CoQ multiprotein complex [3]. Our study points out the importance of the multiprotein complex for CoQ biosynthesis in mammals, which may provide new insights to understand the genotype-phenotype heterogeneity associated with human CoQ deficiency and may have a potential impact on the treatment of this mitochondrial disorder.

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A1-04 Nuclear adaptation to mitochondrial dysfunction

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Mitochondrial DNA is a 16.6 kb double-stranded circular DNA molecule which can be found in various copy numbers in a tissue specific manner. mtDNA encodes for 37 genes which only 13 of them are polypeptides, all having functions in oxidative phosphorylation. The rest of the proteins which have roles in mitochondria are encoded by nuclear genome. Therefore, controlled expression of both genomes (nuclear and mitochondrial) is a very

important process for the well-being of the cell. Furthermore, mitochondria to nucleus cross-talk also known as retrograde response is conserved between species and can interact with most of the intracellular signaling pathways and processes [1].

Interaction between the two genomes of the cell is currently studied by depletion of mtDNA and one of the widely used models for studying effects of mtDNA depletion is rho-zero cells. Mammalian derived Rho^0 cells were firstly characterized in 1989 by G. Attardi [2]. We have decided to use mtDNA depleted cells in order to study details of nuclear involvement in mitochondrial dysfunction. Here, we use RNA Sequencing strategy to compare nuclear gene expression induced by the complete depletion of mtDNA in cell lines of different origin.

Rho^0 cells completely lack mtDNA, therefore they can not form respiratory chain complexes. As a result, these cells cannot synthesize ATP by OXPHOS and they are dependent on glycolysis. However, there are more consequences of complete depletion of mtDNA. For example, mitochondria of Rho^0 cells are mostly fragmented compared to tubular/filamentous mitochondria observed in wild-type cells. Several microarray studies also showed that cellular transcriptome is changed upon mtDNA depletion. However, there is not a complete consensus on the current published results of other groups. This suggests that the details of retrograde response are complex and still waiting to be uncovered [3]. We find common Rho^0 transcriptomic signatures as well as particular modifications associated with the cellular origin.

We are currently performing detailed functional and bioinformatic analysis dissecting basis of common and cell specific responses. We strongly believe the outcome of this study can propose novel ways to treat mitochondrial diseases.

The support of "Mitochondrial European Educational Training, MEET" project of the European Commission's Seventh Framework Programme, FP7-PEOPLE-2012-ITN MARIE CURIE, grant agreement No. 317433 is gratefully acknowledged.

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MiP*art*

A1-05 Impairment of reactive oxygen species defense system affects oxidative phosphorylation and causes early-onset neurodegeneration

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The umbrella term reactive oxygen species (ROS) comprises a wide array of partially reduced forms of oxygen, which are common by-products of cellular metabolism. Most intracellular ROS are derived from mitochondrial superoxide, which results from the monoelectronic reduction of oxygen. Superoxide is efficiently dismutated to hydrogen peroxide via superoxide dismutase, consequently making the mitochondria a major site for H_2O_2 generation. In this context, tight regulation of mitochondrial H_2O_2 levels is critical for their ability to participate in physiological cell signaling and to avoid nonspecific oxidative damage. Therefore, an efficient enzymatic machinery to buffer H_2O_2 levels has developed within the mitochondrial matrix. Key proteins involved in these processes are members of the thioredoxin (TXN) and the glutathione (GSH) systems.
In this study, we describe a 16-year-old adolescent suffering from early-onset neurodegeneration and severe cerebellar atrophy associated with a homozygous stop mutation in *TXN2.* This mutation increases reactive oxygen species levels, impaires oxidative stress defense and leads to secondary mitochondrial dysfunction with reduced cellular respiration and diminished ATP production. Animal studies suggest that TXN2 is essential during embryonic development. Supplementations with antioxidants effectively suppressed cellular ROS production, and lead to moderate clinical improvement during short term follow-up of the patient.

MiP*art*

A1-06 Functional ablation of Tmem70 alters biogenesis of ATP synthase and leads to embryonal lethality in mice

Kovalčíková J[1], Kaplanová Vilma[1],Vrbacký M[1], Nůsková H[1], Chawengsaksophak K[2], Beck I[2], Sedláček R[2], Hozák P[2], Sedmera D[3] and Houštěk J[1]

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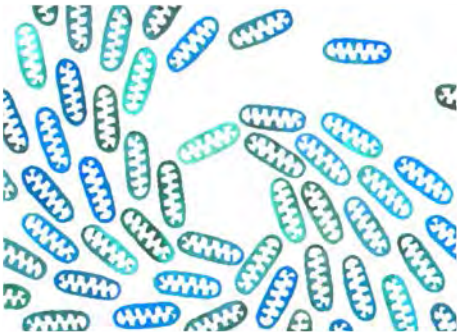
TMEM70 is a transmembrane protein localized in the inner mitochondrial membrane and involved in the biogenesis of the eukaryotic ATP synthase. TMEM70 mutations cause isolated deficiency of ATP synthase often resulting in a fatal neonatal mitochondrial encephalocardiomyopathy, lactic acidosis and 3-methylglutaconic aciduria in patients.
To clarify the exact function of this factor, we generated Tmem70 knockout mice by embryonic stem cell technology. While the heterozygous mice were viable and developmentally normal, the homozygous embryos were distinctly growth retarded and died during the embryonic development about 9.5 days post coitum. Confocal microscopy revealed delayed development of the cardiovascular system and electron microscopy indicated disturbed mitochondrial morphology in the homozygous when compared to the wild type embryos. Blue native electrophoresis demonstrated isolated defect of ATP synthase in the homozygous embryos with the content of fully assembled F1Fo ATP synthase decreased to less than 20% of wild types. In contrast, comparison of the viable heterozygous and wild type mice aged 5 and 14 weeks did not show any significant differences in the heart and liver content of respiratory chain complexes, oxygen consumption, ATP synthase assembly and ATPase hydrolytic activity. On the other hand, we observed decreased fractional shortening, the parameter of the heart function, in heterozygous compared to wild type mice.
In conclusion, this first direct demonstration of the biological role of TMEM70 in experimental animals shows that Tmem70 deficiency in the mouse has lethal consequences that are analogous to TMEM70 dysfunction in humans.

Supported by the Grant Agency of the Czech Republic (P303/11/0970, 14-36804G) and Grant Agency of the Charles University (726214).



MiP*art*

A1-07 Altered mitochondrial ultrastructure, reduced respiration and decreased level of PDH subunits in fibroblasts from 10 patients with Huntington's disease

Rodinová Marie[1], Kratochvílová H[1], Marková M[1], Spáčilová J[1], Tesařová M[1], Klempíř J[2], Lišková I[2,3], Elederová L[2,3], Juhasová J[3], Motlík J[3], Zeman J[1] and Hansíková H[1]

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Huntington's disease (HD) is an autosomal dominant neurodegenerative disease caused due to expansion of the number of CAG repeats on the gene for huntingtin protein (htt). More than 36 CAG repeats leads to pathological extension of glutamine tract of htt which is connected with changes of secondary structure and malfunction of the htt. Mutant htt has been implicated in the disruption of multiple cellular processes, including mitochondrial functions whose impairment is emerging as a contributing factor to the pathogenesis of HD.

The aim of the study was to analyze the impact of HD on selected bioenergetics' functions in cultivated skin fibroblasts of 10 patients with confirmed HD. All patients were heterozygotes in age between 31 and 74 years and the number of CAG triplet repeats on the mutated allele ranged between 40 and 58. All fibroblasts were obtained after informed consents. Fibroblasts obtained from 48 month old minipig boars transgenic for the N-terminal part of human mutated htt (TgHD) and WT controls were analyzed in parallel manner.

Mitochondrial ultrastructure, network and reactive oxygen species were visualized using fluorescent and transmission electron microscopy. Protein analysis of selected mitochondrial proteins was detected by immunoelectrophoretic methods. Functional disturbances were tested using high sensitive polarography.

Pathological changes in mitochondrial ultrastructure, like decreased number of cristae, swollen mitochondria or increased mitochondrial degradation were detected in all patient´s fibroblast lines in comparison to controls. Mitochondrial network was disintegrated and unequally distributed in patient´s cells. Ultrastructural changes of mitochondria and increased content of reactive oxygen species were found in TgHD minipig's fibroblasts in comparison with WT. Protein analysis showed decreased level of pyruvate dehydrogenase complex (PDH) subunits E1-α, and mitochondrial complex I subunit NDUFA9 was detected in 9 patients out of 10.

Our results confirm mitochondrial disturbances in non-neuronal tissue like cultivated skin fibroblasts of HD patients and TgHD minipig model which are similar to phenotypes published in HD human neuronal cells or muscles.

Supported by: Czech-Norwegian Research Programme 7F14308, RVO-VFN64165

A1-08 Mitochondrial respiration in homogenized biopsies from kidney, liver, heart and diaphragm from mice after blunt thoracic trauma and haemorrhagic shock - effects of a novel hydrogen sulfide donor AP39

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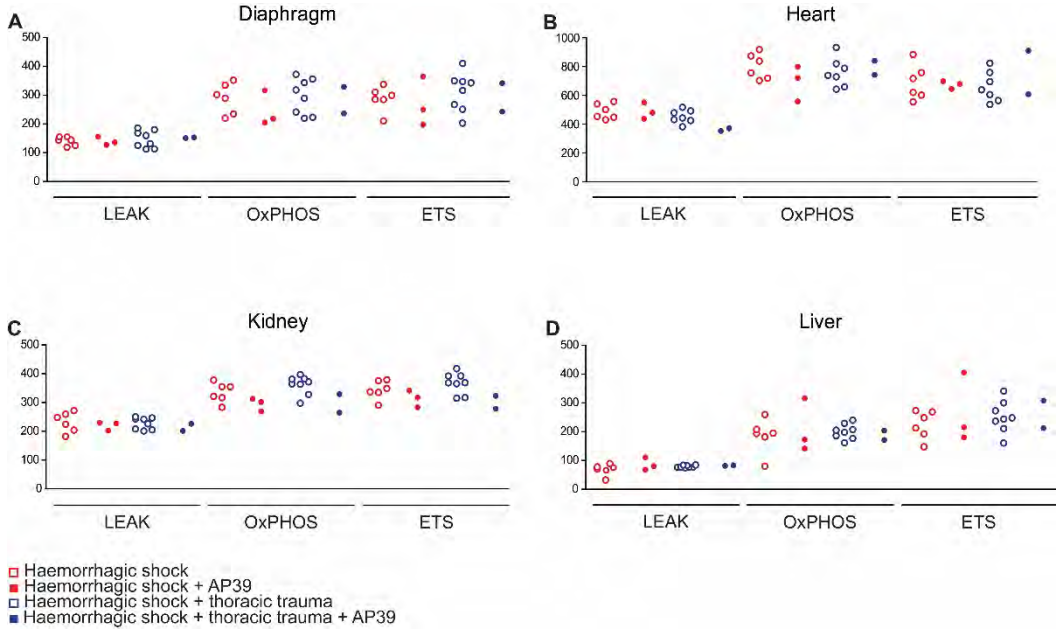
Background: Haemorrhagic shock impairs the perfusion and subsequently the oxygenation of crucial organs as for heart, kidney and liver. The combination of haemorrhagic shock after thoracic trauma increases this effect. Here we tested whether the mitochondrial respiration is impaired by these conditions and whether the treatment with a novel synthesised mitochondrially-targeted hydrogen sulfide (H_2S) donor, AP39, has beneficial effects on mitochondrial respiration. In fact, AP39 has previously been shown to increase mitochondrial activity, however this data has only been obtained in vitro so far [1].

This project illustrates preliminary data in terms of mitochondrial respiration in organs suffering from haemorrhagic shock combined with thoracic trauma and will further reveal the impact of AP39 in this setting.

Methods: Mitochondrial function was measured in small homogenised samples from diaphragm, heart, kidney and liver from 3-6 mice and measured in terms of LEAK-, OxPHOS- and ETS-capacity by using an O2K-Oxygraph (Oroborus Instruments, Austria).



Distinct organ responses following severe haemorrhagic shock or in combination with thoracic trauma. The mitochondrial respiration of crucially affected organs during haemorrhagic shock (diaphragm (A), heart (B), kidney (C) and liver (D)) is variously affected by either single haemorrhagic shock or in combination with thoracic trauma. Notably, the incubation with a novel synthesised mitochondrially-targeted hydrogen sulfide (H_2S) donor, AP39, leads to a decrease of mitochondrial respiration suggesting beneficial effects. Abbreviations: LEAK: Respiration in the absence of ADP due to uncoupled respiration but under maintained oxygen flux; OxPHOS capacity: maximal oxidative phosphorylation activity; ETS capacity: mitochondrial electron transfer system.

Respiratory activity of the samples was simultaneously supported by complex I and II substrates (Malate, Glutamate, Pyruvat and Succinate), as well as by ADP and quantified in terms of oxygen flux [JO2] per wet weight with the unit of pmol O2/ (mg*s). OxPHOS-capacity was obtained as the maximum activity under all substrates and ADP, LEAK-capacity by inhibition of the ATP-synthase obtained by further injection of oligomycine and finally, ETS-capacity by addition of the mitochondrial chain uncoupler FCCP.
Results: Here we present preliminary data with almost descriptive statistical analyses. We can determine minor changes in mitochondrial function upon haemorrhagic shock and in combination with thoracic trauma. However, upon the addition of AP39 the mitochondrial activity seems to decrease, especially in the tissue of liver and heart.
Conclusion: Our test provides reliable data on mitochondrial respiration in various tissues thus allowing an overview of global effects in the whole organism. In fact, our data suggest that organs respond differently to severe haemorrhagic shock and the combination with thoracic trauma, as well to the treatment with AP39. In contrast to previous data, we did not observe an increase of mitochondrial activity under AP39 instead mitochondrial activation tends to decrease after treatment with the sulfide donor. The physiological meaning of this effect required further investigation.

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A1-09 Cellular and animal models for the study of mitochondrial dysfunctions in neurodegeneration with brain iron accumulation

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"Neurodegeneration with brain iron accumulation" (NBIA) comprises a group of progressive neurodegenerative disorders characterized by high content of iron in the brain. Mutations in *PANK2* gene, which encodes for the mitochondrial protein pantothenate kinase type 2, determine an autosomal recessive inborn error of CoA metabolism, called pantothenate kinase-associated neurodegeneration (PKAN). The pathogenesis of PKAN, the most frequent form of NBIA, is still poorly understood. [1]
In our study, we are exploring a *Pank2*-KO mice model, which showed altered mitochondria membrane potential in neurons and defective respiration in the brain. Moreover, we have demonstrated that ketogenic diet, which stimulates lipid utilization by mitochondrial beta-oxidation, was able to reveal a clinical phenotype not present in *Pank2*-KO mice under standard diet [2]. These mitochondrial bioenergetics failure due to the absence of PANK2 protein may result from defects in mitochondrial membrane integrity and consequently in supercomplexes stabilization. Our first results showed a deficiency in complex IV activity in supercomplexes in the brain from *Pank2*-KO mice. In fact, PANK2 by synthesizing CoA required for membrane phospholipids remodeling and repair, indirectly contributes to the synthesis of cardiolipin implicated in supercomplexes stabilization. Thus, phospholipids metabolism could be an interesting target to better explore membrane homeostasis *in vivo*.

In parallel, we are conducting lipidomic analysis on NBIA patients fibroblasts and on PKAN patients red blood cells (RBC). The fibroblasts are an interesting tool to explore lipid metabolism in these diseases. Moreover, the complexity of the blood lipids profile establishes it as a rich source of molecules that can provide clues about human physiology and disease. Our first results showed a difference in fatty acids lipogenesis in fibroblasts and in phospholipids distribution in RBC membranes, mainly a decrease in phosphatidylcholine and sphingomyelin. Thus, lipidomic analysis in NBIA patients' fibroblasts and RBC could provide a powerful biomarker in clinical medicine to understanding lipid biology in NBIA pathogenesis and monitoring therapeutic intervention.

The support of "Mitochondrial European Educational Training, MEET" project of the European Commission's Seventh Framework Programme, FP7-PEOPLE-2012-ITN MARIE CURIE, grant agreement No. 317433 is gratefully acknowledged.

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A1-10 Yeast as a system for modeling mitochondrial disease mechanisms and therapies

Bińko Krystyna [1], Kabala A[1], Niedzwiecka K[1], Dautant A[2], di Rago JP[2] and Kucharczyk R[1]

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Mitochondria, besides the key role in bioenergetics, carry out a lot of functions essential for cell viability, thus impairment of any of them can result in a wide spectrum of severe abnormalities in humans known as mitochondrial diseases. The diagnosis is difficult due to multiplicity of clinical manifestation depending on involved function and affected tissues. Additionally it is complicated by heteroplasmy of mitochondrial DNA (mtDNA) in human cells. The yeast *S. cerevisiae* is the organism of choice to uncover cellular and molecular mechanisms underlying the mitochondrial diseases. The most important is the capability to use fermentable carbon substrates as energy source, resulting in ability to survive even when mtDNA has been completely depleted. What more, site-direct mutagenesis of yeast mtDNA is possible by biolistic transformation and the population of mutated mtDNA will be 100% homoplasmic.
ATP synthase is multi-subunit enzyme located in inner mitochondrial membrane. The enzyme uses the energy provided by the proton electrochemical gradient as a force to drive ATP synthesis. Point mutations in *ATP6* gene were identified in patients suffering the neurological defects.
The mitochondrially encoded Atp6 subunit of ATP synthase is evolutionary conserved, therefore it is possible to create yeast models of human diseases bearing the particular pathogenic mutations for analysis of their consequences. Here we present the results of systematic investigation on cellular effects of 9 pathogenic mutations introduced to *ATP6* gene of *S. cerevisiae* leading in human to Neurogenic Ataxia and Retinitis Pigmentosa

(NARP), Leigh syndrome (LS), Charcot-Marie-Tooth (CMT), NARP or Familial Bilateral Striatal Necrosis (FBSN) syndromes.
Importantly, chemical screens of drugs using yeast have pointed to potential therapeutic targets. Through selection of intragenic revertants in respiratory deficient mutants of *ATP6* gene, the identification of amino acids important for the mechanism of proton transport was possible. Thus from study of the pathogenic mutations yeast has brought us to the fundamental mechanism of the enzyme function.

A1-11 Quantitative live-cell imaging of mitochondrial network morphology in neurodegenerative conditions

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Mitochondrial dysfunction is a well-established hallmark of aging and neurodegenerative diseases. Maintenance of mitochondrial dynamics is essential for mitochondrial health maintenance and disturbances in mitochondrial dynamics have been implicated in a number of neurodegenerative processes. Moreover, patients with mutations in mitochondrial proteins involved in mitochondrial fusion, namely, *Mfn2* and *OPA1* genes have been associated with Charcot-Marie-Tooth disease type 2A, hereditary motor and sensory neuropathy VI, and autosomal optic atrophy (ADOA), respectively. In addition to its major role in mitochondrial fusion, OPA1 is an inner mitochondrial membrane protein which is involved in apoptosis, cristae structure, mtDNA replication maintenance and mitochondrial potential, all potential hallmarks of neurodegenerative conditions [1].
To study the role of mitochondrial fusion in neurodegenerative processes, we have adapted qualitative methods based on subjective classification of organelle morphology into defined categories to a quantitative protocol which uses mitotracker staining of the mitochondrial network followed by live-cell confocal imaging combined with Huygens Essential for deconvolution and image analysis. The output allows a quantitative assessment of mitochondrial length and volume in different physiological and disease conditions [2, 3].
Using this technique, we have successfully imaged the mitochondrial network with a high degree of cell-to-cell reproducibility in both control fibroblasts and patient-derived primary fibroblasts with carrying pathogenic *OPA1* mutations. *OPA1*-mutant fibroblasts showed clear morphological changes when compared with control fibroblasts under both basal and mitochondrial OXPHOS stress conditions.
Our study demonstrates the advantages of in-depth quantitative analysis of mitochondrial network morphology by using a reproducible protocol that is applicable to a wide range of neurodegenerative diseases.

Supported by the Medical Research Council (MRC, UK)

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A1-12 Mitochondrial dysfunction in Niemann Pick type C1 patient´s cells and tissues

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The cholesterol levels in mitochondria are approximately 40-fold lower than in the plasma membrane and 4.5-fold lower than in the endoplasmic reticulum (ER). Therefore mitochondria are sensitive to changes in absolute cholesterol content. Mitochondria require cholesterol for biogenesis and membrane maintenance as well as steroid biosynthesis.
Niemann-Pick type C1 disease is an autosomal recessive neurodegenerative disorder caused by loss-of-function mutations in *NPC1* gene. Mutation in *NPC1* leads to endosomal cholesterol accumulation and defects in cellular cholesterol homeostasis. Mitochondrial cholesterol increase was observed in Niemann-Pick type C1-deficient cells, which affects some mitochondrial function.
The aim of our project was to study impact of altered distribution of cellular cholesterol due to *NPC1* mutation on oxidative phosphorylation complexes and mitochondrial ultrastructure in available cells (fibroblasts) and tissues (brain, liver) from two NPC1 patients.
Filipin test confirmed impaired cholesterol distribution in cultivated NPC1 fibroblast. Moreover altered mitochondrial network and ultrastructure was observed in fibroblast cell lines compared to the control. In brain mitochondria, pronounced deficiency in native amount of complex V and complex III was found in both patient samples. While reduced level of ATP synthase was observed in liver mitochondria only in P2. However levels of complex I, III and IV were increased in all analyzed liver samples. Enzymatic activities of respiratory chain complexes were decreased in liver as well as in brain NPC1 mitochondria. Impaired cholesterol distribution in NPC1 tissues and cells influences steady state levels and function of all OXPHOS complexes.

The study was supported by research project RVO-VFN64165, IGA NT 13114-4, UNCE 204011 and GAUK 1308214.

A1-13 Cofactor deficiency in mitochondrial diseases

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The mitochondrial energy metabolism consists of numerous enzymatic reactions and transport processes. Several of these mitochondrial reactions depend on cofactors. Cofactors are small molecules that can be associated or covalently bound to enzymes. Cofactors are either synthesized de novo or from precursors, several of these precursors are vitamins. Furthermore, cofactors or their precursors have to be transported into appropriate compartments in the cells (e.g. mitochondria). To date, 39 different genes

in the synthesis and transport of cofactors have been reported that manifest clinically as disorders in the mitochondrial energy metabolism. Therefore, the analysis of cofactors and cofactor dependent enzymes is crucial to gain a better understanding of the pathomechanism of these diseases and set the basis for therapeutic interventions.
Thiamin pyrophosphate (TPP) is an essential cofactor for mitochondrial enzyme complexes like pyruvate dehydrogenase and 2-ketoglutarate dehydrogenase and requires thiamin pyrophosphokinase (TPK, EC 2.7.6.2) for its formation [1]. Here we report on two patients with TPK1 mutations providing novel clinical and biological insights into the condition. Two novel homozygous mutations were found c.664G>C (p.Asp222His) leading to decreased TPK1 protein stability, but a high residual enzymatic activity and c.479C>T (p.Ser160Leu) that interferes with TPK dimerization, leading to drastically decreased enzymatic activity [2]. Recombinant mutant or wild type TPK was investigated concerning substrate and Mg^{2+} concentrations. A clear dependence of TPK activity on thiamine and Mg^{2+} was found in both mutant and the wild type TPK. These results hold promise for the clinical use of vitamins/cofactors as pharmacological chaperones in TPK deficient patients harboring thiamine/Mg^{2+} responsive mutations.
The interaction between riboflavin metabolism and the mitochondrial respiratory chain is reported in a large number of human diseases. Our research currently focuses on an important enzyme flavin adenine dinucleotide synthetase (FLAD1, EC 2.7.7.2), involved in intracellular metabolism of riboflavin. FLAD1 catalyzes the adenylation of flavin mononucleotide (FMN) to form flavin adenine dinucleotide (FAD), which is an essential cofactor of e.g. pyruvate dehydrogenase, succinate dehydrogenase, electron transferring flavoprotein dehydrogenase (ETFDH) and many different ETFDH dependent dehydrogenases involved in fatty acid oxidation and branched chain- amino acid metabolism [3]. During these ongoing investigations, we measured the concentrations of riboflavin, FMN, and FAD in different tissues and the distribution in subcellular compartments in patient and control samples. Further functional studies are on the way to elucidate the disease mechanism. Our results point to the importance of exogenous supplementation with cofactor (or vitamin), which can compensate for deficiencies in cofactor biosynthesis/availability.

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A1-14 Novel complex I assembly factor mutation leads to adult phenotype

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Complex I (CI) deficiency is the most common enzymatic defect of the oxidative phosphorylation system in childhood (OMIM 252010) and can cause a wide range of clinical phenotypes [1]. It is often caused by an impaired assembly, a process which is still poorly understood. The enzyme is composed of 44 different subunits and its biogenesis requires chaperones or assembly factors which years were shown to be vital proteins for the process.
Whole exome screening of CI deficient patients led us to a patient with a mutated TMEM126B, a recently discovered assembly factor of CI [2]. By lentiviral complementation we could establish that this protein is the cause of the severe CI reduction, in activity as well as its total amount, likely leading to the patient phenotype. Moreover, two dimensional blue native electrophoresis demonstrated that the mutation leads to an impairment of CI assembly at a specific stage of the assembly process.
These new data allow us to better interpret CI assembly defects, and also to better correlate clinical data with biochemical data providing us a better rationale for possible therapeutic approaches.

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A1-15 Mitochondrial respiration in sequential biospies from skeletal muscles during hemorrhagic shock and over the course of 48h of reperfusion in pigs

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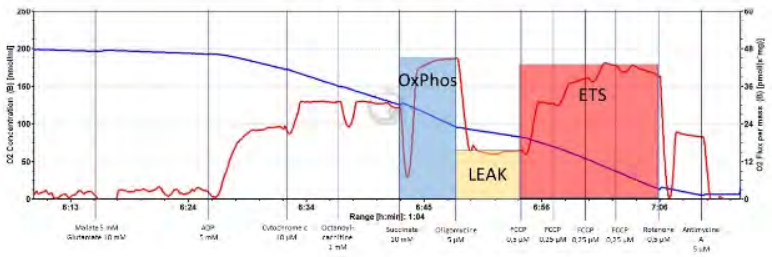
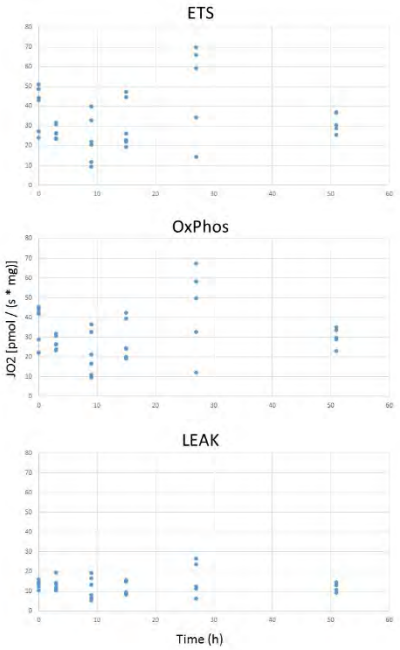
Introduction: Previous data [1] have shown that mitochondrial respiration is severely affected during hemorrhagic shock, but rapidly recovers during reperfusion. Therefore, in our actual experiment we performed sequential measurements of mitochondrial respiratory activity during hemorrhagic shock and for up to 48 hours during reperfusion in skeletal muscles of the pig.
Methods: After approval by the animal ethical committee of our institution we induced hemorrhagic shock in 6 anesthetized, mechanically ventilated pigs (removal of 30% of the blood volume and subsequent blood removal/retransfusion to maintain mean arterial pressure at 40 mm Hg) for 3 hours. Then the shed blood was retransfused and the animal remained under anesthesia and mechanical ventilation for an observation period lasting for 48 hours. Skeletal muscle biopsies were taken before shock, during shock, and after 6, 12,

24, and 48 hours of recovery. The tissue samples were homogenized and put into the chambers of the O2K®-Oxygraph (Oroboros Instruments, Austria) and continuously stirred at 37°C. Mitochondrial respiration was quantified by adding complex I (10 mM Pyruvate, 5 mM Malate, and 10 mM Glutamate) and complex II (10 mM Succinate) substrates and 5 mM ADP. Then 5 µM oligomycine was added to inhibit the ATP-synthase in order to obtain the LEAK-respiration state as an indicator of mitochondrial coupling. This step was followed by the addition of 1 µM of the uncoupler FCCP in order to achive the maximum respiration in the uncoupled state and the coupling (LEAK/ETS)-ratio.

Results: A typical oxygraph tracing as well as the preliminary results are presented in the figures below.





Conclusions: The overall response to hemorrhagic shock and reperfusion seems rather moderate; However, refining the targets of hemorrhagic shock, and, consequently, peripheral perfusion might still reveal a more clear picture of the effects of shock on mitochondrial respiration.

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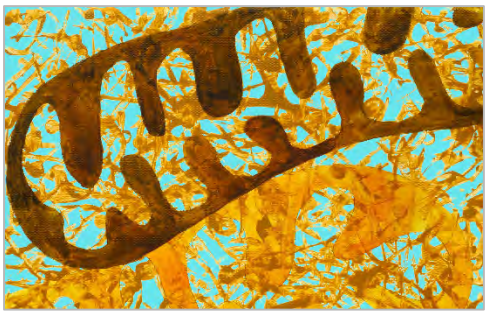

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A1-16 Myocardial iron and mitochondrial function in failing and non-failing human heart: direct tissue analysis

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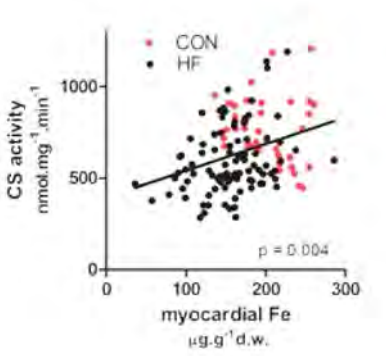
Introduction: Very little is known about the determinants and consequences of myocardial iron (Fe) level in normal or failing human myocardium. We hypothesized that myocardial Fe deficiency (ID) in heart failure (HF) is associated with impaired mitochondrial function.
Methods: LV samples were obtained from 91 consecutive patients undergoing transplantation (HF: LVEF 23±8%, age 53±11y, 83% males, 46% CAD, 24 % diabetes) and from 38 HF-free organ donors (CON: LVEF 57±12%, age 42±15y, 50% males, 14% with diabetes). Abundance of respiratory chain complexes I-V, ROS-protective enzymes and activities of citrate synthase (CS, Krebs cycle) and RC enzymes NADH-cytochrome *c* oxidoreductase (NCCR), succinate cytochrome *c* oxidoreductase (SCCR) and cytochrome *c* oxidase (COX) and tissue respiration (O_2 consumption) were measured in homogenates. Total Fe was measured by inductively-coupled mass spectrometry in lyophilised samples.
Results: Compared to CON, HF patients had reduced total myocardial Fe (156±41 vs 200±38 μg/g dry weight, p<0.001). Myocardial ID (Fe<124.8 μg/g; 2*SD from the mean of CON) was present in 22 % of HF patients. HF+ID patients had more extensive coronary artery disease, less often betablockers and longer duration of HF, but similar age, gender, renal function, haemoglobin concentration, LVEF or BNP as non-ID HF. Respiratory chain complex I-III, activities of CS, COX, SCCR and NCCR and myocardial tissue respiration were all reduced in HF vs. CON (by 21% - 34%, all p<0.001). In HF, Fe correlated with CS (r=0.30, p=0.004) and SCCR activities (r=0.24, p=0.02), but not with COX or NCCR. Iron-deficient HF patients displayed reduced CS activity (516±145 vs 613±19, p=0.03), reduced abundance of respiratory chain complex III, reduced catalase and glutathione peroxidase.
Conclusions: Myocardial Fe content is systematically reduced in advanced HF and is associated with mitochondrial dysfunction, in particular with diminished CS activity and reduced catalase. These relations may lead to reduced substrate flexibility, decreased energetic production and diminished ROS-defense in iron-deficient failing myocardium.



Acknowledgement: Research relating to this abstract was funded by Grant Agency of the Ministry of Health of the Czech Republic (NT14050-3/2013).

A1-17 Derangements of myocardial mitochondrial function in patients with end-stage heart failure is associated with reduced endonuclease G

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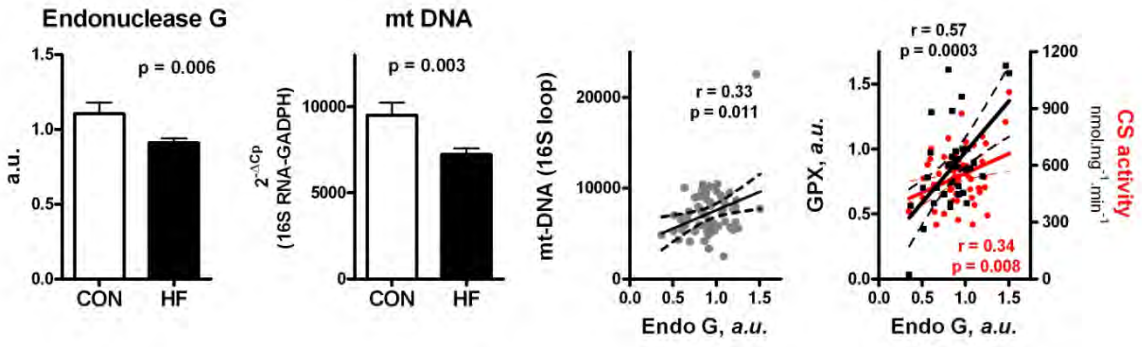
Background: The extent and mechanisms of myocardial mitochondrial dysfunction in patients with heart failure (HF) are poorly understood. Mitochondrial endonuclease G (EndoG) is nuclear-encoded, mitochondria-localised nuclease, experimentally implicated in regulation of apoptosis, mtDNA function, ROS production and cardiac mass, but its relevance to human HF has never been addressed.
Hypothesis: Mitochondrial function is reduced in HF and paralleled by reduction in EndoG activity.
Methods: LV free-wall samples was obtained from 62 consecutive patients undergoing heart transplantation (HF group; LV EF 23±9%, age 51±12y, 80% males, HF aetiology: 41% CAD, 59% non-CAD, 31% diabetes) and from 20 HF-free organ donors (CON group; LVEF 55±12%, age 41±15y, 55% males, 12% with diabetes).
Results: Compared to CON, HF patients had reduced mtDNA content (16S RNA/nDNA-GADPH ratio; -24%, p<0.001). HF patients displayed reduced activity of citrate synthase (CS: -26%, p<0.001) and of oxidative phosphorylation enzymes NADH-cytochrome-*c* oxidoreductase (NCCR: -24%, p<0.01), succinate-cytochrome-*c* oxidoreductase (SCCR: -40%, p<0.001) and cytocrome-*c* oxidase (COX: -38%, p<0.001). High-resolution oxygraphy confirmed a reduction in COX respiration (-21%, p=0.01) and succinate-supported respiration (-25%, p=0.01) in HF. SDS-PAGE western blot showed similar porin (mitochondrial protein abundance), but decreased components of respiratory chain complex I (NDUFA9: -15%), II (SDH70: -17%), III (core2: -20%) and V (F1α: -14%) paralleled by reduction in antioxidant enzymes glutathion-reductase and –peroxidase (GR: -24%, GPX: -20%, all p<0.01). Endonuclease G was downregulated in HF (-17.6%, p=0.01) and correlated with mtDNA (r=0.33, p=0.01, Fig), complex I-III and V abundances (r=0.33-0.42, p<0.01), CS (r=0.34, p=0.008) and strongly with GR and GPX (both r=0.6, p≤0.005, Figure). HF aetiology (ischemic/non-ischemic), presence/absence of diabetes mellitus or age of HF patients had no consistent effect on these alterations.



Conclusions: In the large group of advanced HF patients, we demonstrated systemic reduction of myocardial mtDNA content, lower abundance of mitochondrial respiratory

chain components, lower oxidative phosphorylation activity and reduced mitochondrial respiration. Reduced myocardial endonuclease G may contribute to mitochondrial dysfunction in human HF.

Acknowledgement: Research relating to this abstract was funded by Grant Agency of the Ministry of Health of the Czech Republic (NT14050-3/2013).

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Section A2: Mitochondrial involvement in cancer

A2-01 The energy requirement of metastatic cells

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Many tumor cells show enhanced aerobic glycolysis, even in the presence of oxygen: The so called Warburg effect. This pathway provides substrates for the synthesis of lipids, proteins and DNA. However, the Warburg effect does not necessarily imply mitochondrial dysfunction. Research currently pictures tumors as compositions of different populations of cells with distinct metabolic phenotypes, which are able to adjust to oxygen and nutrient gradients within the tumor mass. Not all cancer cells display a high glycolytic flux as proposed by Warburg. Our results indicate that progression to metastasis requires mitochondrial function. Our research, centered on cell lines that display increasing degrees of malignancy, focuses on metabolic events, especially those involving mitochondria, which could reveal which stages are mechanistically associated to metastasis. The experimental model consisted of murine melanocytes. These cells were subjected to several cycles of adhesion impediment, producing stable cell lines exhibiting phenotypes representing a progression from non-tumorigenic to metastatic cells. These were: non-tumorigenic cells melana (ma), non-tumorigenic cell line 4C (obtained after four cycles of adherence abrogation), non-metastatic 4C11- and metastatic 4C11+ melanoma cell lines [1]. The metabolic profile of each of these different cell lines was investigated by evaluating enzymatic activities and expression of members of the glycolytic and oxidative pathways. Our results show that only metastatic cell line (4C11+) released the highest amounts of lactate may derived from glutamine catabolism. Results from measurements with high-resolution respirometry (HRR) show that 4C11+ intact cells increased (2.8x) oxidative metabolism, with enhanced (2.6x) rates of oxygen consumption coupled to ATP synthesis, when compared to the other pre-malignant stages. We did not observe an increase in mitochondrial content, mitochondrial biogenesis and alterations of mitochondrial morphology. In addition, in 4C11+ cells, we observed an increase in ATP content, succinate oxidation (Complex II activity) and fatty-acid oxidation. In addition, 4C11+ cells exhibited a two fold increase in mitochondrial membrane potential ($\Delta\Psi_{mit}$). Metabolomic analysis revealed that 4C11+ cells could be grouped as a subpopulation whose profile was quite distinct from the other cells investigated here. Furthermore we were able to show that the migration of cells depended on glutaminase activity. The results presented here have centered on how the multiple metabolic inputs of tumor cells may converge to compose the so called metastatic phenotype. Keywords: mitochondria, metabolic profile, metastatic phenotype.

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A2-02 L-type calcium channels prevent mitochondrial network disruption in human cancer cells

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Recently, we reported that tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) induces an excessive fragmentation and clustering of the mitochondria in various human cancer cells, including malignant melanoma, but not in non-transformed cells [1]. TRAIL resistant tumor cells are resistant to this pro-apoptotic mitochondrial structure disruption, and dynamin-related protein 1 (Drp1)-dependent mitochondrial fission contributes to this resistance. Here we identify L-type Ca^{2+} channels (LTCCs) as an important regulator of TRAIL resistance and mitochondrial structure remodeling. We found that melanoma cells expressing high levels of $Ca_v1.2$ and $Ca_v1.3$ were more resistant than cells expressing low levels of $Ca_v1.2$ or $Ca_v1.3$ to spontaneous and TRAIL-induced cell death. In addition, the mitochondria within the latter, but not the former, displayed considerable structural abnormalities, characterized by excessive fragmentation and clustering. Downregulation of either $Ca_v1.2$ or $Ca_v1.3$ by RNA interference increased mitochondrial structural abnormalities and sensitized to spontaneous cell death. On the other hand, downregulation of $Ca_v1.2$, but not $Ca_v1.3$, sensitized to TRAIL-induced apoptosis via the intrinsic apoptotic pathway. Moreover, mitochondrial Ca^{2+} uptake via LTCCs regulated the Drp1-dependent pro-survival mitochondrial remodeling. Altogether, these findings provide the first evidence that LTCCs play an important role in survival, drug resistance, and mitochondrial remodeling in cancer cells.

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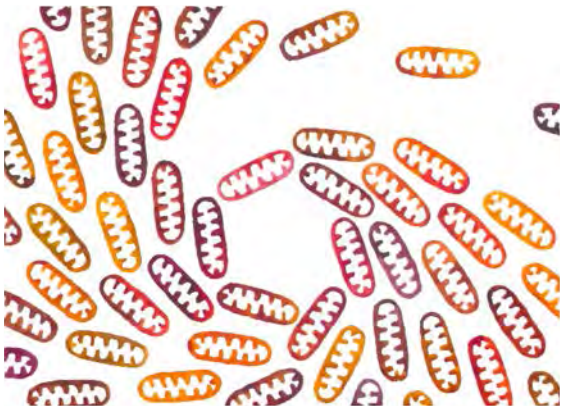

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A2-03 Quantitative characterization of respiratory parameters of human colorectal and breast cancer clinical material

Shevchuk I[1], Koit A[1], Kaldma A[1], Chekulayev V[1], Klepinin A[1], Ounpuu L[1], Timohhina N[1], Tepp K[1], Kutner R[2], Heck K[2], Valvere V [2] and Kaambre Tuuli[1,4*]

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A considerable part of previous studies about tumor bioenergetics were performed on several in vitro models with the conclusion that cancer cells present increased rates of glucose consumption and metabolize it to lactate even in the presence of O2 – a phenomenon called "Warburg effect". In vitro studies cannot give the correct information about the functional activity and significance of OXPHOS versus glycolysis in malignancies and ignore host factors, which could exert significant effects. In our study we compare respiratory parameters of two very prevalent human tumors: breast cancer (HBC) and colorectal cancer (HCC).

Primary tumor samples were provided by the Oncology and Hematology Clinic at the North Estonia Medical Centre and were analysed immediately after surgery. In this work we investigated mitochondrial respiration of tumor and control tissues *in situ* using the skinned sample technique [1, 2]. Rates of O_2 consumption were assayed at 25 °C by an Oxygraph-2k high-resolution respirometer (Oroboros Instruments, Innsbruck Austria). The solubility of oxygen at 25 °C was taken as 240 nmol/ml. All respiration rates were normalized per mg dry weight of tissue.

Multiple substrate-inhibitor titration protocol was used for the measurement of respiratory capacities of different respiratory chain (RC) segments (Fig. 1). To analyze these changes, the respiration rates for different RC complexes and ratios of respiration rates for different substrates were calculated. The HBC is not accompanied with suppression of complex I-dependent respiration as it is shown in colorectal cancer.

Apparent Michaelis-Menten constant (Km) and maximal rate of respiration (Vm) for ADP were calculated to characterize the affinity of mitochondria for exogenous ADP (permeability of mitochondrial outer membrane). Healthy colon tissue displayed low affinity for ADP (apparent Michaelis-Menten constant Km=256 ± 3μM), whereas the affinity for ADP of tumor mitochondria (Km=93.6 ± 7.7μM) and nearby tissue (junction area between cancer and normal mucosa) (Km=84.9 ± 9.9μM) is significantly higher. Average Km value for HBC tissue samples was similar - 114.8±13.6μM. Differences in Vmax correspond, to large extent, to the differences in number of mitochondria in these cell types. Measured rates of O_2 consumption (normalized to Vm) were plotted vs. ADP concentration in medium as double reciprocal Lineweaver–Burk plots (Figure 2 A,B).

This data is showing that formation of colorectal cancer is associated with relative changes in the activities of individual respiratory chain complexes which may be the result of mitochondrial DNA mutations and/or variations in the assembly of respiratory chain supercomplexes.

Two subpopulations of mitochondria in HBC (Fig 2B) confirm the theory of two-compartment metabolism ("reversed Warburg") proposed by several groups of cancer research [3, 4]. During formation of HCC colon smooth muscle can participate in the carcinogenesis like energy reservoir and mitochondria lose the diffusion restrictions in the

outer membrane. From all these results we can conclude that each type of cancer has its own special bioenergetic fingerprint.

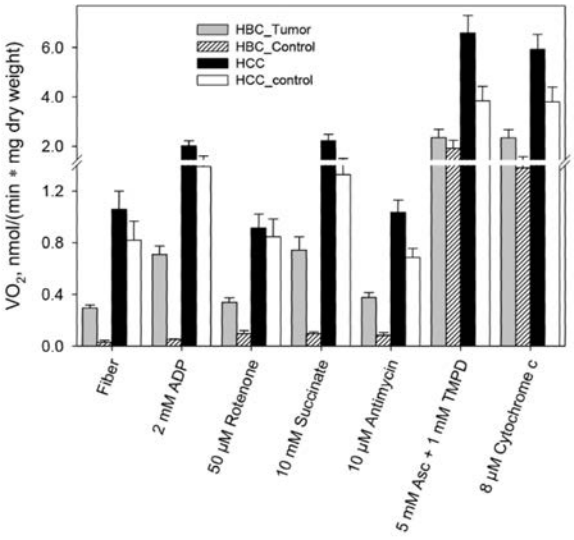

Fig. 1. Respiratory chain analysis of HBC, HCC and healthy control breast and colon tissues.

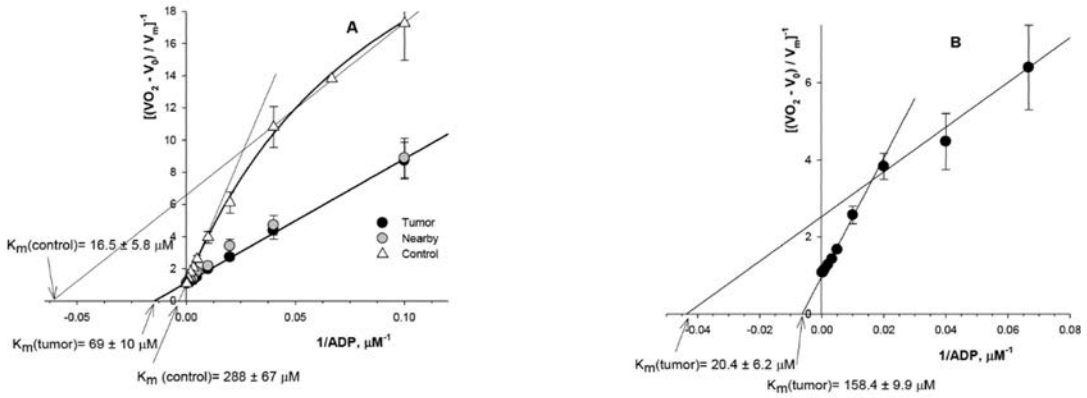

Fig. 2. Analysis of the dependence of the respiration rates on the exogenously added ADP in double reciprocal plots: HCC and two populations of mitochondria in control tissue (A) and two different populations of mitochondria in HBC (B).

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A2-04 Pharmacological inhibition of fatty-acid oxidation synergistically enhances the effect of L-asparaginase in childhood ALL cells

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L-asparaginase (ASNase), a key component in the treatment of childhood acute lymphoblastic leukemia (ALL), hydrolyzes plasma asparagine and glutamine and thereby disturbs metabolic homeostasis of leukemic cells. The efficacy of such therapeutic strategy will depend on the capacity of cancer cells to adapt to the metabolic challenge, which could relate to the activation of compensatory metabolic routes. Therefore, we studied the impact of ASNase on the main metabolic pathways in leukemic cells. Treating leukemic cells with ASNase increased fatty-acid oxidation (FAO) and cell respiration and inhibited glycolysis. FAO, together with the decrease in protein translation and pyrimidine synthesis, was positively regulated through inhibition of the RagB-mTORC1 pathway, whereas the effect on glycolysis was RagB-mTORC1 independent. As FAO has been suggested to have a pro-survival function in leukemic cells, we tested its contribution to cell survival following ASNase treatment. Pharmacological inhibition of FAO significantly increased the sensitivity of ALL cells to ASNase. Moreover, constitutive activation of the mammalian target of rapamycin pathway increased apoptosis in leukemic cells treated with ASNase, but did not increase FAO. Our study uncovers a novel therapeutic option based on the combination of ASNase and FAO inhibitors.

This work was recently published: Heřmanová I, et al., Pharmacological inhibition of fatty acid oxidation synergistically enhances the effect of L-asparaginase in childhood ALL cells. Leukemia. 2015 Aug 4. PMID: 26239197.

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A2-05 Mitochondrial targeting of tamoxifen enhances its activity against Her2^{high} breast cancer

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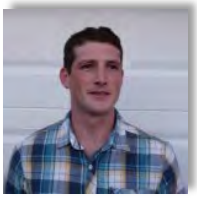
Mitochondria play a crucial role for apoptosis induction in cancer cells. Tamoxifen is an established anti-cancer agent used primarily against hormone-dependent breast cancer. Here we present its mitochondrially targeted analogue, MitoTamoxifen (MitoTAM), generated by addition of the triphenyl phosphonium (TPP^+) group to the parental compound. The mitochondrial delivery resulted in great increase of anti-cancer activity arising from extensive generation of reactive oxygen species. Importantly, in contrast to the parental compound, MitoTAM efficiently kills Her2^{high} cells and suppresses experimental Her2^{high} breast carcinomas in an animal model, such that the treatment leads to near complete disappearance of tumours. As a mechanism of this specificity, we document that Her2^{high} cells comprise high amount of the Her2 protein in mitochondria, which results in increased level of mitochondrial respiratory complex I, the identified molecular target of MitoTAM. Mitochondrial targeting therefore not only improves efficacy of this anti-cancer agent, but also extends its applicability to cancer subtypes thus far recalcitrant to treatment.

A2-06 Targeting complex I as an anticancer strategy



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Tumor cells exhibit profound bioenergetic changes with respect to the original non-transformed cell types [1]. One of the main driving mechanisms leading to such a metabolic alteration is triggered by hypoxia. Hypoxia is experienced by cancer cells during tumor progression and leads to a significant enhancement of glycolysis in order to sustain tumor growth and survival [2].
Notwithstanding low oxygen conditions, cancer cells harboring mitochondrial respiratory complex I (CI) disruptive mutations displayed a chronic destabilization (pseudonormoxia) of hypoxia inducible factor 1α (HIF1α), the main factor driving the hypoxic response. Such genetic lesions are associated to a significant reduction of the tumorigenic potential *in vivo*, suggesting an inability to adapt to environmental changes [3]. Therefore, dissecting the mechanisms by which complex I severe impairment causes HIF1α destabilization will help identifying new targets for potential anticancer strategies.
Using the zinc finger nucleases technology, we have generated NDUFS3-deficient cancer cells which display a marked CI deficiency. Engineered CI-defective cancer cells show a lack of HIF1α stabilization in hypoxic condition together with a significant reduction of the expression of HIF1α-responsive genes involved in the glycolytic machinery and tumor vascularization. These changes are associated with imbalanced Krebs' cycle metabolites

and, in particular, with an accumulation of α-ketoglutarate, known to foster the activity of the prolyl hydroxylases (PHDs) leading to HIF1α proteasomal degradation. Moreover, CI-defective cancer cells show a significant reduction of the tumorigenic potential *in vitro* and *in vivo*.
Hence, targeting the mitochondrial respiratory CI confers antitumorigenic properties by preventing the stabilization of HIF1α, thus hindering cancer cells adaptation to hypoxia. Further analyses will help confirm the pivotal role of PHDs in our current model.

This work is funded by the Italian Association for Cancer Research (AIRC) IG-14242 and by the EU FP7 Marie Curie project MEET 317433.

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A2-07 Modulation of mitochondrial electron transport chain activity differentially regulates cell death in proliferating and quiescent cells

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Mitochondrial electron transport chain (ETC) drives ATP production and is the major source of reactive oxygen species (ROS). We have previously shown that mitochondrially targeted vitamin E succinate (MitoVES) induces cell death by inhibiting complex II of ETC, leading to considerable ROS production. In addition, MitoVES selectively eliminates proliferating but not quiescent (confluent) endothelial cells (ECs) and suppresses tumorigenic angiogenesis *in vivo*. This suggests that modulation of ETC activity in proliferating and quiescent cells might have different outcomes with respect to cell death induction. To investigate the role of ROS generation and inhibition of ATP production (ETC inhibition may result in both), we cultured ECs in low (1 g/L) and high glucose (4.5 g/L) that promotes and suppresses mitochondrial respiration/ATP production, respectively. We exposed these cells to agents that induce ROS without ETC inhibition (phenethylisothiocyanate - PEITC, and hydrogen peroxide), inhibit ETC (rotenone, antimycin A) or directly interfere with mitochondrial ATP production (FCCP, oligomycin). Interestingly, PEITC and hydrogen peroxide induced cell death and ROS preferentially in proliferating cells irrespective of cell culture conditions. In contrast, treatment with the other compounds resulted in more cell death in proliferating than in quiescent cells when glucose was high, but this pattern was reversed when glucose was low. In addition, ROS generation only correlated with cell death induction in high glucose. Respiration measurements showed that cells grown in high glucose slightly reduced, and cells grown in low glucose significantly increased respiration on entering the quiescence state. This occurred despite the fact that ETC was primed for high activity in quiescent cells irrespective of glucose concentration, as evidenced by elevated expression of protein subunits and increased enzymatic activity of ETC complexes as well as enhanced supercomplex assembly. These data suggest that in low glucose interference with ETC

activity is a major factor for cell death induction, whereas in high glucose the level of ROS generation becomes dominant. We therefore propose that ETC inhibition differentially affects proliferating and quiescent cells and might be the key determinant of proliferation-responsive cell death sensitivity. Experiments to confirm this scenario are ongoing.

A2-08 Role of the microRNA-301a in the regulation of mitochondrial function in cancer cells

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MicroRNAs (miRNAs) are 22 nucleotides long, single stranded, non-coding RNAs that negatively regulate gene expression by binding to mRNA, resulting in translation inhibition or mRNA degradation. MiRNAs regulate a wide range of cellular functions, and their abnormal expression is linked with many pathological conditions including cancer. New findings indicate that miRNAs play also an important role in the regulation of mitochondrial function and biogenesis.

MiRNA-301a is an oncogenic miRNA whose expression is associated with tumour development, metastases and overall poor prognosis. MiR-301a as a putative mitochondrial regulator was identified by the analysis of miRNAs expression profile in cells treated with mitochondrially targeted α-tocopheryl succinate (MitoVES), a compound that concentrates in mitochondria, induces profound generation of ROS leading to dysfunction of mitochondria. Treatment with MitoVES caused a significant downregulation of miR-301a compared to control cells.

Web-based miRNA related databases (MiRWalk, microRNA.org, miRBase) identified that genes connected with mitochondrial function, such as *PPARGC1A*, *ESR1* and *PPARG*, possess a putative miR301a-binding seed sequence in their mRNA. We have observed a decrease in the expression of *PPARGC1A*, *ESR1* and *PPARG* in MCF7 cells overexpressing inducible miRNA-301a after addition of doxycycline. Additionally, we detected differences in the expression of genes regulated by ESR1 and PGC1α such as *TTF1A* and *GREB1A*. Importantly, we have found markedly elevated levels of miR-301a in tumour spheres generated *in vitro* that exhibit properties of cancer stem cells in comparison with their normal counterparts.

In summary, we propose that miR-301a plays a role in the regulation of mitochondrial function and biogenesis, describing a novel, so far unknown role of miR301a in mitochondrial biology. Since high expression of miR-301a is associated with higher metastatic potential and poor prognosis, identification of the role of miR-301a in the regulation of mitochondrial function could also shed more light on the role of mitochondria in cancer progression.

A2-09 Mitochondrial metabolite transport in cancer cells

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Carcinogenesis is accompanied by significant remodeling of cellular metabolic pathways, in particular those involving mitochondria. These alterations lead to corresponding changes in metabolite fluxes across the mitochondrial inner membrane. Although the changes in cancer cell metabolism are extensively studied, there are no systematically collected data on mitochondrial transport in these cells.
The mitochondrial inner membrane is not permeable for small hydrophilic molecules. The vast majority of the mitochondrial metabolite transport is mediated by proteins belonging to the mitochondrial carrier family. In this study, we have investigated the abundance and transport activities of mitochondrial carriers in several cancerous and non-cancerous cell lines under normoxic and hypoxic conditions by real-time PCR and Western blotting. Several proteins were significantly upregulated in cancer cells, indicating that their transported metabolites are of special importance for mitochondrial and cellular metabolism under these conditions.
These data give important insights into adaptation of mitochondria to the metabolic challenges of cancer cells. As transport processes are often rate-limiting steps of metabolic pathways, mitochondrial carriers overexpressed in cancer might represent a promising target for future anticancer therapies.

A2-10 Ubiquinone-binding site mutagenesis reveals the role of mitochondrial complex II in cell death initiation

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Respiratory complex II (CII, succinate dehydrogenase, SDH) inhibition can induce cell death, but the mechanistic details need clarification. To elucidate the role of reactive oxygen species (ROS) formation upon the ubiquinone binding (Qp) site blockade, we substituted CII subunit C (SDHC) residues lining the Qp site by site-directed mutagenesis. Cell lines carrying these mutations were characterized on the bases of CII activity and exposed to Qp site inhibitors MitoVES, TTFA and Atpenin A5. We found that I56F and S68A SDHC variants, which support succinate-mediated respiration and maintain low intracellular succinate, were less efficiently inhibited by MitoVES than the wild-type variant. Importantly, associated ROS generation and cell death induction was also impaired, and cell death in the wild-type cells was malonate- and catalase-sensitive. In contrast, the S68A variant was much more susceptible to TTFA inhibition than the I56F variant or the wild-type CII, which was again reflected by enhanced ROS formation and increased

malonate- and catalase-sensitive cell death induction. The R72C variant that accumulates intracellular succinate due to compromised CII activity was resistant to MitoVES and TTFA treatment and did not increase ROS, even though TTFA efficiently generated ROS at low succinate in mitochondria isolated from R72C cells. Similarly, the high affinity Qp site inhibitor Atpenin A5 rapidly increased intracellular succinate in wild-type cells but did not induce ROS or cell death, unlike MitoVES and TTFA that upregulated succinate only moderately. These results demonstrate that cell death initiation upon CII inhibition depends on ROS and that the extent of cell death correlates with the potency of inhibition at the Qp site unless intracellular succinate is high. In addition, this validates the Qp site of CII as a target for cell death induction with relevance to cancer therapy.

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A2-11 Analysis of energy fluxes in colorectal cancer saponin skinned tissues and Caco-2 cells

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To study the problem of the energy metabolism control in cancer cells, Moreno-Sanchez and Westerhoff´s groups have applied the Metabolic Control Analysis (MCA) with the conclusion that the role of OXPHOS in tumor cells should be re-evaluated and experimentally determined for each particular type of tumor cell [1]. Theoretical aspects of MCA have been subsequently analyzed by many researchers, e.g. [2]. MCA helps to understand the mechanisms by which a given enzyme exerts high or low control of metabolic flux and how the control of the pathway is shared by several pathway enzymes and transporters. In oncology, the application of MCA permits to determine the most attractive targets for chemotherapy.

In this work, we have analyzed quantitatively the mitochondrial respiration in post-operational tissue samples taken from 55 patients with colorectal cancer (CRC). Only primary tumor samples were examined; the patients in the study had not received prior radiation or chemotherapy. The high resolution respirometry and the permeabilized cell techniques in combination with MCA were applied to detect possible OXPHOS defects in energy conversion system of this tumor. Rates of O_2 consumption were measured in medium-B supplemented with 5 mM glutamate, 2 mM malate and 10 mM succinate as respiratory substrates by a high-resolution respirometer (Oxygraph-2k, Oroboros Instruments, Austria).

The method of MCA has been applied to human CRC skinned fibers in comparison with healthy tissue of same type and Caco-2 tumor cell line. MCA helps to understand how the control is shared between the enzymes and transporters of the pathway and to identify the steps that could be modified to achieve a successful alteration of flux or metabolite concentration in pathways, and to inhibit cancer cells energy metabolism in selective manner. For the case of irreversible specific inhibitor, an estimation of the value of the coefficient is given by Groen et al. (J. Biol. Chem., 1982,) and Moreno-Sanches et al. (J. Biomed. Biotechnol., 2008). To determine the flux control coefficients (FCC), the flux was measured as the rate of O_2 consumption by permeabilized tissue fibers derived from CRC

patients when all components of OXPHOS system were titrated with specific inhibitors to stepwise decrease of a selected respiratory complex activity in the presence of 2 mM ADP. We quantified the control exerted by different components of the respiratory chain and the ATP synthasome complex in human CRC clinical material as compared with normal tissue and compatible cell culture Caco-2. The control of mitochondrial respiration was distributed across several mitochondrial processes: FCC for complex II (CII) was found to be high for CRC and healthy colon tissue, as well as in the case of Caco-2 cell culture (where the respiratory chain is controlled also by CIV) (Fig. 1). In CRC tissue the value of FCC for ATP/ADT carrier is significantly lowered as compared with healthy intestinal tissue (Fig. 1). It is intriguing that for human healthy colon and CRC tissues *in situ* the sums of the FCCs for ADP activated respiration is close to 5 that significantly exceeds 1 - normally observed in oxidative tissues and isolated mitochondria. It is also well established in theoretical analysis that in an ideal linear system the sum of FCC(s) is 1 [1, 3], but may become higher if the system includes enzyme-enzyme interactions, direct substrate channeling and/or recycling within multienzyme complexes (system becomes non-linear). According to Lenaz and Genova (Antioxid. Redox Signal., 2010, 12, 961-1008) a sum of FCC(s) exceeding 1 indicates the existence of supramolecular association of the respiratory complexes (called as respirasomes) that was confirmed by electron microscopy, native blue-gel electrophoresis, and single particle image processing. Our data suggest that human CRC cells *in vivo* have a different structure of respirasomes as compared with model of Caco-2 cells. The sum of high control coefficients is seems to be characteristic for highly proliferative tissues where the respiratory chain must be someway reorganized.

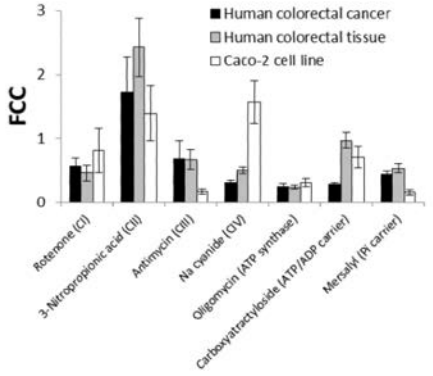

Fig. 1. Flux control coefficients (FCC) determined by the MCA for all mitochondrial respiratory chain and the ATP synthasome complexes for skinned human colorectal cancer, normal large bowel tissues and Caco-2 cell line. FCC(s) were calculated by using non-linear regression analysis by fitting data to the mathematical model, as described by Gellerich *et al.* [4]. The results were verified by a graphical method [2, 4].

From the data obtained we concluded that: 1) human CRC cells *in situ* the main rate-controlling steps of respiration are complex II and III, whereas in normal bowel tissue these are complex II and ATP/ADP carrier; 2) in human colorectal carcinomas some components of the mitochondrial electron transport chain (ETC) are organized into large supercomplexes (respirasomes) that may be a characteristic feature of cells with high proliferative index; and 3) there are strong differences in the function and organization of mitochondrial ETC components between human samples *in vivo* and model cells in culture.

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A2-12 Alterations of the OXPHOS system in rhabdomyosarcomas

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Rhabdomyosarcoma (RMS) is an aggressive neoplasm characterized by metastatic invasion and rapid growth. This type of tumor does not only involve muscle, but also many other tissues with consequently different clinical presentations. There are three main types of RMS: alveolar, embryonal and pleomorphic. Although in the last years the survival of patients affected by RMS substantially improved, many patients still die from advanced disease [1, 2].
Many tumors present a shift of the cellular metabolism from oxidative phosphorylation (OXPHOS) to aerobic glycolysis (Warburg effect) frequently caused by defects in one or more respiratory complexes [3]. The aim of the present study was to investigate whether RMS present alterations in the OXPHOS.
Formalin fixed paraffin-embedded (FFPE) tissue samples from 27 human RMS were stained for porin and complex I to V of the OXPHOS. The intensity of the immunohistochemical (IHC) staining of the proteins was evaluated and compared to normal muscle. Frozen samples of RMS (n=3) were analyzed for enzymatic activity of citrate synthase and OXPHOS complexes I to V. The results were compared to normal skeletal muscle from healthy patients (n=10-28). Transgenic mice expressing a constitutively active Hedgehog (Hh) receptor (Patched, Ptch) develop RMS [4]. Mice were treated with different Hh signaling inhibitors to investigate if these compounds could stimulate or reverse the Warburg effect in the RMS. Hh inhibitors were given either locally or systemically.
Citrate synthase activity, as well as the activity of all OXPHOS complexes, was low in the RMS samples (n=3). IHC analysis of the human RMS revealed that the immunoreactivity of porin was unchanged compared to normal adjacent muscle tissue (n=27). On the contrary, compared to control muscle, specific and significantly lower complex I levels were observed, whereas the amount of the other complexes was similar to unaffected muscle. Only embryonal RMS (ERMS) presented in addition very low complex II levels.
RMSs developed by $Ptch^{+/-}$ mice present many features of human ERMS, among which is also a significantly lower expression of complex I and II, compared to the normal adjacent muscle. Treatment of $Ptch^{+/-}$ mice with Hh inhibitors was not able to alter the OXPHOS system.
In summary RMS are characterized by a normal mitochondrial mass with an isolated complex I deficiency, ERMS also with a complex II deficiency. The low activity of the citrate synthase and the OXPHOS complexes compared to normal adjacent muscle can be explained by the high abundance of stroma/connective tissue. In addition, RMS showed a very small cytoplasm with a very limited space for mitochondria.
Ptch inhibition does not affect OXPHOS protein expression, suggesting that the Ptch receptor in RMS is not involved in the regulation of the Warburg effect, or that RMS cells present other parallel pathways that can overcome the inhibition of the $Ptch^{+/-}$ pathway.

This work was supported by the Children´s Cancer Foundation Salzburg, Cancer Foundation Salzburg, and the Marie Curie International Training Network MEET (317433) of the European Union.

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MiP2014

Section A3: Therapeutic approaches to mitochondrial pathologies

A3-01 Alternative respiratory chain enzymes in research and therapy

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In order to develop a potential therapeutic strategy for mitochondrial disorders, we have transferred genes for non proton-motive alternative respiratory chain enzymes from lower eukaryotes to model organisms, aiming to buffer metabolic stress in the OXOHOS system. Our studies have focused on both widespread and tissue-restricted expression of the alternative oxidase (AOX) from the tunicate *Ciona intestinalis* in human cells, mouse and *Drosophila*, as well as expression of the single-subunit NADH dehydrogenases from Ciona (NDX) and yeast (Ndi1). We were able to generate a robust resistance to OXPHOS toxins at the organellar, cellular and whole organism level. Extensive phenotyping of ubiquitously expressing AOX transgenic flies or mice, or NDX transgenic flies, revealed almost no significant deviations from thw wild-type phenotype under normal physiological conditions.

We determined that AOX is able to compensate for the deleterious organismal phenotypes, up to lethality and including neurodegeneration and locomotor defect, caused by deficiency of cytochrome oxidase in different tissues of *Drosophila*. Similarly, NDX or Ndi1 partially compensated for complex I deficiency. AOX also compensated phenotypes associated with deficiency of *dj-1β*, the fly homologue of a human Parkinson's disease gene, and expression of human *β*-amyloid peptides in a *Drosophila* model of Alzheimer's disease.

However, in our standard *Drosophila* model of mitochondrial disease, tko^{25t}, AOX expression produced no detectable benefit, whilst Ndi1 expression was synthetically lethal with the tko^{25t} mutation.

Even more surprisingly, AOX (but not Ndi1) was able partially to compensate for several phenotypes not previously associated with mitochondrial dysfunction. These include a range of developmental dysmporphologies caused by over-expression of a steroid-binding transcription factor or by deranged cell signalling. Although we do not yet have convincing data to explain these phenomena mechanistically, our findings suggest that mitochondrial dysfunction may play an even wider role in cellular and organismal pathophysiology than hitherto appreciated, and/or that AOX has other properties besides its canonical role as a ubiquinol oxidase.

Acknowledgements: I thank current and former members of my research team whose work I here present, notably Marten Szibor, Eric Dufour, Kia and Esko Kemppainen, Ana Andjelkovic, Suvi Vartiainen, Marcos Oliveira, Alberto Sanz, Giuseppe Cannino, Cagri Yalgin, Daniel Fernandez-Ayala, Eveliina Kaulio and Dmytro Gospodaryov, together with collaborators Pierre Rustin, Thomas Braun, Ines Anderl, Dan Hultmark and colleagues at the German Mouse Clinic.

A3-02 Targeting nutrient signaling pathways for the treatment of mitochondrial diseases

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Mitochondrial diseases are disorders with heterogeneous manifestations, being central nervous system (CNS) and muscle the most severely affected. Despite the advances in the understanding of he pathophysiology of mitochondrial diseases, there are only few cases of effective treatments. To test potential therapies, we recently generated a mouse model of Coenzyme Q (CoQ) deficiency (*Coq9*^{R239X}) that presents a dysfunctional COQ9 protein, which causes widespread CoQ deficiency and mitochondrial encephalomyopaty [1]. Recent studies have shown that inhibition of mechanistic target of rapamycin complex 1 (mTORC1), a protein kinase involved in the control of many anabolic and catabolic process in the cell, by rapamycin administration produces therapeutic benefits in some animal and cellular models of mitochondrial diseases [2, 3]. However, it is not known whether mTORC1 inhibition would be useful in all cases of mitochondrial diseases and the mechanism by which rapamycin delays progression of the disease in the mouse models is not clear. To answer these questions, we have evaluated the effects of rapamycin treatment in the *Coq9*^{R239X} mouse model.
Mice were treated with oral rapamycin in their chow at a concentration of 14 mg/kg food, which corresponds to a dose of 2.24 mg of rapamycin per kg b.w./day (equivalent to a dose of 0.2 mg per kg body weight/day in humans when normalized by body surface area). The treatment started at 1 month of age and we analyzed the animals at 3 months of age. We evaluated the therapeutic effects by immunohistochemistry in different brain sections to determine if rapamycin treatment ameliorates the vacuolization and astrogliosis in *Coq9*^{R239X} mice. Moreover, we carried out a metabolomic analysis and measured CoQ levels and mitochondrial complexes activities. We also evaluated some autophagy markers by western blot.
Our results show that rapamycin produces neurological improvement in *Coq9*^{R239X} mice. These benefits may be due to changes in the metabolic profile of treated *Coq9*^{R239X} mice, while the biosynthetic pathway of CoQ is not affected by rapamycin treatment. Therefore, rapamycin seems to have therapeutics effects in mitochondrial encephalopathy associated to CoQ deficiency. These therapeutic benefits are the result of the modulation of mTORC1 downstream pathways.

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A3-03 GDF15 is a novel biomarker to evaluate efficacy of pyruvate therapy for mitochondrial diseases

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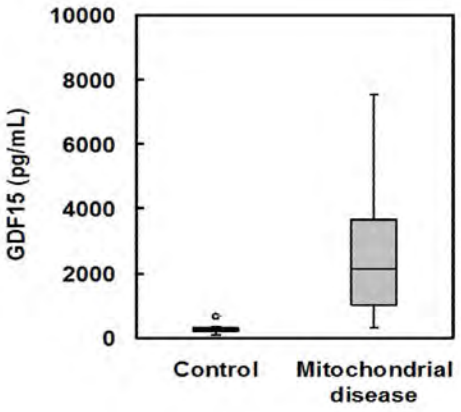
We proposed that the addition of pyruvate would facilitate oxidation of NADH to NAD^+ via the lactate dehydrogenase reaction, which would restore ATP production by the glycolytic pathway even under defective respiratory conditions [1]. Indeed, positive effects of sodium pyruvate on clinical manifestations of mitochondrial diseases have been reported [2]. However, useful biomarkers for evaluating the therapeutic efficacy of pyruvate remain to be developed.

In our earlier study [3], we found that exposure to excessive sodium lactate significantly increases the intracellular L/P and NADH/NAD^+ ratios in cybrid cells harboring the MELAS mutation (m.3243A>G), which implies worsening of lactic acidosis and NAD^+ shortage. On the other hand, we found that treatment with sodium pyruvate facilitates the ATP production and improves the energy status, as indicated by a decrease in the L/P ratio and retention of the NADH/NAD^+ ratio. Taken together, we considered that these experimental conditions would be ideal for identifying biomarker candidate genes, whose expression levels reflect the intracellular energy deficiency and the effect of pyruvate on energy metabolism.

In the present study, we performed a global gene expression analysis of cybrid cells with the MELAS mutation (m.3243A>G: 2SD cells) and control cybrid cells (2SA cells) treated or not with lactate or pyruvate. We identified several biomarker candidate genes, among which we focused on growth differentiation factor 15 (GDF15). The level of GDF15 in the conditioned medium was significantly higher in 2SD cells than in 2SA cells, which level was further increased by lactate but was not affected by pyruvate in 2SD cells. We also demonstrated that the concentration of GDF15 in the serum was markedly elevated in patients with mitochondrial diseases compared with that in those with other pediatric diseases.

Thus, we identified GDF15 as a novel serum marker for the diagnosis of mitochondrial diseases and possibly for monitoring the disease status and progression and for evaluating the therapeutic efficacy of pyruvate.



Measurement of the GDF15 concentration in the serum of patients. The serum GDF15 concentrations in 17 patients with mitochondrial diseases as well as those in 13 patients with other pediatric diseases were determined by ELISA.

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A3-04 Strategies to enhance the endogenous biosynthesis of Coenzyme Q

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Primary CoQ_{10} deficiency is a rare mitochondrial disease caused by mutations in CoQ biosynthetic genes. This syndrome is associated to five major clinical presentations: 1) encephalomyopathy, 2) severe infantile multisystemic disease, 3) cerebellar ataxia, 4) isolated myopathy, and 5) steroid-resistant nephrotic syndrome [1]. The only therapeutic option available for CoQ_{10} deficiency syndrome is the exogenous CoQ_{10} supplementation. However, the results of this therapy are poor in patients with neurologic symptoms due to low absorption and bioavailability of exogenous CoQ_{10} [1]. In those cases, the stimulation of endogenous CoQ_{10} biosynthesis could be an alternative and effective therapeutic option [2]. To do that, it is theoretically possible to bypass the defect in a biochemical pathway providing a metabolic intermediate that is downstream to the defective site. Thus, if the defect is in the *Coq*$_9$ gene, we could assess the ability of 2,4-dihydroxibenzoic acid (2,4-diHB) to rescue CoQ deficiency. Accordingly, we have treated *COQ9*^{R244X} human fibroblasts with 2.5 mM 2,4-diHB, as well as *Coq9*^{R239X} mice with 100-200mg/kg bw*day of oral 2,4-diHB [3, 4]. At those doses, CoQ_{10} levels were significantly increased in *COQ9*^{R244X} fibroblasts. Likewise, CoQ_9 levels were slightly increased in kidneys and skeletal muscle homogenates. In isolated mitochondria, CoQ_9 levels were also increased in kidney and skeletal muscle resulting in an increase of complex I+III activities. However, the levels of CoQ_9 and the CI+III activity were similar in brain of untreated and treated *Coq9*^{R239X} mice and, as a consequence, the histopathological characteristics of *Coq9*^{R239X} mice were unaffected after the treatment. These results point out that it is possible to bypass a defect in CoQ biosynthesis *in vitro* and *in vivo*. However, the results in brain suggest that this tissue has a specific regulation of CoQ biosynthesis or that a higher dose of 2,4-diHB is required to increase CoQ biosynthesis in brain.

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A3-05 Drosophila as a model to study therapeutic approaches for mitochondrial diseases

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Among the wide range of mitochondrial disorders, defects in the oxidative phosphorylation (OxPhos) are the most prevalent. OxPhos deficiencies often lead to early death and are associated with severe and highly variable clinical symptoms. Despite intense efforts in the comprehension of the mechanisms underlying mitochondrial disorders, patients are still without effective treatment. The need of predictive *in vivo* models of the pathology is an important issue in the development of new therapeutics in order to study their therapeutic potential, toxicity and pharmacokinetics. Due to the extreme genetic and phenotypic heterogeneity of OxPhos disorders one cannot rely on a single *in vivo* model.
Here we present the method and strategy we use to create, characterize and validate a set of *Drosophila melanogaster* models of nuclear DNA-encoded OxPhos subunits and preliminary results of systematic evaluation of Khondrion´s lead compound. We primarily focus on complex I by knocking down the core and accessory subunits the most prone to mutation in patients and selecting phenotypes-readouts suitable for drug screening (death at critical stages of development, survival curves, ROS level).
These models will represent a valuable tool with predictive power to evaluate new potential therapeutics as an initial step in the drug development process.

This work was supported by a Marie-Curie Initial Training Networks (ITN) grant MEET (Mitochondrial European Educational Training, FP7-PEOPLE-2012-ITN, Grant Agreement No: 317433), a PM-Rare (Priority Medicines Rare disorders and orphan diseases) grant from ZON-MW (Netherlands Organization for Health Research and Development-Medical Sciences, No: 40-41900-98-033) and the Stichting Energy4All (www.energy4all.eu).

MiP*art*

Section B: Structural basis of mitochondrial physiology

B-01 Superassembly of respiratory complexes: physiological consequences

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B-02 Localized translation near mitochondria: novel factors revive old model

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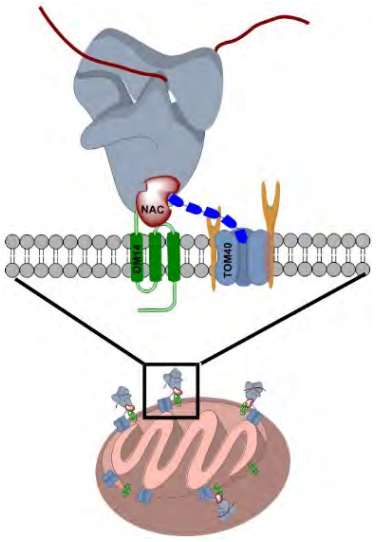
Most of mitochondria proteins are encoded in the nucleus, and need to be imported into this organelle. The predominating, textbooks model for targeting to mitochondria asserts that proteins are translated throughout the cytoplasm and transported after their complete synthesis (i.e. post-translationally). However, recent mRNA localization studies revealed that many mRNAs that encode mitochondrial proteins are localized to the vicinity of mitochondria in a manner that involves translation. These results revived neglected model in which translation of mitochondrial mRNAs is localized to the mitochondria vicinity and import occurs cotranslationally. We are exploring the proteins that coordinate such localized translation. We previously established the involvement of the mitochondrial protein receptor Tom20 and the Hsp70-member Ssa1 in association of translating ribosomes with the mitochondria[1,2]. Herein we further elaborate on a role in localized translation for an additional factor, the conserved ribosome-associated Nascent-chain Associated Complex (NAC). NAC was shown to contribute to ribosomes' association with mitochondria, yet its mitochondrial receptor was unknown. We performed several genome-wide protein complementation assays and detected an outer membrane protein (OM14) of an unknown function as associated with NAC[3]. Mitochondria deleted of OM14 had significantly lower amounts of associated NAC, and ribosomes deleted of NAC had reduced levels of associated OM14.



Model for the role of OM14 in localized translation near the mitochondria[4]

Importantly, mitochondrial import assays revealed a significant decrease in import efficiency into OM14 deleted mitochondria and OM14-dependent import necessitated NAC. Our results identify OM14 as a mitochondrial receptor for ribosomes-associated NAC and reveal its importance for import. These studies re-establish localized translation as an additional mode for protein targeting to mitochondria.

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B-03 pH nanoenvironment sensing in actively respiring mitochondria

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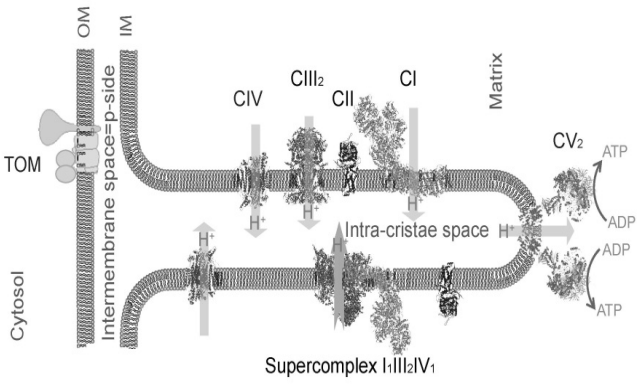
Cristae are flat tube- or disk-like invaginations of the mitochondrial inner membrane extruding into the alkaline matrix space (Figure 1). ATP is mainly produced at the crista membrane by utilization of the proton motive force (pmf = ∆ψ (membrane potential) + ∆pH) across the membrane. The pmf is the coupling parameter between ATP production and electron transport chain (ETC). Proton pumps and the proton-driven ATP synthase can be spatially segregated. Complexes I-IV are mainly found in the flat sheet membrane of cristae, while Immuno-EM and EM-tomography have revealed ribbons of FoF1 dimers lining the highly curved crista rim [1]. These ribbons seem to be involved in folding the crista membrane. It has been proposed that the concave side of the highly curved rim electrostatically up-concentrates protons at CV to augment the local pmf [2].

By attaching the fluorescent ratiometric pH-sensitive GFP variant pHluorin to OXPHOS complex IV and the dimeric FoF1 ATP synthase, we determined the lateral pH profile along the p-side of cristae in the intra-cristae space (ICS) of living HeLa cells [3]. To stimulate an activated oxidative phosphorylation in glycolytic HeLa cells, glucose was replaced by galactose in the glutamine-containing growth medium [4]. Furthermore, we analysed the effect of metabolic adaptation to the galactose-medium on pH from surface to the bulk of the ICS and correlated it to the matrix pH, ∆ψ and basal respiration.

Most interesting, we observed that the local pH at FoF1 dimers (proton sink) is by 0.3 units less acidic than at CIV (proton source) in these cells. This finding is consistent with the calculated pH profile for steady proton diffusion



pH determination in different mitochondrial subcompartments.

from a proton pump in the crista sheet to FoF1 as proton consumer at the rim.
The observed lateral variation in the proton-motive force necessitates a modification to Peter Mitchell's chemiosmotic proposal. The experimental technique can be extended to other pH-dependent reactions in membrane microcompartments.

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B-04 Assembly of subunit F_o-a into mammalian ATP synthase

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The biogenesis of mammalian ATP synthase is complex process believed to proceed via several modules. In the later steps, membranous subcomplex is formed and the final phase is represented by incorporation of the two mtDNA-encoded subunits F_o-a and A6L (Atp6 and Atp8). However, little is known about the position of two newly described F_o accessory subunits DAPIT (also termed Usmg5) and MLQ (also known as c14orf2) in the assembly scheme and about their role in regulation of ATP synthase biogenesis. We have utilised several model systems, namely rho^0 cells lacking mtDNA and thus both subunits F_o-a and A6L, cells harbouring 9205delTA microdeletion, which results in the absence of the subunit F_o-a, HEK293 cells with knockdown of DAPIT protein and HEK293 cells with knockout of MLQ protein and followed the assembly state of ATP synthase among them.
Contrary to previously reported data, we observed normal levels of assembled ATP synthase in DAPIT knockdown and MLQ knockout cells. Our results indicate, that lack of DAPIT protein leads to the assembly of more labile, but complete and functional enzyme. Absence of either F_o-a alone or F_o-a and A6L results into the normal levels of structurally altered, labile, and ~60 kDa smaller enzyme complex, which also lacks DAPIT and MLQ. This complex retains the ATP hydrolytic activity but is unable to synthesize ATP. Cells with the MLQ knockout presented with the phenotype similar to the lack of F_o-a: normal content of smaller and labile complex. In the absence of MLQ, ATP synthase did not contain subunit F_o-a and the total F_o-a content was also decreased, presumably due the degradation of unassembled subunit. This complex also retained ATP hydrolytic activity, while its phosphorylating capacity was affected. Based on our data, we conclude that MLQ and F_o-a closely associate and their incorporation into the enzyme complex depends on each another. On the contrary, DAPIT protein seems to be incorporated at the very last step and its presence stabilises the holoenzyme.

This project is supported by the Czech Science Foundation grants 14-36804G and P303/12/1363.

B-05 Higd1a is a positive regulator of cytochrome c oxidase

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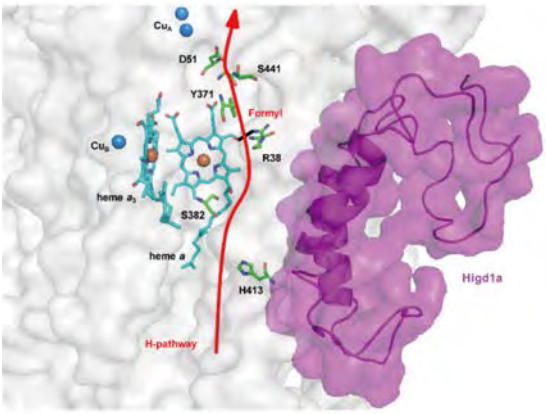
Cytochrome c oxidase (CcO) is the only enzyme that utilizes oxygen to produce proton gradient for ATP production in mitochondrial oxidative phosphorylation. Mammalian CcO is composed of 13 different subunits containing four redox active metal centers [1]. Because CcO is the only enzyme in the body that can utilize oxygen for energy transduction, it has been suggested that regulatory mechanism of CcO is dependent on oxygen concentration [2]. In this study, we aimed to identify CcO regulator which induced under hypoxia.

We screened gene expression profiles of neonatal rat cardiomyocytes and found Higd1a as one of an up-regulating genes in hypoxia. Biochemical analysis revealed that Higd1a directly binds to CcO and structural analysis by resonance Raman revealed that Higd1a caused structural changes in the CcO, especially around heme *a*, the active center that drives proton-pump [3]. Endogenous induction of Higd1a in rat cardiomyocytes under hypoxia increased mitochondrial ATP synthesis. Moreover, exogenous Higd1a successfully improved cell survival in rat cardiomyocytes.

By an identification of Higd1a and its biochemical and structural investigation, we demonstrated that Higd1a could exhibit an increase of ATP production via interaction with CcO.



Higd1a acts on the H-pathway. Model depicting our docking simulation (side view) and its relationship with the H-pathway. The model shows the location of Higd1a (magenta) in the CcO complex (white) and its relationship to R38 of cytochrome c oxidase subunit I and the formyl group of heme *a*, a component of the H-pathway (red arrow).

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B-06 Liposomes simulating the compositions of outer and inner mitochondrial membranes are protected during desiccation by LEA Proteins from *Artemia franciscana*

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Intracellular accumulation of Late Embryogenesis Abundant (LEA) proteins [1] and the disaccharide trehalose [2] is associated with cellular desiccation tolerance in a number of animal species. LEA proteins are a family of intrinsically disordered proteins that are unstructured in solution and adopt secondary structure as water is removed. During drying, LEA proteins protect target enzymes, prevent protein aggregation, and some may form amphipathic alpha-helices capable of interacting with lipid bilayers. Targeting of LEA proteins to different compartments within the cell emphasizes the necessity of protecting organelles from water stress-induced damage. It has been hypothesized that a given LEA protein may preferentially stabilize membranes of a particular lipid composition based on the protein's subcellular location. Here we evaluate the protection of liposomes in the dried state by two LEA proteins from *Artemia franciscana*, by the sugar trehalose, and by LEA protein and trehalose in combination. Using both a cytoplasmic-localized (AfrLEA2) and a mitochondrial-targeted LEA protein (AfrLEA3m) [3,4] allowed us to test the above hypothesis.

Small unilamellar liposomes with compositions that mimicked the inner mitochondrial membrane with cardiolipin (IMM), outer mitochondrial membrane (OMM), and the inner leaflet of the plasma membrane (ILPM) were prepared with a hand held mini extruder. Desiccation-induced damage to liposomes was assessed by carboxyfluorescein leakage after air drying overnight and rehydration. Recombinant AfrLEA3m and AfrLEA2 were purified as described previously [3]. To compare the impact of LEA proteins to a negative control (i.e., a protein predicted to be non-stabilizing), liposomes were also dried with lysozyme at identical protein:lipid mass ratios. Primary amino acid sequences of AfrLEA3m and AfrLEA2 were determined from our existing cDNA library for *A. franciscana* and used for molecular modeling.

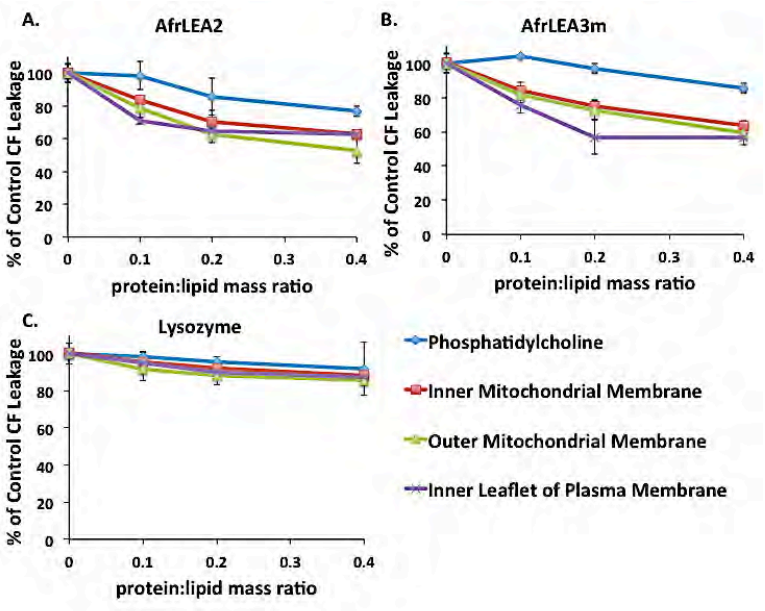

Figure 1. Carboxyfluorescein leakage from liposomes dried overnight and rehydrated in the presence of LEA proteins or a control protein (lysozyme). Data represents the mean ± SD of n = 6 samples.

Both LEA proteins were able to offset damage during drying of liposomes that mimicked the lipid compositions of the IMM, OMM and ILPM (Fig. 1). Thus liposome stabilization by

AfrLEA3m or AfrLEA2 was not dependent on lipid composition, provided physiological amounts of bilayer and non-bilayer-forming lipids were present (liposomes with a non-biological composition of 100% phosphatidylcholine were not protected by either protein). Stabilization by LEA proteins was significantly greater than that afforded by lysozyme for all membranes except 100 % PC liposomes (2-way ANOVA, $p \leq 0.05$, $n = 6$). Additive protection by LEA proteins plus trehalose was dependent on the lipid composition of the target membrane. Consistent with the ability to stabilize lipid bilayers, molecular modeling of the secondary structures for AfrLEA2 and AfrLEA3m revealed bands of charged amino acids similar to other amphipathic proteins that interact directly with membranes (Fig. 2). Amino acids of positive and negative charge align in parallel bands, with acidic (negative) residues flanked to either side by basic (positive) residues. Such organization has been proposed to allow directly interact with the headgroups of lipid bilayers in the case of a plant LEA protein.

LEA proteins and trehalose stabilize liposomes that mimicked biological membranes when desiccated. Neither the cytoplasmic-localized AfrLEA2 nor the mitochondrial-targeted AfrLEA3m exhibits preferential protection of one compositional type of liposome over another. Matrix-resident AfrLEA3m is not more proficient at stabilizing IMM-like liposomes that contain cardiolipin than is AfrLEA2. When trehalose and LEA proteins are used in combination, IMM-like liposomes and ILPM liposomes are protected to a significantly greater degree than when dried with either protectant alone. Modeling of AfrLEA2 and AfrLEA3m as α-helices shows arrangements of charged amino acids that are consistent with other amphipathic proteins capable of direct interaction with lipid bilayers.

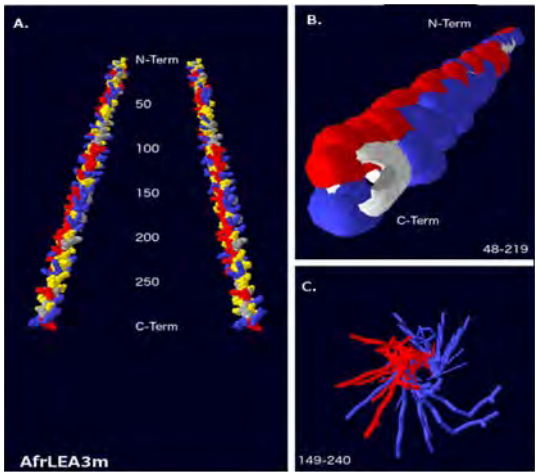

Figure 2. (A) Two views of AfrLEA3m modeled as an α-helix. Charged amino acids are depicted in red (acidic: D or E) or blue (basic: H, K, or R). Hydrophobic residues (A, G, I, L, M, V, or W) are colored gray and hydrophilic residues (N, Q, S, T, or Y) are depicted as yellow. (B) The α-helical backbone (white) is depicted with the charged residues (colored as above) between positions 45-219. (C) End-on view of residues 149-240 with only the charged amino acids visible.

Supported by National Science Foundation grants IOS-0920254 and IOS-1457061/IOS-1456809.

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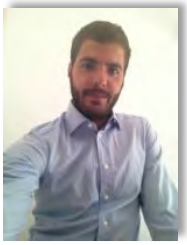
B-07 High-Content Screening of mitochondrial morphofunction in living cells



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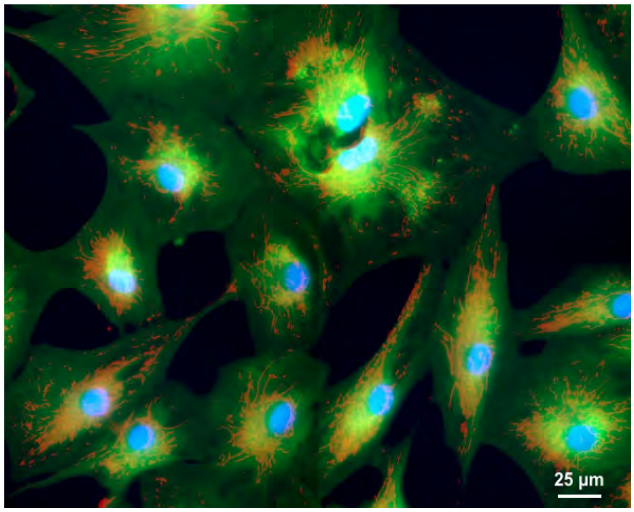
Mitochondrial morphology and functionality ("morphofunction") have been often described in a simplistic binary manner as "normal" or "aberrant" in various pathophysiological conditions. However mitochondrial morphofucntional phenotypes, depending on a balance of countless factors, are not so easily to label. Mitochondrial phiso-pathology usually presents a continuum of morphofunctional states [1].



Typical image. Primary human skin fibroblasts stained with TMRM (in red), Cacein (in green), Hoechst (in blue).

To properly describe this state continuum, simultaneous monitoring of multiple parameters is required. Therefore, here we present an integrated strategy allowing quantification of mitochondrial morphofunction in single intact living cells using, rather than single measurements, multivariate data sets. To this end, three fluorescent reporter molecules are combined into a multispectral fluorescence microscopy assay. In contrast to manual classification the presented approach allows combined high-content and high-throughput analysis of multi-well plates (High-content Screening). The automated analysis of large data sets drastically reduces bias, provides strong statistical power, and enable reliable analysis between various cell lines and conditions.

The proposed HCS technology is able to discriminate drug-induced modulation of mitochondrial phenotypes. Moreover, the assay can clearly individuate and quantify pathophysiological morphofunctional phenotypes of cell lines carrying or not pathogenic mutations. Therefore, the technology is proposed as an ideal platform to perform library drug screening [2] and mode-of action studies but also to address fundamental questions in mitochondrial research.

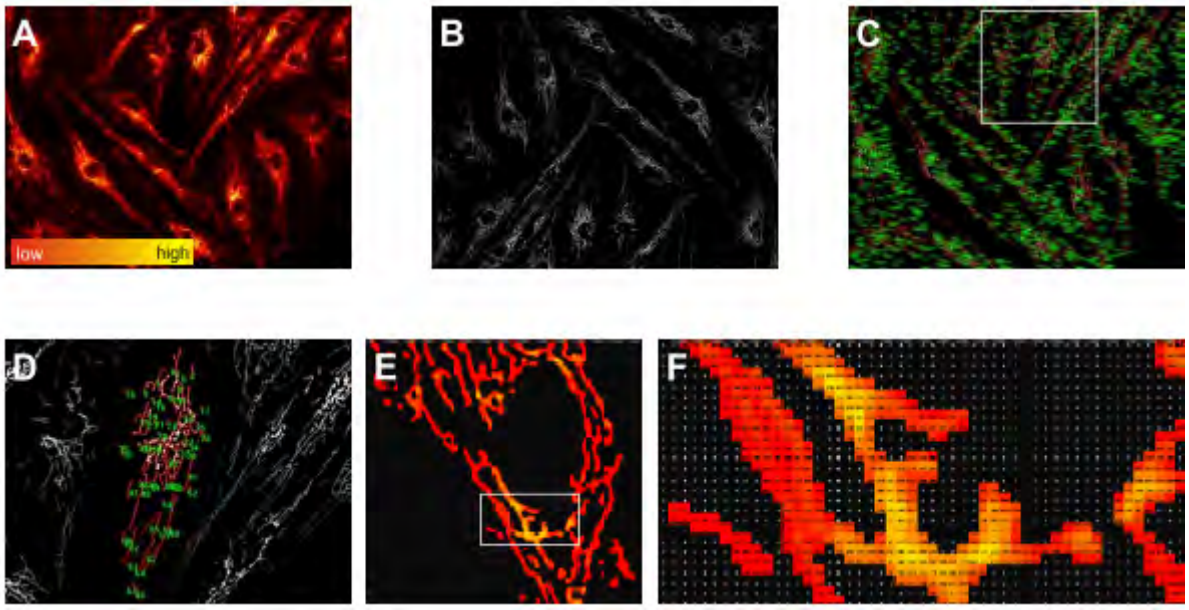


Image processing and data extraction. (A) TMRM RAW image. Fluorescence intensity is color-coded using a red to yellow scale. (B) MASKED image obtained by applying an automated image processing algorithm. (C) Computer-assisted identification of mitochondrial objects. Each object is described by 31 morphological and functional descriptors; these parameters are automatically extracted and provide a multidimensional phenotypic quantification of the mitochondrial network morphology and functionality. (D) Magnification of a region of interest in panel C. (E) Magnification of a region of interest in panel D. (F) Bitmap of a region of interest in panel E.

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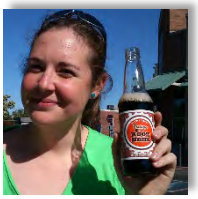
B-08 Insufficient energy provision or increased oxidative stress – what matters more in ATP synthase deficiencies?



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Mitochondrial F_1F_o-ATP synthase is responsible for most of the ATP production in aerobic organisms. Its deficiencies are associated with severe pathologic phenotypes. To shed light on the functional consequences of ATP synthase deficiencies, we utilised a model of HEK293 cell line and explored the effect of RNAi mediated knockdown of the three subunits (γ, δ and ε) forming the central stalk of the enzyme, which results in an isolated decrease of ATP synthase content.

For functional evaluations of ATP synthase deficiencies, 9 stable knockdown clones with down-regulated subunits γ (*ATP5C1* gene), δ (*ATP5D* gene), or ε (*ATP5E* gene) have been selected. The residual oligomycin-sensitive ATPase hydrolytic activity in these clones ranges between 2 and 78 % as compared to controls, which is paralleled by a decrease in the content of fully assembled ATP synthase complex.

Examination of cellular respiration and glycolytic flux, using the Seahorse XFe24 analyser, revealed that the clones with less than 30 % of residual ATPase activity switched their metabolism to enhanced glycolysis. There is a decrease in their basal respiration rate relatively to their respiratory capacity (47 vs 61 % in controls) and in parallel, their basal glycolytic rates utilise by up to 20 % more of their glycolytic capacity. These findings clearly demonstrate metabolic adaptations of these cells. On the other hand, the clones with more than 30 % residual ATPase activity showed no change either in the respiration or in their basal glycolytic rate.
As a result of ATP synthase deficiency, the knockdown clones exhibit reduced dissipation of mitochondrial membrane potential ($\Delta\Psi_m$) under ADP stimulation (by up to 20 mV compared to controls). The increase of $\Delta\Psi_m$ might then stimulate the production of reactive oxygen species (ROS) that is, indeed, elevated by 20 % in the knockdown clones with the lowest ATPase residual activity. The content of antioxidant enzymes, on the other hand, did not display any correlation to ATPase activity or ROS production.
In conclusion, our data indicate two pathogenic mechanisms of ATPase deficiency – energetic deprivation and increased oxidative stress. Generally, the threshold for defect manifestation and subsequent metabolic remodelling equals to approximately 30 % of ATPase activity.

This project is supported by the Grant Agency of Charles University (grant 1160214) and the Czech Science Foundation (P303/12/1363, P303/11/0970).

B-09 Tissue- and species-specific differences in cytochrome *c* oxidase assembly induced by *SURF1* defects

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Introduction: In this study we focused on distinct biochemical phenotype of cytochrome c oxidase (COX) deficiency in mouse and humans due to the absence of SURF1 protein, an important ancillary factor of COX biogenesis, which exact function is not known yet. While mutations in *SURF1* gene lead to a fatal neurodegenerative mitochondrial disorder in humans, the Leigh syndrome, *SURF1*$^{-/-}$ knockout in mouse results in surprisingly mild COX deficiency and no neurodegenerative disorder [1, 2]. The aim of our study was to find out interspecies differences in the impaired process of COX biogenesis, from early assembly intermediates to formation of COX supercomplexes with other respiratory enzymes. This was achieved by investigating *SURF1*$^{-/-}$ mouse tissues and fibroblasts in comparison with patient fibroblasts lacking SURF1 protein due to SURF1 gene mutations.
Methods: Isolated mitochondria from control (*SURF1*$^{+/+}$) and *SURF1*$^{-/-}$ mouse tissues and fibroblasts and from human control and SURF1 patient fibroblasts were analyzed using 2D BNE/SDS PAGE, activities of COX and citrate synthase were measured. Doxycycline reversible inhibition and pulse-chase metabolic labeling of mitochondrial DNA encoded subunits were used for investigating of COX biogenesis in *SURF1*$^{+/+}$and *SURF1*$^{-/-}$ mouse fibroblasts and in control and SURF1 patient fibroblasts.

Results: Our study revealed considerably decreased COX monomer and COX activity in *SURF1* patient fibroblasts compared to *SURF1*$^{-/-}$ mouse tissues/fibroblasts. *SURF1*$^{-/-}$ mouse tissues/fibroblasts also showed much lower accumulation of COX assembly intermediates on one hand and very low amount of I-III2-IVn COX supercomplex on the other. In contrast, assembled COX was present mainly in I-III2-IVn supercomplex in *SURF1* patient fibroblasts where the prominence of COX assembly defect was also apparent from accumulation of incomplete COX assembly intermediates. We subsequently characterized kinetics of COX biogenesis in *SURF1* patient and *SURF1*$^{-/-}$ mouse fibroblasts by doxycycline reversible arrest of mitochondrial translation and ^{35}S-labeling of mtDNA encoded proteins. Doxycycline inhibition and gradual recovery to steady state revealed rather stable proportion between COX monomer and supercomplexes in human control cells, while in *SURF1* patient cells COX monomer markedly decreased and formation of supercomplexes was preferred. In *SURF1*$^{+/+}$ and *SURF1*$^{-/-}$ mouse cells, however, the recovery proceeded mainly to the level of COX monomer. Pulse-chase metabolic labeling clearly showed higher stability of COX monomer and faster proteolytic degradation/depletion of accumulated COX assembly intermediates in *SURF1*$^{-/-}$ mouse fibroblasts, while more persistent COX assembly intermediates prevailed over the gradually decreasing signal of COX monomer in *SURF1* patient cells.
Conclusions: Our experiments clearly demonstrate crucial importance of the *SURF1* protein for effective COX biogenesis in human cells, whereas its absence is much better tolerated in mouse cells and tissues with faster COX turnover.

This work was supported by the Grant Agency of the Czech Republic (14-36804G), Ministry of Education, Youth and Sports of the Czech Republic (ERC CZ: LL1204, RVO:67985823), the Grant Agency of the Ministry of Health of the Czech Republic (NT12370-5) and ERC Advanced Grant FP7-322424.

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Luční Bouda, Giant Mountains National Park

Section C: MITOEAGLE

C-01 Mitochondrial function of cryopreserved HEK 293T cells: development of a reference sample for high-resolution respirometry

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An increasing number of metabolic and other diseases is recognized as being linked to mitochondrial physiology and dysfunction of oxidative phosphorylation (OXPHOS), which can be analyzed effectively and quantitatively by high-resolution respirometry (HRR). Instrumental quality control is a fundamental component of HRR applied in many laboratories [1]. Beyond the instrumental level, a standardized mt-laboratory quality management system (QMSmtf [2]) is required for establishing a global data base on mitochondrial function (mtf) in human cells and tissues, taking into account the variables of evolution, age, gender, life style and environment (EAGLE). A QMSmtf requires the availability of a mt-reference sample which is functionally stable over time and across geographical space, as a basis of standard proficiency tests within and between reference laboratories. Mammalian mt-preparations (isolated mitochondria, tissue preparations) appear to be neither suitable for prolonged storage nor large scale production. Therefore, we focused on cryopreserved human cells [3]. Using the widely applied cell line HEK 293T we optimized cryopreservation to maintain cell viability and stabilize respiratory characteristics of intact and permeabilized cells over variable periods of time.

HEK 293T cells were cultured under standard conditions with DMEM, were cryopreserved by cooling cells suspended in a freezing medium with fetal calf serum (FCS) and 10% dimethyl sulfoxide (DMSO), and stored at -80 °C for variable periods of time. Cells were thawed by addion of and careful mixing in pre-warmed PBS or MiR05. Cell counts and viability were assessed by determination of Trypan blue exclusion using an automated Countess cell counter. Respiration was measured in the OROBOROS O2k applying standard substrate-uncoupler-inhibitor-titration (SUIT) protocols for permeabilized cells in MiR05. For HRR with intact cells, cyropreserved cells were suspended in pre-warmed DMEM. Results were compared with tests on cells cryopreserved with addition of the powerful antioxidant melatonin at 10 nM or 25 µM.

Cryopreservation did not impair cell viability for storage times up to 311 days. In intact cells both ROUTINE and ETS (*E*) but not LEAK respiration (*L*) were depressed, with a decline of c. 20% after 3 weeks of cryopreservation. Subsequently, respiration was stable after prolonged storage for up to 311 days. Short-term preservation for 1 to 3 weeks did not affect respiration examined in permeabilized cells with or without melatonin, but OXPHOS (*P*) and ETS capacities were reduced compared to controls in the presence of fluorescence probes applied for simultaneous detection of mt-membrane potential (TMRM; CI- and CI&II-linked respiration) or H_2O_2 production (Amplex Red; tested for CII [4]). Storage for 1 to 12 months was without effect on CI_L and CII_E compared to cells maintained in culture, but CI_P, $CI\&II_P$, and $CI\&II_E$ were reduced. CI&II-linked OXPHOS capacity of these permeabilized control cells was not stimulated by cytochrome *c* (10 µM c), compared to a

c-flux control factor in the cryopreserved cells as low as 0.02±0.01 (SD; *N*=5 cultures measured in duplicate). Therefore, damage of the outer mt-membrane was not the mechanism responsible for the changes observed in these OXPHOS analyses.

In summary, cryopreserved HEK 293T cells maintained full cell viability but showed some impairment of respiration upon prolonged storage. Such impairments may even be observed after short-term storage in the presence of fluorescence probes typically applied to examine mitochondrial function. However, the overall decline of respiration in permeabilized and intact cells appear to be rather limited even after a period of up to 311 days, suggesting that with careful characterization of these changes, maintenance of strictly controlled conditions and further optimization, cryopreserved HEK 293T cells may provide a validated reference sample for HRR, applicable for standardized proficiency testing and a QMSmtf without geographical borders.

Supported by projects K-Regio MitoFit and NextGen-O2k, Tiroler Innovationsförderung.

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O2k ethanol dose response protocol

C-02 Mitochondrial respiration of peripheral blood mononuclear cells in patients with borderline personality disorder

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Borderline personality disorder (BPD) is characterised by a pervasive pattern of instability of interpersonal relationships, self-image, affects, and marked by impulsivity [1]. Beside the pronounced psychological stress, patients with BPD show an increased risk for somatic disorders and an impaired immunity. The resulting high burden of patients suffering from BPD is associated with conditions of chronic stress, which negatively influences the reactivity of the cellular immune system [2, 3]. So far, the underlying pathophysiological processes and long-term consequences of BPD on cellular immunity and energy metabolism are hardly explored.
Here, we report first data on mitochondrial functioning and the quantity of mitochondria in immune cells from female patients with BPD (n = 24), which were compared to an age- and gender-matched group of healthy controls (n = 13). The severity of BPD symptoms was measured by the self-report questionnaire *Borderline Symptom List* (BSL), the severity of depressive symptoms by the *Beck Depression Inventory* (BDI). Peripheral blood mononuclear cells (PBMC) were isolated from whole blood (15 ml) using Ficoll dense gradient centrifugation. Total PBMC were cryopreserved in Mannheim and after thawing in Ulm, the respiratory activity was assessed in living cells in a high-resolution oxygraph 2k. Characterization of mitochondrial activity included the following parameters: Routine, Leak, Uncoupled, and Residual oxygen consumption (ROX). Respiration was controlled for the intracellular amount of mitochondria, which was assessed with the citrate synthase activity (CSA) assay, a spectrophotometric technique [4].
We found no statistically significant alterations of mitochondrial activity in patients with BPD compared to controls. Interestingly, within the BPD group ATP turnover-related oxygen consumption was significantly correlated with both the severity of BPD (BSL sum score, r = 0.592, p = 0.010) and depressive symptoms (BDI sum score, r = 0.735, p = 0.001). Furthermore, there was a significant effect of depressive symptoms (BDI sum score) on residual oxygen consumption (ROX), the amount of oxygen consumed independently from ATP-production (r = 0.450, p = 0.053). Finally, the CSA assay revealed no significant difference in the amount of mitochondria between the two groups.
Chronic stress associated with BPD seems to negatively affect the homeostasis of immune cells, which has to be counteracted by a higher production of ATP. The increase of ROX subject to the severity of depressive symptoms provides evidence for the production of reactive oxygen species (ROS) in a dose-dependent manner. Consequently, the severity of depressive symptoms seems to have a stronger impact on mitochondrial functioning in immune cells than the severity of BPD. To address the question of a possible usage of mitochondrial respiration in immune cells as a new marker for the biological effects of BPD treatment, follow-up intervention studies with a longitudinal design are necessary.

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C-03 Mitochondrial function and ROS homeostasis in young mouse heart and brain: The sex factor!

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Gender-specific differences in mitochondrial function and free radical homeostasis are consistently reported in the context of aging and associated deficits. However, little is known about the gender-related roles of these parameters in the pathogenesis of neurological and cardiovascular disorders that occur early in life. Aim: To test the hypothesis that gender disparity in mitochondria function and ROS homeostasis starts early in life and hence can be implicated in sexual dimorphism in some cardiac as well as neurological disorders. Approach: We investigated heart and brain mitochondrial respiratory function in young (2-4 months) male and female wild-type C57BL6J mice, by high-resolution respirometry. Parallel productions of ROS by respiring mitochondria or active NADPH oxidases (NOXs) were also assessed using high-resolution oxymetry, fluorescence assays, and electron paramagnetic resonance (EPR) spin trapping techniques. Results: Although mitochondrial respiratory activity in the heart didn't significantly vary between genders, female brains exhibited enhanced activity during state 3, state 4, and maximally uncoupled respiration. This was associated with lower rates of hydrogen peroxide production in female cardiac and brain tissues. Furthermore, no gender differences have been detected in Nox2 and Nox4 proteins or activities in brain homogenate or freshly isolated synaptosomes. However, a strong trend of increased EPR-detected NOX-superoxide in male synaptosomes hinted at gender-specific discrepancy in antioxidant enzymes. Indeed, we found that superoxide dismutase (SOD) activity was higher in female brains using two independent approaches. Conclusion: Taken together, our results indicate that gender differences in mitochondrial bioenergetics and ROS production occur at young age, and that differences in superoxide dismutation capacity may be primarily responsible for gender differences in ROS homeostasis. These findings may eventually assist in the understanding of sexual dimorphism in some disorders that occur early in life.

C-04 High intensity interval training (HIIT) induces specific changes in respiration and electron leakage in the mitochondria of different rat skeletal muscles

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High intensity interval training (HIIT) is characterized by vigorous exercise with short rest intervals. Hydrogen peroxide (H_2O_2) plays a key role in muscle adaptation. This study aimed to evaluate whether HIIT promotes similar H_2O_2 formation via O_2 consumption (electron leakage) in three skeletal muscles with different twitch characteristics. Rats were assigned to two groups: sedentary (n=10) and HIIT (n=10, swimming training). We collected the tibialis anterior (TA-fast), gastrocnemius (GAST-fast/slow) and soleus (SOL-slow) muscles. The fibers were analyzed for mitochondrial respiration, H_2O_2 roduction and citrate synthase (CS) activity. A multi-substrate (glycerol phosphate (G3P), pyruvate, malate, glutamate and succinate) approach was used to analyze the mitochondria in permeabilized fibers. Compared to the control group, oxygen flow coupled to ATP synthesis, complex I and complex II was higher in the TA of the HIIT group by 1.5-, 3.0- and 2.7-fold, respectively. In contrast, oxygen consumed by mitochondrial glycerol phosphate dehydrogenase (mGPdH) was 30% lower. Surprisingly, the oxygen flow coupled to ATP synthesis was 42% lower after HIIT in the SOL. Moreover, oxygen flow coupled to ATP synthesis and complex II was higher by 1.4- and 2.7-fold in the GAST of the HIIT group. After HIIT, CS activity increased 1.3-fold in the TA, and H2O2 production was 1.3-fold higher in the TA at sites containing mGPdH. No significant differences in H2O2production were detected in the SOL. Surprisingly, HIIT increased H2O2 production in the GAST via complex II, phosphorylation, oligomycin and antimycin by 1.6-, 1.8-, 2.2-, and 2.2-fold, respectively. Electron leakage was 3.3-fold higher in the TA with G3P and 1.8-fold higher in the GAST with multiple substrates. Unexpectedly, the HIIT protocol induced different respiration and electron leakage responses in different types of muscle.

MiP*art*

C-05 Mitochondrial 16S rRNA is methylated (m1A) throughout vertebrate evolution to maintain protein synthesis and cell growth

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Canonical RNA-DNA-Differences (RDDs), i.e. A-to-G and C-to-U, are important for mammalian sequence diversity. However, non-canonical RDDs have been questioned. Recently, we identified both canonical and non-canonical RDDs (A-to-U and A-to-G) in human mitochondrial 16S rRNA position 947, and suggested that they echo RNA modification. Here, using mass spectrometry and primer extension of 16S rRNA transcripts in human TRMT61B-silenced cells, we show that the RDDs reflect a 1-methyladenosine (m1A) modification. Since these 16S rRNA RDDs were found in all tested human mitochondrial genomes (mtDNAs, ~10,000) and tissues, as well as in 90% of all available vertebrates (N>1700), the m1A modification is likely important. Moreover, the m1A alters a bacteria-to-human structurally conserved interface between the small and large mitoribosomal subunits. However, this mtDNA base is a thymine in 10% of the vertebrates, and guanine in most (95%) bacteria (N>1300), suggesting functional evolutionary alternatives. Since human mtDNA cannot be modified in vivo, we tested this hypothesis in mutant *Escherichia coli*. Strikingly, bacterial strains with the mtDNA base (adenine) had impaired protein synthesis and growth as compared to strains with a thymine or a guanine. Modeling m1A, thymine or guanine in the mitoribosome, demonstrated stabilized structure, in contrast to the mtDNA base (adenine). Hence, either 16S rRNA m1A modification, or thymine or guanine in the DNA, are evolutionary alternatives that stabilize mitoribosomes for proper mitochondrial translation. Furthermore, our findings offer a testable model for the occurrence of non-canonical RDDs throughout the human genome.

C-06 Ancient out of Africa mitochondrial DNA variants associate with distinct mitochondrial gene expression patterns

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Mitochondrial DNA (mtDNA) variants have been used as markers for ancient population migrations. The past decade revealed association of a subset of these variants with altered susceptibility to various disorders, suggesting their functional importance. However, only little is known about the direct genotype-phenotype relationship of ancient mtDNA variants, especially in terms of transcript regulation. Here, by analyzing RNA-seq data from 454 lymphoblast cell lines from unrelated individuals representing major global populations, we show that the variants defining the ancient African genetic background L haplogroup have distinct expression pattern from the rest of the world. This correlation was independent of mtDNA copy number, suggesting that the effect is due to transcript level regulation and not due to variation in mitochondrial quantities. As mtDNA transcription and post-transcription are regulated by nuclear DNA (nDNA) genes we sought for nDNA genes whose expression pattern associated with the L haplogroup. Strikingly, this analysis revealed the best correlation with RNA-binding proteins, of which some are known mitochondrial proteins, thus suggesting post transcriptional candidates to modulate this phenomenon. Our results underline marked change in the regulation of mtDNA transcripts as humans left Africa to populate the rest of the world.

MiP2014

Section D: Mitochondrial physiology in life and death of the cell

D-01 The channel function of the F-ATP synthase complex and its role in the mitochondrial permeability transition

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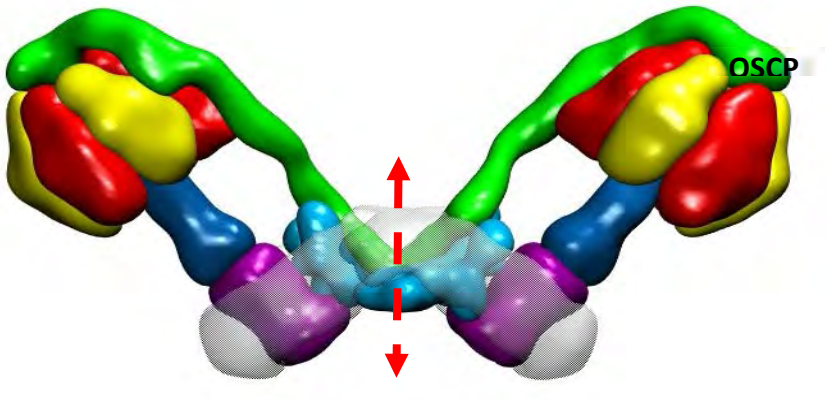
Mitochondria are fundamental to cell life and death because they not only supply the bulk of cellular ATP through oxidative phosphorylation, but also have an essential role in free radical signalling and harbour both pro-apoptotic and anti-apoptotic proteins.
In the presence of oxygen mitochondria operate the exergonic flow of electrons along the respiratory complexes, which is coupled to proton pumping from the matrix to the intermembrane space. The resulting proton motive force drives the backflow of protons through the c-ring in the F_O sector of ATP synthase, leading to the rotation of the F_1 γ, δ, and ε subunits within the F_1 $\alpha_3\beta_3$ subcomplex, thereby supporting the synthesis of 3 ATP molecules for each 360° rotation. Mitochondria also harbor a regulated channel, the permeability transition pore (PTP), whose radius in mammalian mitochondria is estimated to be about 1.4 nm. PTP opening requires matrix Ca^{2+} and oxidative stress and is modulated by many effectors including reactive oxygen species, matrix cyclophilin D, Pi, and matrix pH. When PTP opening becomes long-lasting, it causes collapse of the proton gradient preventing ATP synthesis, as well as equilibration of ionic gradients and solutes leading to mitochondria swelling, cristae unfolding, and eventually rupture of the mitochondrial outer membrane accompanied by release of pro-apoptotic proteins [1].
The nature of the PTP has remained a mystery for 60 years until we recently demonstrated that ATP synthase dimers can reversibly undergo a Ca^{2+}-dependent transition to form the PTP [2]. This finding was made possible by 2 sets of critical observations. The first was that CyPD interacts with the ATP synthase at the lateral stalk connecting F_O to F_1, and that CyPD interaction is favored by Pi and counteracted by CsA with matching effects on the catalytic activity [3]. The second insight was the identification of subunit oligomycin sensitivity conferring protein (OSCP) as the binding site of CyPD and of the F-ATP synthase inhibitor Bz-423. After the demonstration that Bz-423 is a PTP inducer, we showed that in planar lipid bilayer experiments purified dimers of F-ATP synthase form channels activated by Ca^{2+}, Bz-423, and oxidative stress with currents typical of the PTP [2]. Channel formation by F-ATP synthase has been demonstrated in *B. taurus* [2], *S. cerevisiae* [4], and *D. melanogaster* [1] and appears to be a novel property of the eukaryotic complex.
These findings demand an assessment of the modifications of ATP synthase that determine the transition of ATP synthase from an energy-conserving into an energy dissipating device. Our working hypothesis is that the channel forms after a conformational change that would follow replacement of Mg^{2+} with Ca^{2+} at the catalytic sites located in the β subunits. In mammalian mitochondria, binding of CyPD or Bz-423 to OSCP would increase the accessibility of Ca^{2+} to the catalytic sites, resulting in onset of the permeability transition. Once the conformational change has occurred, permeation would take place at the interface between dimers (Figure 1), consistent with the inhibiting effect on PTP

formation of genetic ablation of the e and g subunits in yeast, which also inhibits ATP synthase dimerization [4].



ATP synthase dimers and permeability transition pore formation.
The putative region of channel formation at the interface between dimers (broken arrow) is shown. The F_1 α and β subunits are colored in red and yellow, respectively. The F_1-rotor γ, δ, and ε subunits are colored in shades of blue, the peripheral stalk subunits b, d, F6, and oligomycin sensitivity conferring protein (OSCP) in shades of green. The position of OSCP is indicated. The c-ring and the remaining F_O subunits a, e, f, g, A6L are colored in purple and light blue, respectively. The image (lateral view) has been built starting from the yeast dimer molecular model (PDB ID 4b2q) and superimposing the cryo-electron microscopy map of bovine F-ATP synthase (EMD ID EMD-2091). The molecular model for bovine F-ATP synthase was obtained by superimposing the 3-dimensional structure of the bovine F1-c-ring complex (PDB ID 2xnd) onto each corresponding monomer of the yeast dimer.

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D-02 Kv1.3 channels in mitochondria: Are they important in cellular proliferation?

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Kv1.3 is a member of the delayed rectifier family of voltage-activated potassium channels and has become a major therapeutic target because of its role in autoimmune diseases, in leukaemia, atherosclerosis and obesity and type 2 diabetes. Kv1.3 is not only expressed on the plasma membrane but also on the inner mitochondrial membrane [1] suggesting that some of its actions might be via modulation of mitochondrial function.
This was investigated in HEK293/Kv1.3 cells and human saphenous vein smooth muscle cells (HSVSMCs), using proliferation assays, immunocytochemistry and high resolution respirometry.

HEK293/Kv1.3 cells had significantly increased rates of proliferation compared to WT HEK293 cells. PAP-1, a selective, cell permeant Kv1.3 inhibitor, reduced proliferation in both HEK293/Kv1.3 and HSVSMCs. Channel expression in both the plasma membrane and mitochondria was confirmed using mitotracker in conjunction with immunocytochemical detection of Kv1.3. Mitochondrial expression of the channel was confirmed in both cell types. In addition, the functional expression of the Kv1.3 channel in the plasma membrane was confirmed using patch clamp electrophysiology. High resolution respirometry demonstrated that HEK293/Kv1.3 cells were significantly more metabolically active than WT HEK cells with both increased OXPHOS and glycolytic activity.
Thus mitochondrial Kv1.3 may contribute to increased mitochondrial respiration. This will be further investigated using additional permeant and impermeant inhibitors of the Kv1.3 channel.

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D-03 Mitoenergetic dysfunction triggers a rapid compensatory increase in steady-state glucose flux

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ATP can be produced in the cytosol by glycolytic conversion of glucose (GLC) into pyruvate (PYR). The latter can be metabolized into lactate (LAC), which is released by the cell, or taken up by mitochondria to fuel ATP production by the tricarboxylic acid (TCA) cycle and oxidative phosphorylation (OXPHOS) system. Altering the balance between glycolytic and mitochondrial ATP generation is crucial for cell survival during mitoenergetic dysfunction, which is observed in a large variety of human disorders including cancer [1].
To gain insight into the kinetic properties of this adaptive mechanism we here determined how acute (30 min) inhibition of OXPHOS affected cytosolic GLC homeostasis. GLC dynamics were analyzed in single living C2C12 myoblasts expressing the fluorescent biosensor FLII12Pglu-700μδ6 (FLII, [2]). Following *in situ* FLII calibration, the kinetic properties of GLC uptake (V1) and GLC consumption (V2) were determined independently and used to construct a minimal mathematical model of cytosolic GLC dynamics [3].
After validating the model, it was applied to quantitatively predict V1 and V2 at steady-state (*i.e.* when V1=V2=Vsteady-state) in the absence and presence of OXPHOS inhibitors. Integrating model predictions with experimental data on LAC production, cell volume and oxygen consumption revealed that glycolysis and mitochondria equally contribute to cellular ATP production in control myoblasts. Inhibition of OXPHOS induced a 2-fold increase in Vsteady-state and glycolytic ATP production flux. Both in the absence and presence of OXPHOS inhibitors, GLC was consumed at near maximal rates, meaning that GLC consumption is rate-limiting under steady-state conditions.
Taken together, we here demonstrate that OXPHOS inhibition increases steady-state GLC uptake and consumption in C2C12 myoblasts [3]. The latter activation fully compensates for the reduction in mitochondrial ATP production, thereby maintaining the balance

between cellular ATP supply and demand. The underlying mechanistic aspects and further consequences of this phenomenon [*e.g.* 4,5] are currently investigated.

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D-04 Moscow news: two more representatives of sodium motive force generators (Na^+-*cbb*$_3$ oxidase and Na^+-bacteriorhodopsin); natural delay of the aging program (neoteny) in mammals, namely in naked mole rat and "naked ape" (human)

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M.S. Muntyan and coworkers [1] in our group succeeded in describing Na^+ - motive terminal oxidase from extremely alkalophilic bacterium *Thioalkalivibrio versutus* living in alkaline Siberian soda lake at saturating salt concentration. At such conditions, this respiring bacterium cannot employ Mitchellian H^+ cycle since two constituents of proton motive force, Δψ and ΔpH, are oppositely directed. It was found that (i) respiration and Δψ in *Th. vesutus* show pH optimum at pH 9, (ii) both respiration and Δψ at this pH are stimulated by Na^+, whereas K^+, Li^+ and $choline^+$ are ineffective, (iii) respiration is coupled to extrusion of Na^+ from the cell or right-side out vesicles, the extrusion being stimulated by protonophorous uncouplers, (iv) *Paracoccus denitrificans* mutant lacking all terminal oxidases and displaying no respiration-driven Na^+ pumping became capable of such a pumping after expression of *Th.versutus cbb*$_3$. The above listed data directly demonstrated for the first time existence of a terminal oxidase pumping Na^+ instead of H^+.

In 2013-2015, a Na^+ - pumping bacteriorhodopsin was described in several marine flavobacteria [2]. In our group, Bogachev and coworkers expressed Na^+ - bacteriorhodopsin (NaR) from *Dokdonia sp.* PRO95 in *E.coli* [3]. It was found that such *E.coli* cells show light-dependent Na^+ pumping which is stimulated by a protonophores. *E.coli*-expressed NaR was incorporated into proteoliposomes that were attached to phospholipid – impregrated collodion film. Illumination of such a film by a single 15 ns laser flash resulted in generation of Δψ up to 200 mV, directly measured by two electrodes separated by the film. The following four steps were identified in the NaR photocycle: $NaR_{519} + hv \rightarrow K_{585} \rightarrow (L_{450} \rightarrow M_{495}) \rightarrow O_{585} \rightarrow NaR_{519}$. The first step was too fast to be separated from the second one. Contributions of steps (2), (3) and (4) to the total Δψ proved to be 15, 15 and 70 %, respectively. Step (3) was the only one which was Na^+

dependent. Li^+, but not K^+, substituted for Na^+. An interesting possibility consists in that NaR was evolutionary primary energy generator in biosphere.

For several years, our group is studying the role of mitochondria and mitochondrial reactive oxygen species (mROS) in aging program of mammals. Naked mole rat represents the most interesting model to study aging of mammals. This rodent is as small as a mouse but lives at least 10 times longer. For a naked mole rat, cancer, cardiovascular and brain pathologies, diabetes, infections and other aging-stimulated diseases are absent from the list of reasons of the death, mortality is very low and age-independent, fertility seems to not decrease with age. E. Rüppell who discovered naked mole rat stressed that its adult form resembles in some aspects newborn rodents of the same *Bathyergidae* family, being much smaller and having, like *newborn* rodents, no fur, auricles and scroptum. Later some other features typical to newborn mammals were also revealed, i.e. inability to maintain stable body temperature below thermoneutrality, underdevelopment of vomeronasal organ, low expression of insulin and IGF1 and high expression of IGF2 genes. FAS – activated proinflammatory serine/threonine kinase (FASTK) is absent from naked mole rat. This suggests that "inflammoaging" is suppressed in these animals. mROS seem to play a key role in aging: $O_2 \rightarrow$ mROS $\rightarrow H_2O_2 \rightarrow$ apoptosis, necrosis $\rightarrow$ decrease in cellularity of organs $\rightarrow$ decay of functions. It was found that extracellular concentration of potent antioxidant hyaluronan is very high in naked mole rat cell cultures, which explains (a) why added H_2O_2 fails to induce apoptosis of these cultures and (b) very high resistance of hippocampal neurons of naked mole rat to anoxia / reoxygenation. Experiments performed by our group in cooperation with the group of Th. Hildebrandt (Berlin) showed that heart mitochondria from naked mole rat (i) contain lower concentration of adenine nucleotides and (ii) respire much faster after exhaustion of added ADP than before ADP addition. The effect (ii) might be regarded as a mechanism lowering mROS generation. Both effect (i) and (ii) are inherent in mitochondria from embryo and neonatal rodents [4]. Generally, longevity of naked mole rats can be considered as the precedent of neoteny, i.e. extension of youth and delay of aging, a phenomenon known in amphibian (the axolotl / salamander case).

Neotenic aspect of human ontogenesis was investigated since 1926 when L. Bolk suggested that this process differ from ape ontogenesis by extension of childhood and youth. The main argument in favor of such a possibility consisted in that ape embryos and young apes resemble much more humans than apes. In other words, apes are brutalized from a human-like child when they are transformed from young to adult. The following neotenic traits were found to be inherent in the adult humans and young apes but not in adult apes: relatively large skull, thin skull bones, small fangs, flattered and broadened face, ear shape, small nose, hairless body, absence of baculum, short limbs compared to torso, longer legs compared to arms, smaller mass of skeletal muscles. An important discovery was recently done by Ph. Khaitovich and coworkers. The authors showed that initiation of transcription of a large group of genes encoding proteins of prefrontal brain cortex in prenatal and early postnatal periods occurs much later in humans than in chimpanzee and rhesus macaques. Among these genes, there are those encoding synaptic proteins. Nevertheless, the size of the adult brain is larger in humans than in apes. Finally, humans become much more developed in cognitive aspects but underdeveloped in such aspects as physical (muscular) force. In particular, construction of some parts of skeleton (e.g. feet) is clearly more primitive in humans than in apes, as if the common ancestor of humans and apes was more human-like than ape-like. Curves of mortality vs. age for humans start at extremely low mortality in youth (it is much lower than for young apes). However, in elderly the human mortality values eventually become higher than for apes. An impression arises that in humans a mechanism controlling results of operation of aging program stimulates this program in the end of our life.

In conclusion, extension of youth by delay of aging is impossible to imagine within the framework of the concept of stochastic (non-programmed) aging but can be explained if aging is programed [4].

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D-05 Regulation of mitochondrial respiration and apoptosis by cytochrome *c* phosphorylation

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Mammalian cytochrome *c* (Cyt*c*) is a small globular protein and functions as a mobile electron carrier between complexes III and IV of the electron transport chain (ETC). In addition, Cyt*c* participates in type II apoptosis, during which it is released from the mitochondria. Considering the key role of Cyt*c* in cell life and death, it can be expected to be tightly regulated. We have previously shown that cell signaling pathways target mitochondrial proteins including Cyt*c*, which is phosphorylated on two distinct tyrosine residues in heart and liver. We show here by mass spectrometry that Cyt*c* isolated from mammalian kidney tissue is phosphorylated on a novel residue, threonine 28. To functionally study this phosphorylation we used in vivo-phosphorylated Cyt*c* and phoshomimetic Thr28Glu Cyt*c*. The latter replacement leads to a reduction of the Cyt*c* redox potential and a partial inhibition of respiration, or 'controlled respiration' in the reaction with cytochrome *c* oxidase compared to unphosphorylated wild-type Cyt*c*. These results fit our model that under healthy conditions ETC proteins are phosphorylated to limit electron flux in the electron transport chain, which in turn prevents a hyperpolarization of the mitochondrial membrane potential, a known cause of reactive oxygen species (ROS) production and trigger of apoptosis.

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D-06 High-resolution measurement of mitochondrial membrane potential and respiration – comparison of potentiometric and fluorometric methods

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The relationship between mitochondrial (mt) membrane potential ($\Delta\Psi_{mt}$) and respiration remains poorly understood due to methodological limitations and the complexity of interrelations between fluxes (respiration) and forces (mt-membrane potential as the electric component of the protonmotive force). $\Delta\Psi_{mt}$ reporter cations inhibit and uncouple mitochondrial respiration depending on type, concentration, and the specific electron transfer segment under investigation. Potentiometric signals based on ion-sensitive electrodes (ISE) and optical signals from fluorophors require different calibrations (linear and non-linear), transformations (log-lin), corrections for unspecific binding, and conversion to electric units [mV] of mt-membrane potential. Signals of ISE reflect the log concentration of the free reporter ion (TPP^+) outside of mitochondria, providing a quantitative basis for calculation of $\Delta\Psi_{mt}$ [mV] in the range of high membrane potential. On the other hand, fluorescence may comprise a mixture of signals from a fluorescent dye in its free state and bound to membranes or proteins, and corrections may be difficult for obtaining the concentration of the free dye [1]. A direct comparison of results with these two approaches, application of different $\Delta\Psi_{mt}$ reporter molecules and combination with respirometry is required for a critical analysis of mitochondrial membrane potential.
In this study of isolated mouse brain mitochondria (37 °C, MiR05), we used the Oxygraph-2k with the O2k-TPP^+ ISE-Module with tetraphenylphosphonium (TPP^+) as a reporter ion, or with the O2k-Fluo LED2-Module with safranin or tetramethylrhodamine methyl ester perchlorate (TMRM). All signals required quantitatively important corrections for chemical background effects (responses to titration of substances such as ADP in the absence of mitochondria). The sensitivity of the potentiometric or fluorometric signal of these probes to inhibition of mt-respiration was compared in Complex I- (CI-) or CII-linked substrate states in the absence of adenylates (LEAK, *L*), at saturating ADP (OXPHOS, *P*), and in uncoupler titrations (ETS capacity, *E*).
CI-linked OXPHOS respiration was inhibited by TPP^+ by <5% up to 3 µM, without any inhibitory effect on CII-linked OXPHOS capacity. Both fluorescent dyes, 2 µM Safranin and 1.5 µM TMRM, inhibited substantially (more than 30%) CI-linked OXPHOS respiration, and to a lower extent (around 10%) CII-linked respiration. 1.5 µM TMRM gradually uncoupled CII_L respiration, which is particularly problematic for evaluation of $\Delta\Psi_{mt}$. The TPP^+ electrode was very sensitive to inhibition of respiration in states CI_L and CI_P by rotenone, and in state CII_L by malonate. However, the TPP^+ signal was practically insensitive to inhibition in the CII_P state when the mt-membrane potential is low already in the uninhibited state. The increase of TPP^+ concentration from 1.5 to 3 µM did not change the sensitivity of the response. In contrast, the fluorescence signal of 2 µM safranin responded well also to the inhibition of CII-linked OXPHOS respiration [2]. When the concentration of safranin was decreased to 1 µM, the fluorometric sensitivity was lost to detect the response to inhibition of CII_P. The fluorescence signal of 1.5 µM TMRM was less sensitive to inhibition of CI_P and CII_L in comparison to safranin or TPP^+, and less sensitive to inhibition of CII_P respiration when compared to safranin. The signal of 1 µM TMRM was insensitive to inhibition of CII_P.

Based on the these results, we recommend using the TPP^+ electrode for evaluation of $\Delta\Psi_{mt}$ with CI-linked and CI&II-linked respiration and 2 µM safranin for evaluation of $\Delta\Psi_{mt}$ with CII-linked respiration, which is more sensitive in the range of low $\Delta\Psi_{mt}$ compared to TPP^+. The fluorometric method should be elaborated for evaluation of $\Delta\Psi_{mt}$ for each type of mitochondrial preparation and protein concentration [3] that has to be kept constant for comparison of different samples in any set of experiments. Before reporting results uncritically as $\Delta\Psi_{mt}$ [mV], inhibitory and uncoupling effects on respiration, sensitivity and linearity, and confounding effects of unspecific binding need to be taken into account for various mt-preparations (isolated mt, homogenate, permeabilized cells and fibres), and for various probe/sample concentration ratios.

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D-07 Simultaneous measurement of mitochondrial respiration, hydrogen peroxide production, and NADH autofluorescence to assess mitochondrial function

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An increasing number of studies point to mitochondria as key regulators of many physiological and pathological conditions, related to life style (including physical exercise and nutrition), neurodegenerative diseases, metabolic disorders, inflammatory diseases, cancer, heart failure, and aging. Moreover, mitochondria are an important source of reactive oxygen species (ROS), which are needed for cell signaling. However, an increase in ROS production generates an oxidative stress which is implicated in the pathogenesis of many diseases. In particular, the NADH redox state is related to ROS production. Succinate is a substrate of succinate dehydrogenase (CII). For analysis of mitochondrial function with CII- linked substrates, rotenone (inhibitor of CI) is added to prevent accumulation of oxaloacetate (Oa), which is a strong competitive inhibitor of CII [1]. Ischaemic accumulation of succinate has been related to mitochondrial ROS production during reperfusion by reverse electron transfer [2]. In the present study, we used succinate with and without rotenone as a model for pathophysiological mitochondrial ROS-production.
We investigated the effect of succinate (10 mM) alone, S, or succinate (10 mM) with rotenone (0.5 µM), S(Rot), on mitochondrial respiration, hydrogen peroxide (H_2O_2) production and NADH redox state in cardiac isolated mitochondria from C57BL/6 mice. Respiration media (37 °C) were optimized for the specific protocols. High-resolution respirometry (HRR) was applied with the O2k-Fluorometer (OROBOROS, Innsbruck,

Austria). H_2O_2 production was measured simultaneously using Amplex Ultrared [3]. NAD(P)H autofluorescence was monitored in a prototype NextGen-O2k, which combines HRR with O2k-Spectrofluorometry. Step changes of the fluorescence signal were calibrated with NADH and corrected for changes observed in chemical background tests. These methods allow analyzing simultaneously relevant bioenergetic parameters to assess mitochondrial function.

Oxygen consumption levels were similar for S and S(Rot) in the LEAK state (without adenylates). However, H_2O_2 production was substantially higher with S in LEAK state. Adding ADP (2 mM) to S(Rot) to induce OXPHOS capacity, mitochondrial respiration increased by 70%. In contrast ADP titration to S induced a decline in respiration by 30% with respect to the LEAK state, which is the so-called "succinate paradox" [4]. H_2O_2 production, however, declined to similar low levels after addition of ADP in protocols S and S(Rot). Rot added after the "succinate paradox" state displayed a stimulatory effect on mitochondrial respiration, restoring OXPHOS capacity comparable to S(Rot). When Rot was added to isolated mitochondria in the absence of succinate and ADP, NADH levels increased, as expected when endogenous substrates support dehydrogenase activity at a low level of residual oxygen consumption. Addition of S to mitochondria increased NADH levels with and without Rot in the LEAK state. Addition of ADP to S(Rot) induced a significant increase of the NADH fluorescence signal, which was entirely explained by the chemical background effect of ADP titration, such that NADH levels remained identical in the LEAK and OXPHOS states. In contrast, NADH levels declined significantly upon ADP without Rot. Further titration of malate inhibited S(Rot)-OXPHOS capacity (with an unexpected concomitant decline of NADH), and inhibited further respiration with S alone without change in NADH.

Fluorometry and spectrofluorometry integrated into the NextGen-O2k provide bioenergetically relevant parameters to analyze mitochondrial function. Accumulation of Oa in the presence of S and absence of rotenone causes reverse electron transfer, which induces a pathological increase of ROS production. After addition of ADP to S(Rot), NADH did not change significantly, indicating that the malate dehydrogenase equilibrium was maintained at constant low Oa concentration, supporting high activity of CII. However, the decrease of NADH after addition of ADP to S indicated a shift to higher Oa levels, which then may explain the observed inhibition of CII-linked respiration. On the other hand, antioxidant systems are challenged in the S protocol and thus may contribute to the depletion of NAD(P)H. Taken together, the "succinate paradox" represents a relevant model for the study of physiological and pathological control of ROS production, redox and respiratory control.

The project NextGen-O2k is supported by the "Technologieförderungsprogramm - Tiroler Innovationsförderung" of the Tyrolean Government.

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D-08 Mitochondrial complexes I and II behave differently in two anoxia-tolerant species: adaptive mitochondrial plasticity may be related to the ability to undergo metabolic depression

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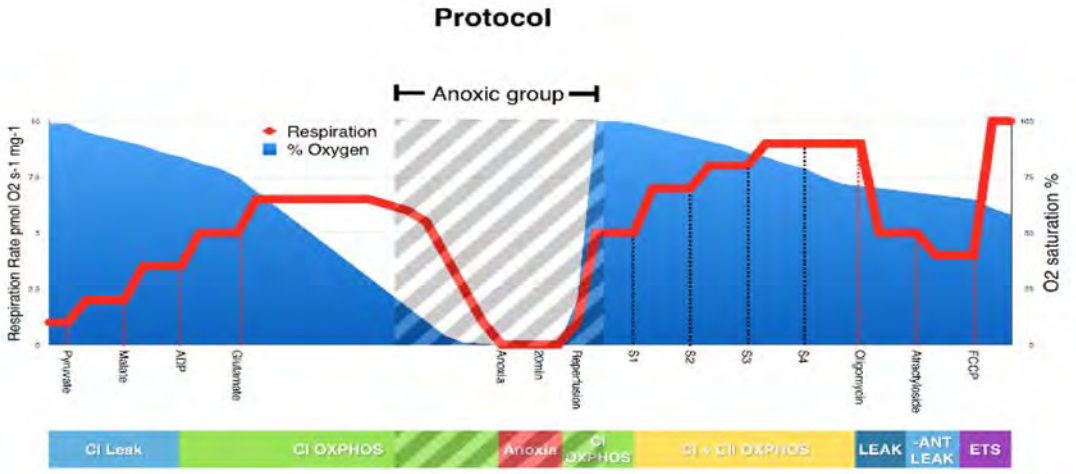
An adequate oxygen supply is crucial for vertebrate survival because ATP is almost exclusively produced by mitochondria though oxidative phosphorlyaltion (OXPHOS). Anoxia leads to ATP depletion and altered metabolic pathways including succinate accumulation which during re-oxygenation triggers a reverse of electron flow to CI accompanied by an enhanced deleterious reactive oxygen species (ROS) production [1]. A few species are able to survive prolonged periods of hypoxia and anoxia at tropical temperatures [2,3]. Two closely related elasmobranchs have very different adative responses to anoxia [2] while both can survive prolonged periods of anoxia the epaulette shark enters a phase of metabolic depression in response to hypoxia or anoxia while the grey carpet shark maintains its metabolic rate but releases additional red blood cells to prolong survival. Mitochondria in heart fibers from the anoxia tolerant epaulette shark maintained mitochondrial efficiency with a low ROS release in response to oxygen limitation, including anoxia [4]. The extent of mitochondrial plasticity in response to diminished oxygen (hypoxia or anoxia) and the triggers involved in this adative process are currently being investigated in our laboratories.



Our aim was to determine the response of mitochondrial complexes to elevated succinate after a bout of diminished oxygen in two closely related anoxia tolerant species. Sharks were acclimated in aerated sea-water (100L tanks) held at 22°C and were not fed 24 hours prior to sampling. Sharks were euthanised, the cerebellum isolated and homogenised in cold MiRO5. Mitochondrial respiration was measured using high resolution respirometry (OROBOROS) with a SUIT protocol. Respiration flux (pmol O_2 s^{-1} mg^{-1}) was determined using DatLab 6.0 and statistical analysis were performed using SPSS™. Calculations were made to determine CI capacity, CII activation and coupling efficiency with and without a 20 min episode of anoxia followed by reoxygenation and stepwise succinate titrations (using a TIP2K microPump).

CI OXPHOS was examined under i) normoxia; ii) hypoxia (PO_2 = 1%); and iii) after 20mins of anoxia (followed by reoxygenation). Respiration rates were normalised to the respective normoxic CI OXPHOS for each species in order to estimate the potential loss of CI capacity. Exposure to hypoxia or anoxia/reoxygenation induced a decrease of CI capacity in both species. Post-anoxia, the CI capacity of mitochondria from the grey carpet shark was significantly less than those of the epaulette shark. Furthermore, the coupling efficiency of mitochondria from the epaulette shark increased significantly after anoxia/reoxygenation compared to those from the grey carpet shark.
After 20 mins of anoxia followed by 5 minutes of reoxgyenation, the CII substrate succinate was titrated from physiological concentrations (0-2mM) until OXPHOSmax was reached. Succinate-stimulated mitochondrial respiration was normalised to the ETSmax measured for each species. Analysis revealed that succinate stimulated CII respiration following 20 mins of anoxia compared to CII respiration in normoxia (controls) was significantly lower in the epaulette shark, reaching a 60% decrease in CII respiration at low levels of succinate (0.5mM). In contrast, in the grey carpet shark the succinate stimulated CII respiration following 20 mins of anoxia compared was significantly higher than the CII respiration in their controls, reaching a 40% increase at 2.5mM.
Taken together these data indicate that the ES but not the GCS was able to maintain coupling efficiency and CI capacity even after 20 mins of anoxia followed by reperfusion. The ES but not the GCS responded to succinate accumulation by significantly decreasing CII respiration. Taken together these data suggest that the adaptive plasticity of ES mitochondria would support entry into metabolic depression as a protective response to anoxia/reoxygenation

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D-09 Mitochondrial responses to thermal stress: is the ability to withstand anoxia-induced stress associated with cross-tolerance to thermal stress?

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The thermal limit of cardiac mitochondrial efficiency could be a major determinant of species distribution [1]. The effect of high temperature on brain mitochondria has been less well explored. We examined the effect of 6 temperatures (20°C, 25°C, 30°C, 37°C, 40°C and 45°C) on brain mitochondrial function in homogenates from two Orectolobiform sharks: the Epaulette shark (*Hemiscyllium ocellatum*), which undergoes metabolic depression in response to the stress provided by

oxygen limitation (anoxia) [1, 2] and the Grey carpet shark (*Chilloscyllium punctatum*) which does not respond to anoxia-induced stress by depressing its metabolism [3]. Both of these sharks can occupy shallow reef flats and estuarine habitats potentially exposing them to severe temperature-induced stress during summer low tides. We measured the effect of each temperature on: i) mitochondrial coupling efficiency; ii) non-phosphorylating proton leak from mitochondria; and iii) changes in substrate utilisation for complex I and complex II.

High resolution oximetry with a multiple substrate-inhibitor protocol [4] revealed that for both species: mitochondrial coupling (efficiency) was greatest at 25°C, and maintained at 30°C but was 25% lower at 37°C and 50% lower at 40°C. Mitochondria in both species were totally uncoupled at 45°C.

Despite an exponential increased in proton leak as temperature increased, Epaulette mitochondria maintained their electron transport system in coupled mitochondria at 25-37°C, while Grey carpet shark mitochondria showed a 30% decrease in mitochondrial efficiency at 37°C compared to 25°C. Examination of substrate utilisation revealed that mitochondria from Epaulette shark, which undergoes metabolic depression in response to the stress of oxygen limitation (hypoxia and anoxia) had a more stable complex 1 utilisation than Grey carpet sharks, especially at 37°C. It is possible that the mitochondria from the Epaulette shark have adaptations, associated with the ability to enter a state of metabolic depression, that enable them to withstand other stressors.

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D-10 A new look at the bioenergetics of the bloodstream *Trypanosoma brucei* mitochondrion

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The infective bloodstream stage (BS) of *Trypanosoma brucei* possesses a single reduced mitochondrion that lacks the proton pumping respiratory complexes III and IV. Interestingly, the essential mitochondrial (mt) membrane potential ($\Delta\psi_m$) is maintained by a reverse activity of F_oF_1-ATPase, which translocates H^+ into the intermembrane space at the expense of ATP. Meanwhile, dyskinetoplastic (Dk) trypanosomes lacking the mt encoded A6, an essential subunit of the F_o proton pore, alternatively maintain their $\Delta\psi_m$ by combining the hydrolytic activity of the matrix-facing F_1-ATPase and the electrogenic exchange of ATP^{4-} for ADP^{3-} by the ADP/ATP carrier (AAC). While the AAC protein expression levels do not significantly differ between BS and Dk trypanosomes, the sensitivity of these cells to AAC inhibitor carboxyatractyloside was approximately 40-folds higher for Dk cells compared BS trypanosomes. This result would suggest that AAC activity is not as important for BS as for Dk cells and thus the ATP for maintaining the $\Delta\psi_m$ in BS cells is provided by mt substrate

phosphorylation pathway(s). Indeed, RNAi silencing of AAC in BS trypanosomes has neither effect on growth in vitro nor on $\Delta\psi_m$. Which of the mt substrate phosphorylation pathway(s) for ATP production are important in BS trypanosomes is being further investigated and the revisited mt energy metabolism map of the infectious stage of the parasite will be presented.

MiP2014

Section E: Mitochondria in whole body physiology

E-01 Mitochondrial respiration in homogenized small tissue biopsies from the M. Vastus lateralis of patients with Huntington's Disease before and after cycling exercise

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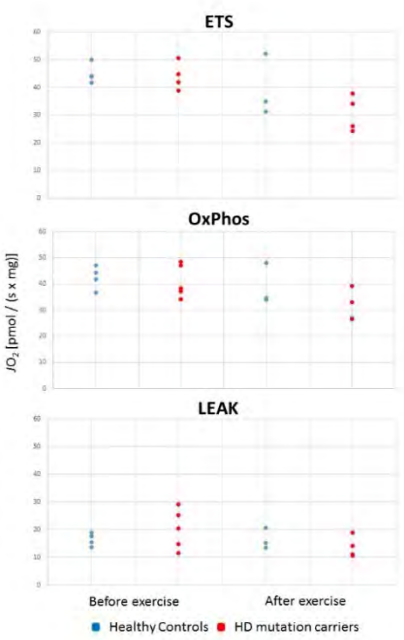
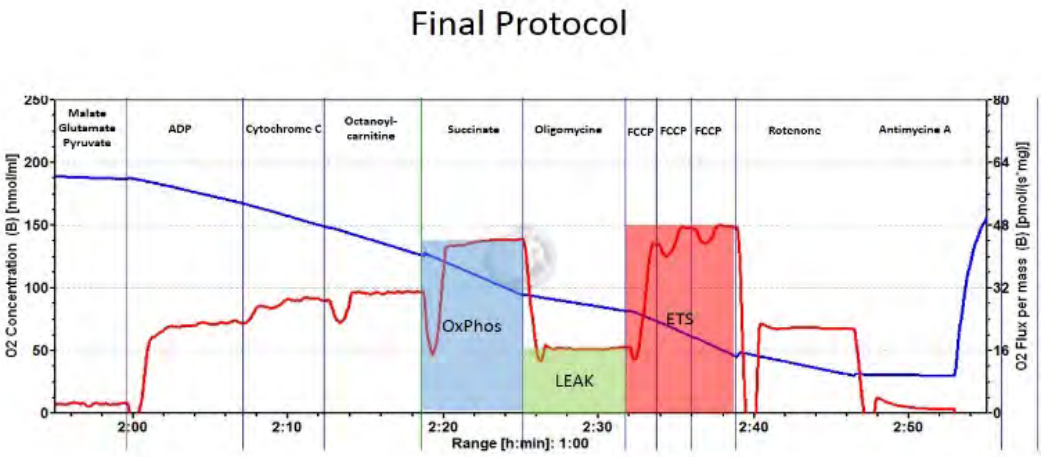
Introduction: Mitochondrial respiration is assumed to be severely affected by the presence of mutant huntingtin in HD patients. However, mitochondrial function remains difficult to be quantified in-vivo. Therefore, we used minimal volume tissue biopsies (4-8 mg) obtained from the m. vastus lateralis of HD subjects (mutation carriers) who volunteered to participate to our study, for quantifying mitochondrial respiration by means of the *high-resolution respirometry* before and after a standardized cycling exercise.

Methods: all patients gave written consent to participate to our study; the study protocol has been approved by the ethical committee of our institution. The tissue samples homogenates were put into the chambers of the O2K®-Oxygraph (Oroboros Instruments, Austria) and continuously stirred at 37°C. Mitochondrial respiration was quantified by adding complex I (10 mM Pyruvate, 5 mM Malate, and 10 mM Glutamate) and complex II (10 mM Succinate) substrates in the medium containing the homogenate and 5 mM ADP. Then 5 µM oligomycine was added to inhibit the ATP-synthase in order to obtain the LEAK-respiration state as an indicator of mitochondrial coupling. This step was followed by the addition of 1 µM of the uncoupler FCCP in order to achive the maximum respiration in the uncoupled state and the coupling (LEAK/ETS)-ratio. After blocking mitochondrial respiration by 0.5 µM rotenone and 5 µM Antimycine A, the complex IV activity was selectively quantified by adding 2 mM Ascorbate and 0,5 mM TMPD. Here we present preliminary data from the first 3 patients included into the study.



Results: a typical experiment as well as the preliminary data obtained yet in healthy controls and HD mutation carriers are presented in the figures below.



Conclusions: Our preliminary data do not allow definitive conclusions yet but they suggest that mitochondrial respiration can be reliably quantified in minimal volume needle biopsies from the m. vastus lateralis of human subjects. This test may be therefore used in the to quantify mitochondrial dysfunction as well as the effects of physical training during the course of the disease.

E-02 The metabolically inert environmental pollutants perfluorinated fatty acids directly activate uncoupling protein 1 in brown-fat mitochondria

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The environmental pollutants perfluorooctanoic acid (PFOA) and perfluorooctane sulfonate (PFOS) are degradation products of polyfluorinated surfactants and polymers that due to their unique surface-active properties, the stability of their C-F bonds and their thermal resistivity, are utilized in a wide variety of products, including fire-fighting foams, lubricants, paints, cosmetics and ski-wax. PFOS/PFOA cause a dramatic reduction in the size of the major adipose tissue depots when they are added to the food of mice. A large part of the effect of PFOA/PFOS on food intake was dependent on the presence of the uncoupling protein 1 (UCP1) in the brown adipose tissue [1].
We have examined here the possibility that PFOA/PFOS can directly (re)activate UCP1 in isolated mouse brown-fat mitochondria. In wildtype brown-fat mitochondria, PFOS and PFOA overcame GDP-inhibited thermogenesis, leading to increased oxygen consumption and dissipated membrane potential. The absence of this effect in brown-fat mitochondria from UCP1-ablated mice indicated that it occurred through activation of UCP1. A competitive type of inhibition by increased GDP concentrations indicated interaction with the same mechanistic site as that utilized by fatty acids. The stimulatory effect of PFOA/PFOS was not secondary to nonspecific mitochondrial membrane permeabilisation or to ROS production.

Thus, metabolic effects of perfluorinated fatty acids could include direct brown adipose tissue (UCP1) activation. The possibility that this may lead to unwarranted extra heat production and thus extra utilization of food resources, leading to decreased fitness in mammalian wildlife, is discussed, as well as possible negative effects in humans. However, a possibility to utilize PFOA/PFOS-like substances for activating UCP1 therapeutically in obesity-prone humans may also be envisaged.

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E-03 Changes in energy transfer regulation during development and aging

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In adult cardiomyocytes the main energy transfer pathway from mitochondria to the energy consumption sites is creatine kinase-phosphocreatine (CK-PCr) shuttle, while in the situation of high workload or pathology adenylate kinase (AK) and hexokinase (HK) pathways could compensate the energy requirements in some extent [1]. During postnatal period quick structural and functional changes in energy metabolism take place – rearrangement of mitochondria into regular pattern, distinctive for cardiomyocytes, and formation of Intracellular Energetic Unit (ICEU). The CK-PCr system activation is the last step of the formation of adult bioenergetic metabolism [2]. The alterations taking place in the energy transfer and kinetics of OXPHOS during healthy aging is till now have been less studied. In pathology of the heart the level of PCr have prognostic value in diagnosis [3]. Therefore, it is important to study the alteration of CK shuttle in aging as well as to find out the role of the alternative energy transfer systems.
The main methods used were confocal microscopy, high resolution respirometry with Oxygraph-2K, and real time quantitative PCR and western blot analysis.
Results of the study showing that on the seventh postnatal day AMP activated mitochondrial respiration achieve the equal level with maximal OXPHOS capacity (with 2mM ADP). During the same period mitochondrial CK activates. The adult energy metabolism has formed by the age of 3 month. In parallel isoforms levels of AK1 and AK3 increase significantly. HK expression profile changes from HK I, which is the main HK isoform in neonatal cardiomyocytes, to HK II that is predominant in the adult heart. These results demonstrate that the AK phosphotransfer system plays an important role in ATP turnover during CK system maturation.
In the aging cardiomyocytes gender related differences could be followed. Compared to adult the values of the apparent Michaelis-Menten constant Km(ADP) for 12- and 18-month male cardiac cells increases, as for females the value has even slightly decreased. At the same time creatine-stimulated respiration rate decreases in the cardiomyocytes of both sexes.
Results of the study allow to conclude that maturation of the ICEU is closely related to the formation of phosphocreatine/creatine kinase system that ensures the increasing energy

demands. In developing cardiomyocytes AK pathway plays an important role in energy transfer till the CK-mediated phosphotransfer system is completely formed and functional. During aging the energy transfer regulation is influenced by gender differences and the CK phosphotransfer pathway efficiency is declined.

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E-04 Individual variation in whole-organism performance is related to mitochondrial properties at high temperatures

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As global temperatures rise, there is a growing need to understand the proximate causes that determine the boundary of an organism's thermal niche. Individuals can vary in how they respond to temperature increases, but the mechanisms responsible for this inter-individual variation are unclear.

Here we tested the hypothesis that individual performance at high temperatures depends on mitochondrial respiratory properties. We assessed the food intake in an *ad libitum* diet, rate of growth in mass and length, and mitochondrial function in liver and white muscle of juvenile brown trout *Salmo trutta* gradually acclimated to the high testing temperature (19°C).

Food intake and growth rate were highly variable amongst fish: food intake varied by 10 among individuals, some fish did not grow, some lost body weight whilst others grew and increased body mass. Around 50% of the individual variation in food intake was explained by liver and muscle mitochondrial function. Individuals with the highest leak respiration in liver and muscle exhibited the lowest food intake. Moreover, food intake was worst in individuals with a lower muscle phosphorylating respiration, and in turn a lower respiratory control ratio (RCR). After accounting for food intake, no aspect of mitochondrial function could explain individual variation in growth.

Our results demonstrate that individuals with higher leak respiration and lower coupling in mitochondria (as estimated by the RCR) had the poorest performance, suggesting that their capacity for ATP production at 19°C could not support an adequate foraging. Our findings suggest that differences in the ability of mitochondria to generate ATP could shape the boundary of an individual's thermal niche.

E-05 Comparative mitochondrial physiology: OXPHOS and ETS capacity in permeabilized fibres of canine superathletes

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Comparative mitochondrial physiology strongly relies on quantitative data sets for comparison of OXPHOS capacities and respiratory control patterns between species and tissues. Combination and interpretation of a wide variety of studies requires standardization of respiratory protocols, implementation of quality control criteria, and consistency of normalization. Previously we described a reference method for the application of a cytochrome c threshold as exclusion criterion in mitochondrial OXPHOS analyses [1]. Alaskan sled dogs (N=6) were studied 72 to 120 h after finishing a competitive 1,000 mile race within less than nine days. Permeabilized fibres (0.81-1.28 mg ± 0.12 SD wet weight per assay) were prepared from needle biopsies and immediately studied by high-resolution respirometry [2] using 12 chambers in parallel (OROBOROS Oxygraph-2k). Compared to human skeletal muscle fibres, the canine samples were more delicate to handle, highly sticky and appeared to be fragile, disintegrating to various degrees during substrate-uncoupler-inhibitor titration (SUIT) protocols in mt-respiration medium MiR06Cr. Two substrate-uncoupler-inhibitor titration protocols were applied (Fig. 1). SUIT1 emphasized substrate control with fatty acid oxidation (FAO) versus carbohydrate oxidation capacity, whereas the focus of SUIT2 was on coupling control with CI-linked substrates. Both protocols were designed to provide a common reference state of CI&II-linked ETS capacity, in comparison to separate Complex I- and Complex II-linked substrate states (CI versus CII).

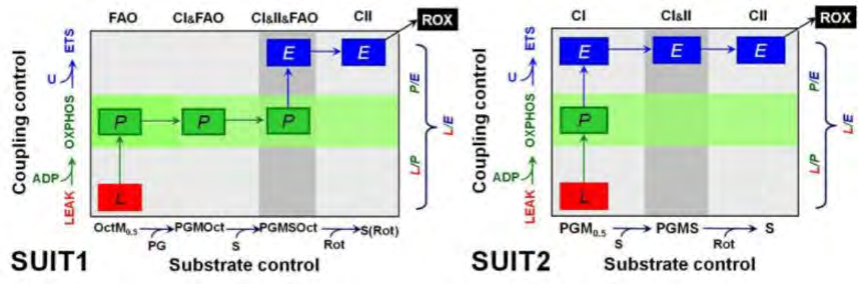

Figure 1. Coupling/substrate control diagrams. Coupling states: LEAK, *L*; OXPHOS, *P*; ETS or electron transfer system capacity, *E*. Substrate states differ in SUIT1 and SUIT2. Octanoylcarnitine, Oct 0.2 mM; malate, M 0.5 mM (OctM: FAO); pyruvate; P 5 mM; glutamate, G 10 mM (PGM: CI); succinate, S 10 mM (CI&II); rotenone, Rot 0.5 µM (CII); residual oxygen consumption, ROX with malonate, 5 mM, and antimycin A, 2.5 µM.

CI&II-linked ETS capacity was 262±41 pmol·s^{-1}·mg^{-1} W_w independent of the presence or absence of 0.2 mM octanoyl carnitine (FAO). This is the highest value so far reported for mammalian skeletal muscle. Top human endurance athletes have a CI&II-linked ETS capacity approaching 200 pmol·s^{-1}·mg^{-1} W_w [3], compared to 153±19 pmol·s^{-1}·mg^{-1} W_w in competitive racing horses [4].

Supported by K-Regio project MitoFit.

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E-06 Effects of an ultramarathon on mitochondrial respiration, oxidative damage and repair in human platelets

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Introduction: Acute strenuous exercise is linked to severe inflammatory responses [1], alterations of mitochondrial function of human skeletal muscle and increased oxidative stress [3]. Due to the invasive nature of muscle biopsies, minimally-invasive alternatives to study mitochondrial function in tissues such as blood cells are gaining significance. While mitochondrial function in human platelets and lymphocytes has been characterized in various disease states, the effect of strenuous exercise on this cell type, is limited to one study [2]. Therefore, we investigated the influence of an ultramarathon on mitochondrial respiration and H_2O_2-production in human platelets.
Methods: After informed consent, 10 male recreational athletes [mean age: 39.9 yrs; BMI 24.9] who participated in a competition over 67 km and approximately 4500 m of ascent, were included in our study. Baseline measurements were performed on the day before the competition and follow-up sampling was performed up to 15 min after finishing the race. To address potential effects of recovery, a third sampling was performed 24 h after finishing. Additionally, neutrophils, monocytes and lymphocytes (inflammatory response), creatine kinase (CK; muscular damage) andplasma markers of oxidative damage and repair were assessed at baseline and after the race. Experiments of mitochondrial respiration and simultaneous H_2O_2 production were performed on several Oroboros Oxygraph 2Ks including the LED2 module.
Results: Concentration of all leukocyte subgroups as well as creatine kinase were increased significantly. No significant changes were found in respiratory control ratios (CI/CI+II; CII/CI+II), neither when comparing baseline and after race, nor between after race and recovery. However, R/E ratio (routine state divided by max. stimulated electron transfer system) was changed significantly ($p<0.05$), indicating changes in platelet metabolism. We found a significant ($p<0,05$) influence of BMI on CI/CI+II ratio, whereas age and training time per week was not affecting metabolism.
Conclusion: It has been shown that alterations in leukocyte content are a consequence of an inflammatory response due to strenuous exercise. Interestingly, our results demonstrate no significant alterations of the CI/CI+II and CII/CI+II ratios and therefore do not confirm existing findings [2], indicating just minor changes between single enzymatic complexes. However, a significant change of the R/E ratio indicates changes in human platelet metabolism. Platelets may therefore be a suitable tissue to assess mitochondrial function in response to strenuous exercise in a minimally-invasive way.

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MiP2014

E-07 UCP3-related changes in brown and brite/beige adipose tissue in cold-acclimated mice

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Uncoupling protein 3 (UCP3) is a member of mitochondrial inner membrane carrier family. Earlier we have demonstrated that UCP3 is upregulated in skeletal muscle of the constantly shivering mice that lack the thermogenic protein UCP1. This UCP3 upregulation was correlated with enhanced lipid oxidation [1]. UCP3 is also upregulated in brown adipose tissue (BAT) and inguinal white adipose tissue (ingWAT) during britening/beiging process upon cold acclimation [2]. The function and regulation of UCP3 in brown and brite/beige adipose tissues is unknown.
We acclimated wild-type and UCP3-knockout (UCP3 KO) mice at 4 °C and addressed features of brown and brite/beige adipose tissues: morphology, thermogenic capacity (amount of UCP1 protein by Western blot and respiration of isolated mitochondria), as well as lipid metabolism (expression of genes involved in lipid synthesis and catabolism).
In mice lacking UCP3, both BAT and ingWAT displayed higher total protein content and a morphology with smaller lipid droplets. In BAT, these changes were associated with upregulation of expression levels of several genes involved in lipid droplet formation and lipid composition. In ingWAT, the higher total protein content was correlated with higher number of mitochondria estimated by VDAC content. Neither in BAT nor in ingWAT was thermogenic capacity (UCP1 content) upregulated in the UCP3 KO mice. The thermogenic function of isolated mitochondria did not differ between wild-type and UCP3 KO mice.
Thus, the lack of UCP3 induces changes in lipid metabolism concerning lipid droplet formation in brown adipose tissue. In brite adipose tissue, the lack of UCP3 upregulates mitochondriogenesis.

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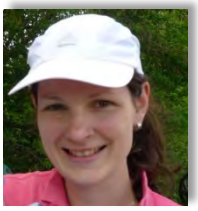
MiP*art*

E-08 CD36 does not directly participate in mitochondrial fatty acid transport and oxidation



Pravenec M[1], Karbanová Vendula[1], Tauchmannová K[1], Zídek V[1], Landa V[1], Kazdová L[2], Vrbacký M[1], Drahota Z[1], Mráček T[1] and Houštěk J[1]

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Introduction: CD36/FAT permease of plasma membrane is the key transmembrane transport protein for long chain fatty acids (FA). In the last years, conflicting results have been published regarding the localization of CD36 in mitochondria and its direct role in mitochondrial FA transport and oxidation.
Methods: We used the spontaneously hypertensive rat (SHR) that harbors mutant CD36 and transgenic SHR expressing wild type *Cd36* (SHR-*Cd36*) and compered parameters of lipid metabolism in brown adipose tissue (BAT).
Results: CD36 protein in BAT was high, comparable to heart and present mostly in a glycosylated form. Of all tissues the *Cd36* transcript was the highest in BAT (2.9 x higher than heart). Most of the CD36 signal was in microsomal fraction and only traces in mitochondria, most likely due to contamination. We also compared palmitate transport and oxidation in BAT and in primary cultures of brown adipocytes from SHR and SHR-*Cd36* to test whether palmitate transport and oxidation is affected by mutant CD36. The import of palmitate into BAT was reduced in the SHR when compared to SHR-*Cd36* rats (24.1±0.8 vs. 29.0±1.6 nmol palm/mg prot/2h, $P<0.05$), confirming that FA transport across plasma membrane mediated by mutant CD36 is less effective. In contrast, there was no significant difference in palmitate oxidation in BAT from SHR and SHR-*Cd36* rats (2.1±0.1 vs. 2.1±0.1 nmol palm/mg prot/2h), suggesting that CD36 is not important for FA transport into mitochondria.
Conclusion: Our results demonstrate important role of CD36 in transport of long chain FA across plasma membrane but not into mitochondria. We were not able to detect a significant amount of CD36 in isolated mitochondria and CD36 does not seem to directly participate in mitochondrial FA transport and oxidation.

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MiP*art*

E-09 Artificial hypothermia of rats, as opposed to natural hibernation of ground squirrels *Spermophilus undulatus*, is not accompanied by inhibition of mitochondrial respiration in liver

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In liver mitochondria of hibernating animals is observed the suppression of oxidative phosphorylation, which is irrespective of temperature and remains in experiments at 37°C. The mechanisms of implementation of this phenomenon are still under active debate. One of the assumptions is that the main cause of the suppression of oxidative phosphorylation is the inhibition of succinate dehydrogenase - complex II of the respiratory chain [1]. Presented data in our work maintain another standpoint of the inhibition of respiratory chain in the segment of complex III [2]. We demonstrate on the liver mitochondria of ground squirrels *Spermophilus undulatus* significant inhibition of phosphorylation rate and the maximum rate of respiration in hibernating animals in comparison with the active, using substrates oxidize both through complex I (glutamate, pyruvate) and the complex II (succinate), but found no change in the complex IV, under the oxidation of the artificial substrate TMPD, oxidized via cytochrome C. This indicates that the point of inhibition of mitochondrial respiration localized in the area after the complex II and prior to cytochrome *C*. It is unclear whether such inhibition is necessary for occurrence of hibernation state and is the property unique to the natural hypometabolic state or is the consequence of any hypothermia. To find it out, we used normal homoeothermic animals (rats), which were immersed in artificial non-drug hypometabolism under conditions of hypoxia, hypercapnia and low ambient temperature, leading to a decrease in body temperature up to 15°C, and ground squirrels having the same temperature during entering in the hibernation. We found no difference in states of respiration of liver mitochondria between control and hypothermic rats. An artificial hypometabolism caused by hypothermia in nonhibernator mammals, is not accompanied by specific inhibition of mitochondrial respiration of liver, unlike in the natural hypometabolic state in hibernating ground squirrels.

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E-10 Autocrine effects of transgenic resistin reduce palmitate and glucose oxidation in brown adipose tissue

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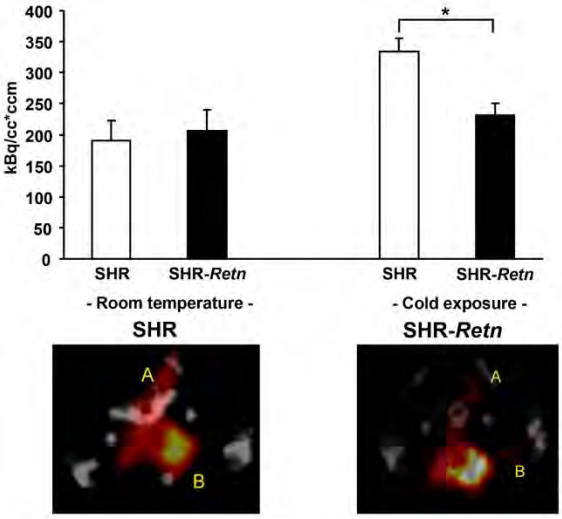
Introduction: Resistin has been originally identified as an adipokine that links obesity to insulin resistance in mice. In our previous studies in spontaneously hypertensive rats (SHR) expressing a nonsecreted form of mouse resistin (*Retn*) transgene specifically in adipose tissue (SHR-*Retn*), we observed an increased lipolysis and serum free fatty acids, ectopic fat accumulation in muscles and insulin resistance. Recently, brown adipose tissue (BAT) has been suggested to play an important role in the pathogenesis of metabolic disturbances by its ability to dissipate energy excess.

Results: In the current study, we analyzed autocrine effects of transgenic resistin on BAT glucose and lipid metabolism and mitochondrial function in the SHR-*Retn* versus nontransgenic SHR controls. We observed that interscapular BAT isolated from SHR-*Retn* transgenic rats when compared to SHR controls showed a lower relative weight (0.71±0.05 vs. 0.91±0.08 g/100 g body weight, P<0.05), significantly reduced both basal and insulin stimulated incorporation of palmitate into BAT lipids (658±50 vs. 856±45 and 864±47 vs. 1086±35 nmol/g/2h, P≤0.01, respectively), and significantly decreased palmitate oxidation (37.6±4.5 vs. 57±4.1 nmol/g/2h, P=0.007) and glucose oxidation (277±34 vs. 458±38 nmol/g/2h, P=0.001). In addition, *in vivo* microPET imaging revealed significantly reduced ^{18}F-FDG uptake in BAT induced by exposure to cold in SHR-*Retn* versus control SHR (232±19 vs. 334±22 kBq/cc*ccm, P<0.05). Gene expression profiles identified differentially expressed genes involved in skeletal muscle and connective tissue developmental and inflammation, as well as MAPK and insulin signaling.



Conclusions: These results provide compelling evidence that autocrine effects of resisitin in BAT play an important role in the pathogenesis of insulin resistance in the rat.

Acknowledgement: Research relating to this abstract was funded by ERC CZ LL1204 and Grant Agency of the Czech Republic (14-36804G).

Upcoming MiP events

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MiP*school* on Mitochondrial Physiology

Zewail City of Science and Technology

Giza, ET

Organizer: Sameh S. Ali (*Helmy Institute of Medical Sciences, Zewail City of Science and Technology*), The MiP*society*.

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MiP2017

12th Conference on Mitochondrial Physiology

Ilha Grande, BR

Organizers: Antonio Galina, Nivea D. Amoêdo (*Medical Biochemistry Institute, Federal University of Rio de Janeiro*), The MiP*society*.

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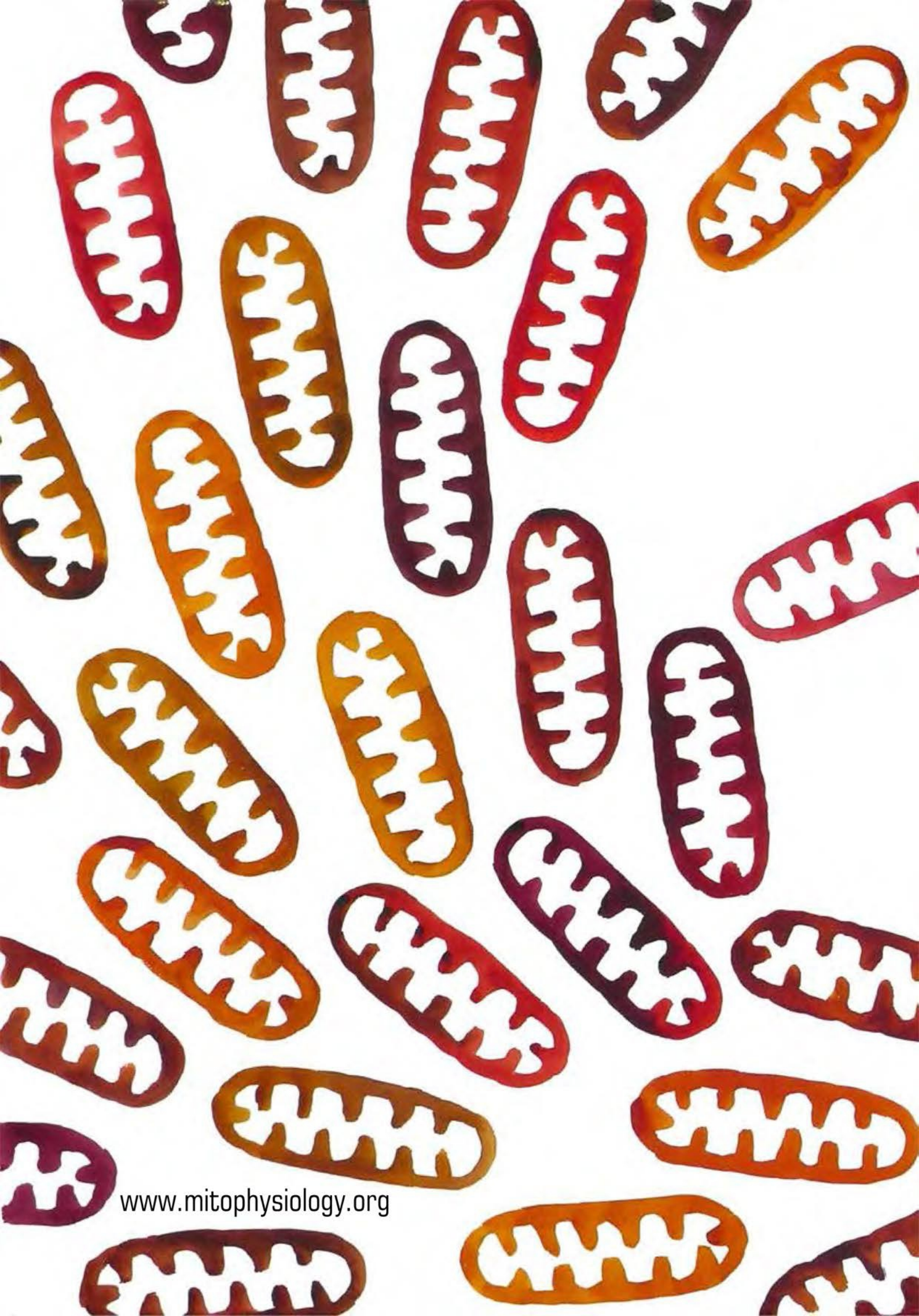
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