Mitochondrial Physiology

Joint IUBMB/MiP Symposium

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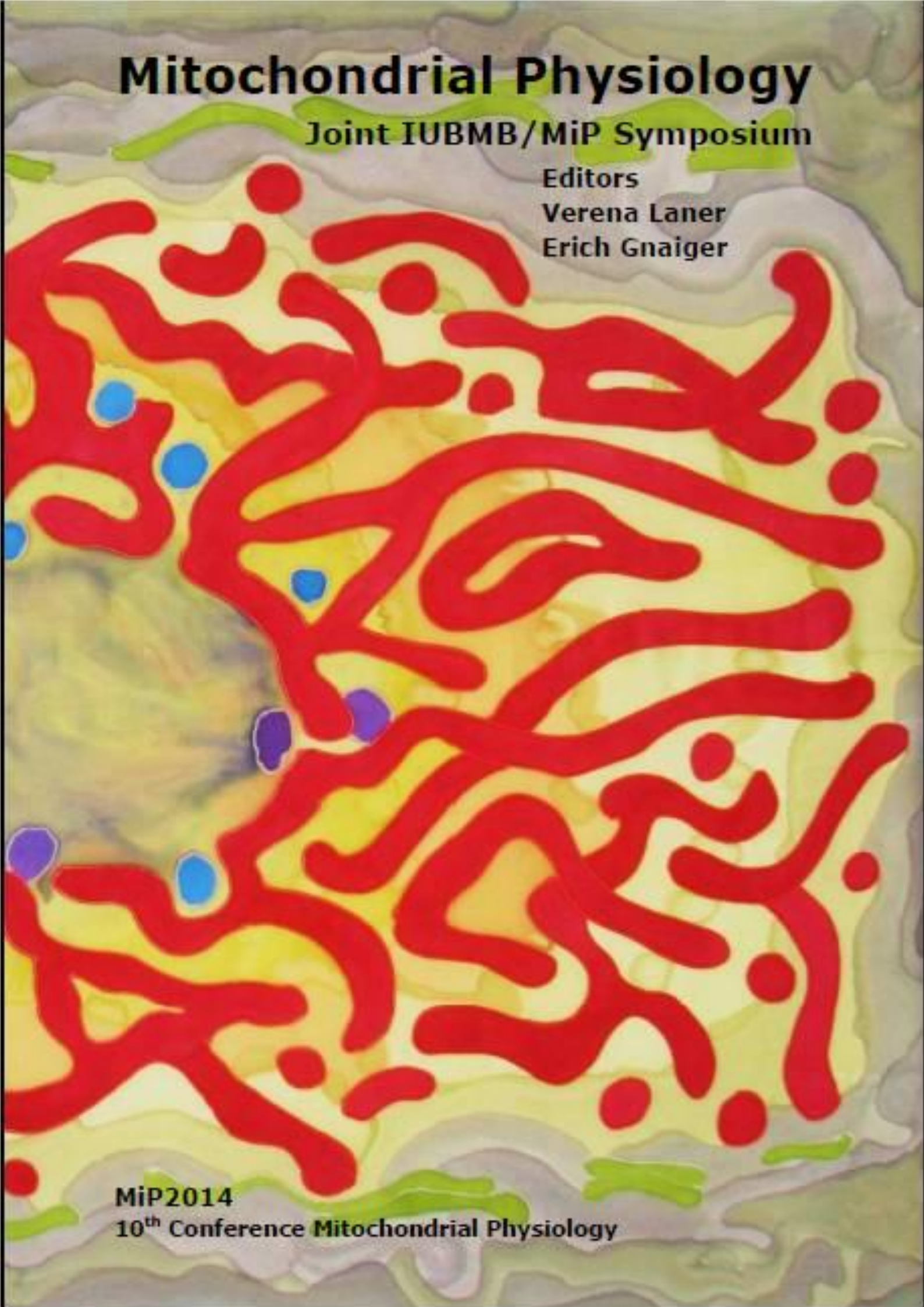
Verena Laner
Erich Gnaiger



MiP2014
10th Conference Mitochondrial Physiology

MiP 2014

Fission and fusion in the LEAK state

Odra Noel (London) Bioblast 2012

MiPArt Gallery (Innsbruck)

MiP2014 Organization

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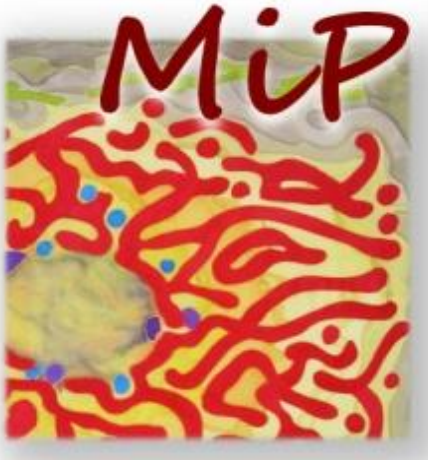

Citation

Laner V, Gnaiger E, eds (2014) Mitochondrial Physiology – methods, concepts and biomedical perspectives. MiP2014. Mitochondr Physiol Network 19.13: 88 pp.

ISBN 978-3-9502399-9-7

Printed in Austria
Steiger Druck GmbH, Axams, Austria
steigerdruck@tirol.com

Mitochondrial Physiology Network 19.13: 88 pp (2014)

Mitochondrial Physiology

Methods, concepts and biomedical perspectives

Editors
Verena Laner
Erich Gnaiger

MiP2014
Joint IUBMB/MiP Symposium

Obergurgl, Tyrol, Austria

08 – 12 Sep 2014

10th Conference on Mitochondrial Physiology

Joint IUBMB/MiP Symposium
The Mitochondrial Physiology Society

The Mitochondrial Physiology Society

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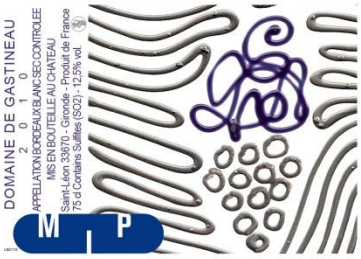

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10:00	**R**	Coffee	Coffee	Coffee	**E**
10:30	**R**	**Session A2**	**Session B2**	**Session C2**	**P**
12:00	**I**	Lunch	Lunch	Lunch	**A**
13:30	**V**	Walks and talks	Excursion	**Session C3**	**R**
15:30	**A**	**Session A3**	**Session B3**	**Session C4**	**T**
17:00	**L**	Coffee	Coffee	Coffee	**U**
17:30	Welcome reception	**Session A4**	**Session B4**	**Conclusions**	**R**
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20:30	Opening	Programme committee meeting Round tables / Posters	Project groups Posters	Social evening	Postconference Workshop

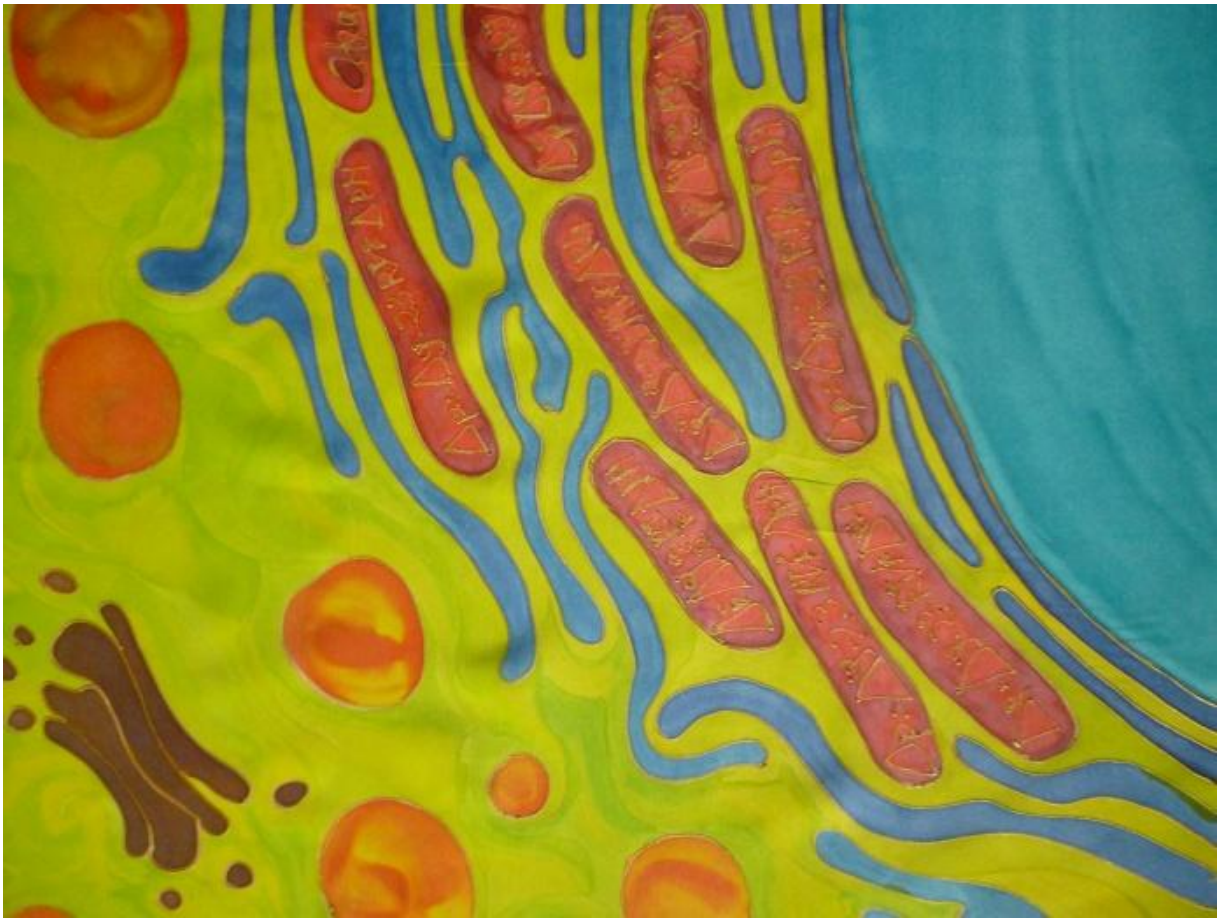
By arranging 10+5 talks, posters and 7+2 P-flashes in sequence of logically linked presentations, each of the 12 Sessions of MiP2014 may be conceived as a team approach to elaborate a chain of connected topics from different points of view, proceeding on a common pathway towards a deeper understanding of mt-physiology.

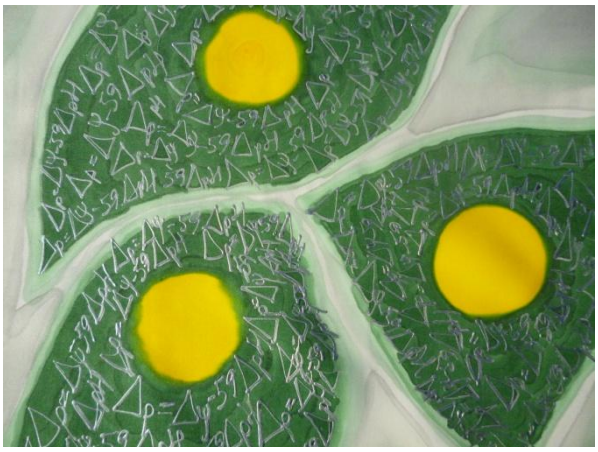
The MiP*conference* carries *physiology* in the title and received a prestigeous support by the *biochemical* INTERNATIONAL UNION OF BIOCHEMISTRY AND MOLECULAR BIOLOGY (IUBMB). This provides excellent testimony not only to the interdisciplinary nature but also to the recognition of the MiP*society* as an initiative building bridges.

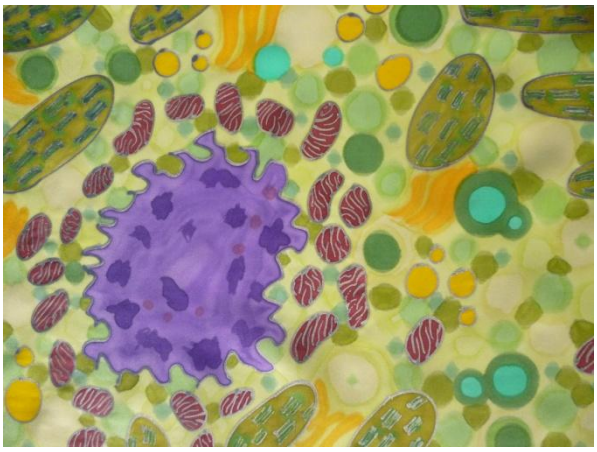
MiPArt – Mitchell's dream

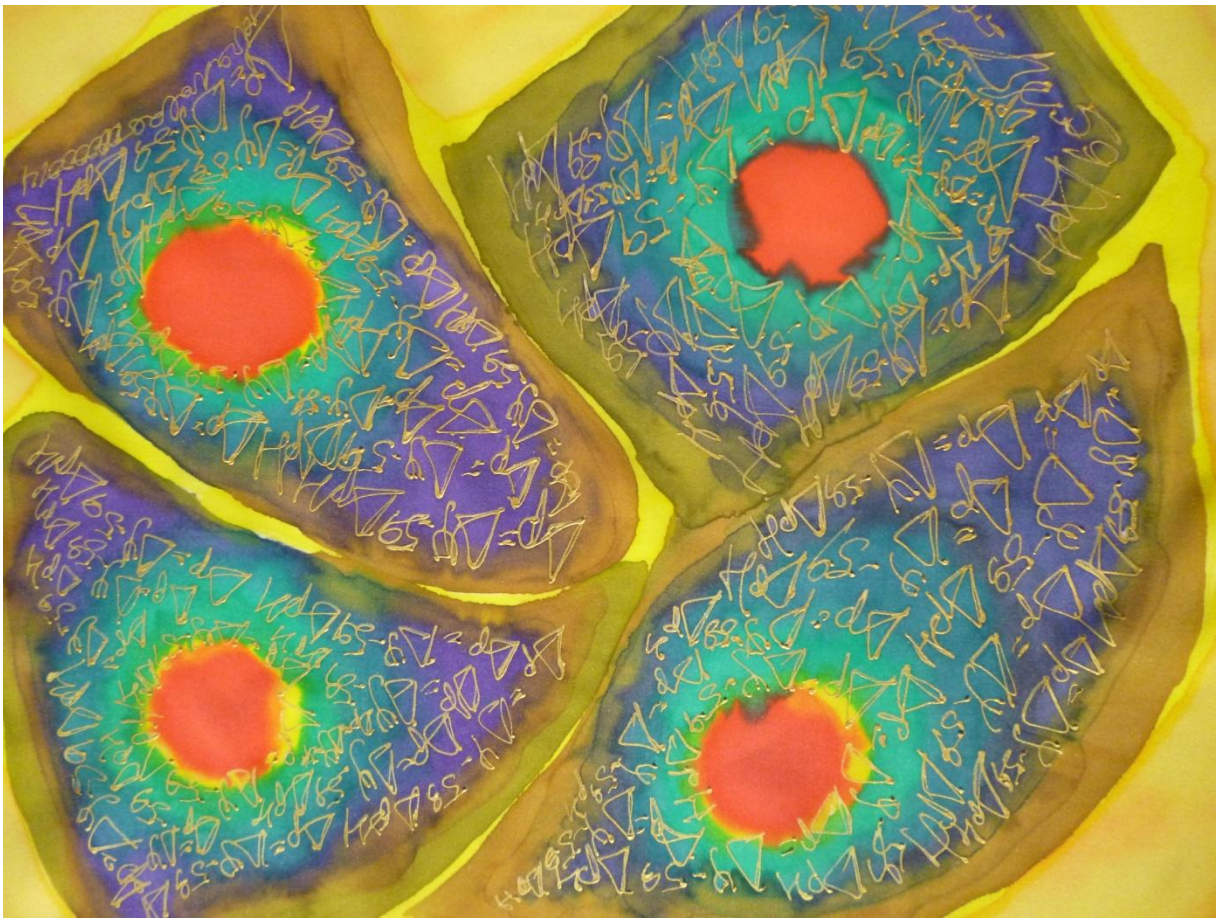
by Odra Noel
http://odranoel.eu/category/mitochondrial-art

*Science is beautiful: it has truth, it has drama, it is full of wonder. The **Mitchell's dream** series is a vision of some aspects of science through art. I hope it explains, inspires or makes you curious to find out more.*









MiPArt – Mitchell's equation

by Erich Gnaiger

Do you ever dream about an equation?

The *Mitchell's dream series* by Odra Noel is a dream on equations and shows a dream on the equation that penetrates all of biology since Peter D Mitchell started publishing on the protonmotive force equation [1]. Can we imagine how many dreaming was required until the chemiosmotic hypothesis emerged on energy coupling by the protonmotive force of oxidative phosphorylation in the bioblasts, which comprise the mitochondria, chloroplasts, bacteria and archaea? Seeing Odra Noel's pictures on *Mitchell's dream* provides insights into the equations of biophysics and biochemistry: these equations do not just belong to our books. They do belong to our cells, our bioblasts, to the living world. It is the mitochondria that help us to understand these equations, since the equations are in the mitochondria, they are the visible parts of the mitochondria and open in*sights* into function beyond the visible form – this is mitochondrial physiology.

Do you feel part of the equation?

An equation (or it's opposite) connects two sides by the equal (or unequal) sign,

$$=\qquad (1)$$

The left side may show simply a symbol,

$$\Delta p_{mt} =$$

This symbol is defined as being equal to a combination of various parts on the right side,

$$\Delta p_{mt} = \textit{electric}\ \text{part} + \textit{chemical}\ \text{part} \quad (2)$$

and these parts may again be shown simply as symbols,

$$\Delta p_{mt} = \Delta\Psi_{mt} + \Delta\mu_{H+}/F \quad (3)$$

The *electric* part is the potential difference across the inner mitochondrial (mt) membrane, $\Delta\Psi_{mt}$. This suggests that the protonmotive force in the form of Equation 3 should be expressed in the electric unit of volt [V]. *Electric* force of the mitochondrial membrane potential is the electric energy change per 'motive' electron or per electron moved across the transmembrane potential difference, with the number of 'motive' electrons expressed in the unit coulomb [C].

Therefore, the *chemical* part, $\Delta\mu_{H+}/F$, which stems from the difference of pH across the mt-membrane, contains a factor that bridges the gap between the *electric* force [J/C] and the *chemical* force [J/mol]. This factor is the Faraday constant, *F*, for conversion between *electric* force expressed in joules per coulomb or Volt [V=J/C] and *chemical* force with the unit joules per mole or Jol [Jol=J/mol],

$$F = 96.4853\ \text{kJol/V} = 96{,}485.3\ \text{C/mol}$$

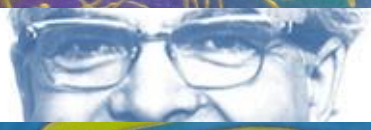
Generally, a force is the change of potentially available or 'free' energy (exergy) per 'motive' unit [2]. The *chemical* force or chemical potential of the 'motive' proton is the exergy change [J] per 'motive' amount of substance [mol]. Protonmotive means that the proton is moved across the mt-membrane at ΔpH maintained across the mt-membrane,

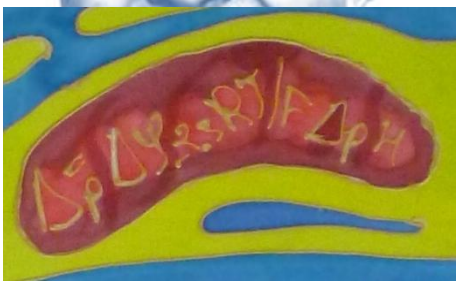
$$\Delta\mu_{H+} = -2.3 \cdot RT \cdot \Delta\text{pH} \quad (4)$$

This chemical force is the *difference* (Δ) of chemical potential across the inner mitochondrial membrane. Mitchell's equation and Odra Noel's pictures don't show proton *gradients* or membrane potential *gradients* – this dream belongs to another group.

The right side of Equations (2) and (3) helps us to separate the different parts, which require different methods of measurement, are expressed in different units, confuse us with different sign conventions and scientific nomenclature with terminological incompatibilities. On which side of the equation are you at home? Which part is more your part? Do you feel part of the equation?

On the other hand, the left side of the equation brings the different parts together in a unifying concept. With full focus on the equation, do we still see the mitochondria? Odra Noel places the sides and parts of the equation where they belong: They are parts of the bioblasts, they are the essence of the mitochondria themselves. Unification is brought to the limit of reduction in the form of Equation 1:





We have to write $\Delta\Psi_{mt}$ to point out that the mitochondrial membrane potential difference is in our mind. However, $\Delta\Psi$ in Odra Noel's pictures ***is*** the mitochondrion, the mt does not have to be written *into* the pictures, mt is essentially shown *by* the pictures. The *Mitchell's dream series* illustrates the importance of putting a symbol in the right form at the right place for understanding an equation and easily identifying the meaning of the symbol. *Mitchell's dream* is a symbol of form and function – form and function is the mitochondrial physiologist's dream.

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2. Gnaiger E (1993) Nonequilibrium thermodynamics of energy transformations. Pure Appl Chem 65: 1983-2002.

A1 From genotype to mitochondrial phenotypes in health and disease

A1-01 Cardiolipin is a key determinant for mitochondrial DNA stability and segregation.

Luévano-Martínez LA, Forni FM, dos Santos VT, Souza-Pinto NC, Kowaltowski Alicia J

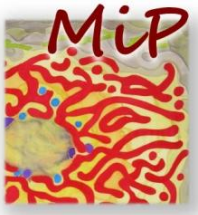
Dept Bioquímica, Inst Química, Univ São Paulo, Brazil. - alicia@iq.usp.br

Mitochondria play a key role in adapting to stress. Cardiolipin, the main anionic phospholipid in mitochondrial (mt) membranes, is expected to be a determinant in this adaptive mechanism, since it modulates the activity of most membrane proteins.

Here, we used *Saccharomyces cerevisiae,* subjected to conditions that affect mitochondrial metabolism, as a model to determine the possible role of cardiolipin in stress adaptation. Interestingly, we found that thermal stress promotes an increase in cardiolipin content, modifying both surface charge and the physical state of mt-membranes. These changes have effects on mtDNA segregation and mt-morphology, thus adapting cells to thermal stress. Conversely, since a cardiolipin synthase-null mutant strain is unable to adapt to thermal stress, this effect is cardiolipin-dependent. Interestingly, we found that the loss of cardiolipin specifically affects the segregation of mtDNA to daughter cells, leading to a respiratory deficient phenotype after replication. Furthermore, we provide evidence for a physical interaction between cardiolipin and the mitochondrial nucleoid.

In summary, our results demonstrate that the mitochondrial lipid cardiolipin is a key determinant in the maintenance of mtDNA stability, morphology and segregation.

Funded by FAPESP, CNPq and NAP-Redoxoma.



A1-02 Complementation of nuclear-encoded proteins which maintain mitochondrial DNA in *Saccharomyces cerevisiae* with homologous proteins from other fungi species.

Kruszewski Jakub[1], Golik P[1,2]

[1]Inst Genetics Biotech, Univ Warsaw; [2]Inst Biochem Biophysics, Polish Acad Sc, Warsaw; Poland. - j.kruszewski@biol.uw.edu.pl

Yeast mitochondrial DNA polymerase (Mip1) and RNA polymerase (Rpo41) are nuclear-encoded proteins, crucial for proper homeostasis of mitochondria and quality of mitochondrial DNA (mtDNA). Whereas organization of mitochondrial genome is different, even between related species, mitochondrial polymerases are highly evolutionary conserved in various organisms [1,2]. Complementation of *Saccharomyces cerevisiae* Mip1 and Rpo41 activity with proteins from other fungal species may help to understand mechanisms of mitochondrial polymerases specificity, their interactions with substrate and the general complexity of the mitochondrial-nuclear network [3,4].

We replaced activity of endogenous Mip1 and Rpo41 (separate experiments) from *S. cerevisiae* with their homologs from *S. bayanus*, *S. paradoxus*, *Kluyveromyces lactis, Candida glabrata* and *Candida albicans* [5]. We analyzed efficiencies of maintaining mtDNA quality in chimeric strains and compared them to wild type. To establish how efficiently non-native polymerases could process their substrates in *S. cerevisiae,* chimeric strains with homologs from other fungi species were created. Phenotype analysis of obtained strains included drop assay and petite frequencies analysis. For *K. lactis*, *C. glabrata* and *C. albicans* we created strains with promoters and terminators from those species and other strains with native versions from *S. cerevisiae.*

Our results show that it is possible to complement activity of those polymerases with homologous proteins from related species, and that promoters and terminators from another yeast species are recognized by *S. cerevisiae* transcription factors. Therefore, we suggest that the described chimeric strains of baker's yeast can be used as a model for studying mutual evolution of mtDNA and nuclear-encoded proteins.

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A1-03 YER077C encodes a novel PPR protein essential for mitochondrial genome expression in *Saccharomyces cerevisiae*.

Zapisek Bartosz[1], Golik P[1,2]
[1]*Inst Genet Biotechn, Univ Warsaw;* [2]*Inst Bioch Biophysics, Polish Acad Sc, Warsaw; Poland.* - *bzapisek@biol.uw.edu.pl*

Mitochondria possess a residual genome which encodes only a few proteins, including some key proteins of oxidative phosphorylation (OXPHOS) complexes. Therefore, the mitochondrial genome maintenance and expression is highly dependent on a variety of proteins encoded by the nuclear genome [1,2]. Pentatricopeptide repeat (PPR) proteins form the largest known RNA-binding protein family and are found in all eukaryotes, where they play an essential role in organellar genome expression [3]. The budding yeast *Saccharomyces cerevisiae* has traditionally been a leading model for the study of mitochondrial gene expression and the biogenesis of OXPHOS complexes [4].

We report a new nuclear gene, reading frame Δyer*077C* on chromosome V, encoding a mitochondrial PPR protein required for the proper expression of mitochondrial genetic information on the RNA level in *S. cerevisiae*. Regardless of the presence of introns in mtDNA, the lack of Δyer*077C* gene product results in a complete loss of respiratory capacity and increased mtDNA instability leading to conversion to ρ^-/ρ^0 petites. Northern analysis of mitochondrial RNA in the Δyer*077C* strain revealed the abolished levels for the transcripts encoding essential subunits for the mitochondrial cytochrome bc_1 and ATPase complexes (i.a. accumulation of pre-RNA and strongly decreased levels of mRNA). Sucrose gradient sedimentation analyses suggest that yer*077cp* might bind to a small subunit of mitochondrial ribosome and also to fully assembled mitoribosomes.

In view of our results and the RNA-binding properties of known PPR proteins, we conclude that yer*077cp* is an important factor for mitochondrial RNA maturation and stability and thus an essential component of the mitochondrial gene expression system. The interaction with mitoribosomes might also reveal yer*077cp* as a potential mRNA translational activator which has already been annotated for other yeast PPR proteins [5].

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A1–04 Balanced ratio of anionic mitochondrial phospholipids is important for mitochondrial functions in the yeast *Saccharomyces cerevisiae*.

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Phosphatidylglycerol (PG) is a metabolic precursor to the anionic mitochondrial phospholipid, cardiolipin (CL). The typical feature of a mutant without CL synthase (crd1Δ) is a lack of CL and accumulation of PG. Deletion of the *PGC1* gene encoding PG specific phospholipase C also causes accumulation of PG, especially in inositol-free media [1]. The major difference in phospholipid composition between *S. cerevisiae crd1Δ* and pgc1Δ mutant mitochondria is a lack of CL in the crd1Δ mutant strain. In the present work we investigate the impact of PG accumulation on mitochondrial functions to better understand how controlling anionic phospholipid levels affects cellular functions.

Our results indicate that accumulation of PG in mitochondria of the pgc1Δ mutant with normal levels of CL causes growth defects at increased temperature, decreased respiratory control ratio, increase of respiration rates 3- and 4-fold compared to the wild type. These results complement already published data [2], which suggests that a lack of CL in the crd1Δ mutant results in defects in cell wall biosynthesis, in reduced survival at increased temperature and in mitochondrial DNA instability. Recently, it was shown that the absence of CL in the *crd1Δ* mutant causes reduced respiratory control ratio and destabilization of supercomplexes of the respiratory chain [3].

Taken together, our results indicate that not only a lack of anionic phospholipids but also the excess of PG or unbalanced ratio of anionic phospholipids in mitochondrial membranes has harmful consequences for mitochondrial functions.

Supported by the Slovak Grant Agency of Science (VEGA 2/0168/14) and the Slovak Research and Development. Agency contracts No. LPP-0291-09 and APVV-0123-10.

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A1-05 Proteomic analysis of mitochondrial Complex I deficient mouse model - impact of Complex I deficiency on p66Shc-Ser36 phosphorylation pathway in NDUFS4$^{-/-}$ mouse tissues.

Wojtala Aleksandra[1], Koopman W[2], Willems PH[2], Smeitink JA[2], Duszynski J[1], Wieckowski MR[1]
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Key mitochondrial energy-providing reactions are carried out by the oxidative phosphorylation system (OXPHOS), involving the electron transfer and phosphorylation systems including F_1F_o-ATP synthase. The most common OXPHOS disorder in humans is associated with Complex I deficiency, leading to fatal encephalomyopathies of early childhood- Leigh-like syndrome [1]. The growth factor adaptor protein p66Shc has a substantial impact on mitochondrial metabolism through regulation of cellular responses to oxidative stress. A low level of p66Shc protein or its complete ablation protects against numerous age-related disorders and may partially prevent pathologies caused by reactive oxygen species (ROS). On the other hand, a high level of p66Shc phosphorylation is correlated with increased intracellular ROS production [2,3].

Organs from NDUFS4$^{-/-}$ mice with Complex I deficiency were used as a model of self-propelling intracellular oxidative stress. The status of the antioxidant defense system, oxidative stress markers and the p66Shc-Ser36 phosphorylation pathway were measured

in these tissues. Mass spectrometry analysis was also performed for selected NDUFS4$^{-/-}$ mouse tissues.

In our study, mice with defective Complex I were characterized by attenuated intracellular oxidative stress, connected with increased p66Shc phosphorylation. Mass spectrometry revealed aberrations in the level of Complex I proteins and oxidative stress- related proteins, as well as other proteins involved in metabolic processes.

Supported by the Statutory Founding from Nencki Institute of Experimental Biology and Polish Ministry of Science and Higher Education grant W100/HFSC/2011.

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A1-06 Reversible mitochondrial DNA mutagenesis.

Wang W[1,2], Scheffler K[1,2], Esbensen Y[3], Strand JM[1,2], Stewart JB[4], Askeland G[1], Bjørås M[1,2], Eide Lars[1]

[1]Dept Medical Biochem; [2]Dept Microbiol; Oslo Univ Hospital, Univ Oslo, Norway; [3]Dept Clinical Molec Biol Lab Sc (EpiGen), Div Medicine, Akershus Univ Hospital and Univ Oslo, Lørenskog, Norway; [4]Max Planck Inst Biology of Ageing, Cologne, Germany. - lars.eide@medisin.uio.no

Mitochondrial mutations can result in mitochondrial dysfunction, depending on the fraction of mutated molecules as well as the type of the mutation. We have established high-resolution techniques to quantify mutation frequency in mitochondrial DNA (mtDNA) and to measure errors in mitochondrial RNA (mtRNA). Comparing mtDNA mutation frequency with mtRNA integrity allows us to estimate the tolerance level for mtDNA mutation frequency.

The mtDNA mutation frequency in embryonic stem (ES) cells is higher than in the aged brain, indicating that mutated mtDNA molecules are deselected during development. mtRNA polymerase introduces errors at a frequency that exceeds the high mutation frequency in ES mtDNA, thereby providing an explanation for the apparent high tolerance for ES mtDNA mutations.

In order to characterize the dynamics in mtDNA mutagenesis further, we evaluate mtDNA mutagenesis during induced pluripotent stem (iPS) cell reprogramming and subsequent re-differentiation. Our results show that iPS cell reprogramming induces mutated mtDNA molecules, which are subsequently removed upon re-differentiation.

We are currently investigating the role of mtDNA repair in this reversible mtDNA mutagenesis.

A1-07 Quantitative regulation of nuclear gene expression by mitochondrial DNA heteroplasmy.

Picard Martin[1], Zhang J[2], Hancock S[3], Derbeneva O[1], Golhar R[4], Golik P[5], O'Hearn S[6], Levy SE[7], Potluri P[1], Lvova M[1], Davila A[1], Lin CS[1], Perin JC[8], Rappaport EF[8], Hakonarson H[3], Trounce I[9], Procaccio V[10], Wallace DC[1]

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Mitochondrial disorders caused by mtDNA mutations result in heterogeneous and organ-specific symptoms, but the cellular basis for this phenomenon is unknown [1]. Beyond a central role in energy production, mitochondrial metabolism involves key molecular substrates that modify transcriptional processes and the epigenome, suggesting that mitochondria actively regulate nuclear gene expression in complex ways [2]. To address this question, we introduced different proportions of normal and mutant mitochondrial DNA (termed heteroplasmy) of the most common human pathogenic mtDNA point mutation ($tRNA^{Leu(UUR)}$ 3243A>G) [3] to a series of human syngenic cybrid cell lines. Increasing mutation load from 0 to 100% resulted in a progressive loss of electron transfer system subunits and a range of respiratory system impairment from mild to severe. In contrast, RNA sequencing revealed broad genome-wide expression profiles and specific functional pathways strongly affected in a dose-response, but biphasic manner. These notably included chromatin components such as histone variants, chromatin-remodeling factors of the SWI/SNF family and the DNA methyltransferases.

Likewise, mtDNA transcript levels were modified, following a similar bi-phasic pattern. Gene ontology and functional pathway analysis indicated that, consistent with clinical disease presentation, mild bioenergetic defects mainly downregulated gene families associated with energy metabolism and intracellular signaling, whereas more severe bioenergetic defects activated distinct non-overlapping pathways previously associated with neurodegeneration. Increasing heteroplasmy levels also modified cellular and nuclear volumes, mitochondrial morphology and ultrastructure, and mtDNA copy number. Thus, the same mtDNA mutation can result in a multifinality of cellular and transcriptional phenotypes, depending upon its intracellular levels. Overall, these data establish that even mild mitochondrial defects triggered by mtDNA heteroplasmy induce broad transcriptional changes throughout the nuclear genome. This, along with cell-specific, may contribute to explain the heterogeneous and organ-specific nature of mitochondrial disorders.

Supported by NIH, Simons Foundation.

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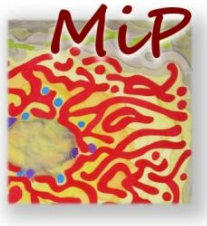

A1-08 Homozygous missense mutation in the human NARS2 gene results in reduced homodimerization of mitochondrial AsnRS enzyme in a patient with Alpers syndrome.

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Mitochondrial function in energy production and biosynthesis is dependent on coordination of nuclear and mitochondrial gene expression. Defects in either genome can result in a wide spectrum of disease phenotypes. Alpers syndrome is a progressive, neurodegenerative disorder that is characterized by psychomotor regression, seizures and diffuse degeneration of cerebral grey matter [1]. Although the genetic etiology is unknown in most patients, several mutations targeting components of mtDNA maintenance and expression have been described [2]. Using whole-exome sequencing, a patient with Alpers syndrome was found to have a homozygous missense mutation in the asparaginyl-tRNA synthetase gene, encoding mitochondrial asparagine-tRNA ligase (AsnRS) [3]. AsnRS is a class II aminoacyl tRNA-synthetase whose function is poorly characterized. By similarity to other class II enzymes, it is predicted to function as a homodimer. The crystal structure of the *P. horikoshii* [4] AsnRS homolog and *in silico* modeling of AsnRS suggest that the affected Pro214 residue is located at the base of a loop that protrudes out from the main body of the folded protein, suggesting it may participate in monomer-monomer interaction. In this study, we aimed to characterize the AsnRS p.P214L mutation in fibroblasts derived from the patient.

Mitochondrial polarography, performed previously on isolated mitochondria from skeletal muscle, revealed decreased oxygen consumption in the presence of Complex I and IV substrates as well as decreased respiratory enzyme activities. Protein abundance, measured by Western blot on fibroblast lysates, of a marker of Complex II was unaltered, whereas markers of Complex IV (COX IV – nuclear gene, and MT-CO1 – mitochondrial) were both increased in the patient compared to the control. Abundance of AsnRS protein was reduced by 63% in the patient, suggesting altered regulation of protein stability. Finally, preliminary results from size exclusion gel filtration under non-denaturing conditions indicated a shift in motility of AsnRS in the patient, suggestive of reduced dimerization.

In combination with previous clinical data, our results show that the AsnRS p.P214L mutation found in the patient is correlated with altered mitochondrial protein expression and regulation. Awaiting confirmation of our results, we hope to demonstrate that AsnRS indeed exists as a homodimer in the mitochondria. Future experiments should address the mechanistic link between disturbed dimerization and tRNA aminoacylation.

Supported by LUA/ALF grant from Sahlgrenska University Hospital.

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A1–09 Alteration of structure and function of ATP synthase and cytochrome *c* oxidase by lack of F_o-a and Cox3 subunits caused by mitochondrial DNA 9205delTA mutation.

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Mitochondrial disorders due to maternally transmitted dysfunction of ATP synthase are frequently caused by missense mutations in the mtDNA *MTATP6* gene. A different type of *MTATP6* mutation is represented by a unique 9205delTA microdeletion which disrupts the STOP codon of the *MTATP6* gene and affects the cleavage site in the *MTATP8/MTATP6/MTCO3* polycistronic transcript. This change interferes with the processing and translation of mRNAs for the ATP6 subunit (F_o-a) of the ATP synthase and Cox3 subunit of the cytochrome *c* oxidase (Complex IV, CIV). Two cases, described so far, presented strikingly different clinical phenotypes – mild transient lactic acidosis or fatal encephalopathy [1,2]. To gain more insight into the pathogenic mechanism, we prepared 9205delTA cybrids with a mutation load between 50–100% and investigated changes in the structure and function of ATP synthase and CIV.

We found that 9205delTA mutation diminishes the synthesis of both F_o-a and Cox3 proteins, alters the structure but not the content of ATP synthase, decreases the content of CIV and prevents most of the mitochondrial ATP production. The ATP synthase complex was assembled without F_o-a subunit but it was rather labile. It retained ATP hydrolytic activity but was unable to synthesize ATP. The biochemical effects displayed a pronounced threshold effect above 85% of mutation heteroplasmy. Since the relationship between the reduction of subunit F_o-a or Cox3 content and functional impairment was linear, the threshold effect originated primarily from a gene-protein level.

Supported by the Grant Agency of the Czech Republic (14-368046) and Ministry of education, youth and sports of the Czech Republic (LL1204).

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A1-10 Dysregulation of muscle ATP synthesis in hypophosphatemic mouse models and in a patient with hypophosphatemia due to a mutation in NaPi2c.

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Previous studies have demonstrated that young lean insulin resistant offspring of parents with type II diabetes have decreased insulin-stimulated rates of muscle mitochondrial ATP production, but the mechanism responsible for this phenomenon remains unclear [1]. In this study we examine the potential role of decreased insulin-stimulated phosphate transport in this process.

To examine this hypothesis we assessed the impact of hypophosphatemia on basal and insulin-stimulated rates of muscle ATP synthesis *in vivo,* using ^{31}P NMR saturation transfer experiments. We applied this ^{31}P NMR method to assess rates of muscle ATP synthesis in two mouse models of hypophosphatemia: a diet-induced mouse model of hypophosphatemia and a renal phosphate transporter knock out mouse model of hypophosphatemia (NaPi2a$^{-/-}$). Using this approach, we observed a strong association between serum phosphate levels with P_i-ATP flux (J_{ATP}) in skeletal muscle in both mouse models of hypophosphatemia. Low plasma P_i levels in these animals led to decreased J_{ATP}, while restoration of P_i to normophosphatemic levels concomitantly normalized J_{ATP}. Furthermore, insulin-stimulation increased muscle J_{ATP} by ~20% (P<0.05) in wild type mice but did not increase J_{ATP} in the hypophosphatemic NaPi2a$^{-/-}$ mice, indicating an important role of P_i mediating insulin's anabolic effects on ATP synthase flux.

The same pattern was observed in a patient with hypophosphatemia due to a mutation in the renal phosphate transporter NaPi2c gene. This patient, presented with hypophosphatemia and manifested low muscle J_{ATP}, assessed by ^{31}P NMR saturation transfer experiments, compared to normophosphatemic age-weight matched control subjects. Furthermore, the low J_{ATP} in this patient normalized following supplementation with oral phosphate salts and normalization of his plasma phosphate concentrations.

Taken together, these results demonstrate an important role for phosphate in the regulation of basal and insulin-stimulated rates of muscle ATP synthesis and may, in part, explain the symptoms of muscle fatigue in patients with genetic disorders of phosphate metabolism.

DP was supported by the Austrian Science Fund (FWF), project number J 3267.

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MiP2013: Dominik Pesta, Dominique Votion, Marcus Oliveira

A2 Mitochondrial structure and function: fission and fusion to supercomplexes

A2-01 Metabolic implications of mitofusin 2 dysfunction.

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Mitochondrial fusion and fission are key processes, regulating mitochondrial morphology. Mitochondrial fusion is catalyzed by Mfn1, Mfn2 and OPA1 in mammalian cells, whereas mitochondrial fission is operated by another set of proteins. Mfn2 protein seems to play a specifically complex role in mitochondria. It regulates mitochondrial morphology, and, in addition, it also controls endoplasmic reticulum morphology and function. Mfn2 expression is exquisitely regulated in skeletal muscle. It is up-regulated in skeletal muscle as response to chronic exercise and upon cold exposure. In contrast, Mfn2 is repressed in muscles from high-fat fed mice or in obese or type 2 diabetic individuals.

Changes in Mfn2 expression have a marked impact on mitochondrial metabolism. Skeletal muscles obtained from Mfn2 KO mice show a reduced respiratory control ratio, which occurs in the absence of significant changes in OXPHOS capacity and LEAK respiration. Mfn2-ablated soleus muscles also show reduced glucose oxidation and reduced expression of some OXPHOS subunits. Additional evidence, indicating that Mfn2 deficiency causes mitochondrial dysfunction, comes from studies in muscle cells in culture. The content of hydrogen peroxide was also enhanced in skeletal muscle upon Mfn2 depletion or in Mfn2-silenced muscle cells. These results occurred in the presence of a normal antioxidant defense.

Skeletal muscle Mfn2 KO mice also show susceptibility to develop insulin resistance in response to a high fat diet or to aging. In keeping with this, defective insulin signaling is detected in Mfn2-deficient mice treated with a high fat diet in response to *in vivo* insulin administration. Mfn2 knockdown muscle cells also show an impaired capacity to respond to insulin.

In summary, available data indicate that Mfn2 regulates metabolism and insulin signaling in skeletal muscles and may contribute to the pathophysiology in obesity and type 2 diabetes.

A2–02 Mitochondrial dynamics and quality control, a conflict of interest.

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As our relationship with mitochondria evolves, we remain fascinated by the impact of this organelle in two seemingly unrelated conditions: aging and metabolic diseases. While aging involves insufficiency of mitochondrial quality control and turnover mechanisms (such as autophagy), type II diabetes and obesity are influenced by the ability of the organism to deal with excess nutrient environment. The observation that both conditions are impacted by the duration of exposure to excess nutrient environment raises the question: Are the tasks of handling nutrients in excess and maintaining quality control ever in conflict? Mitochondria go through continuous cycles of selective fusion and fission, referred to as the "mitochondrial life cycle", to maintain the quality of their function [3-5]. Changes in mitochondrial architecture can represent an adaptation of mitochondria to respire according to the bioenergetic needs of the cell [2]. Conditions requiring high mitochondrial ATP synthesis capacity and/or efficiency, such as limited nutrient availability, are associated with mitochondrial

elongation [1]; reviewed in [2], while conditions of excess energy supply and relatively low ATP demand, such as beta-cells exposed to excess nutrients, induce mitochondrial fragmentation [6]. This raises the possibility that mitochondrial fragmentation supports uncoupled respiration and thus increases energy expenditure by promoting nutrient oxidation towards heat production, rather than towards mitochondrial ATP synthesis.

To test this hypothesis, we explored a system where a robust shift from coupled to uncoupled respiration and increased energy expenditure can occur. The brown adipocyte offers a unique system where transition to uncoupling can occur within minutes and in a physiological rather than pathological context. Therefore, it represents an attractive model for studying the regulation of energy expenditure induced by hormones.

Our results indicate that norepinephrine induces changes to mitochondrial architecture that serve as an amplification pathway for uncoupling in brown adipocytes. Remarkably, we now have evidence that similar changes, though at a longer time scale, occur in the beta cells under excess nutrient environment. In the beta cell, nutrient–induced fragmentation is associated with increased uncoupling and the enhanced consumption of excess nutrients, thereby serving as an adaptive mechanism.

Placement of bioenergetic adaptation and quality control as competing tasks of mitochondrial dynamics might provide a new mechanism, linking excess nutrient environment to progressive mitochondrial dysfunction, common to age-related diseases.

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A2-03 Redox regulation of mitochondrial dynamics and function.

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Within living cells, individual mitochondria are motile and continuously divide and fuse (mitochondrial dynamics). It appears that these processes allow dissipation of metabolic energy, complementation of mtDNA mutations, separation of mitochondrial content between daughter cells, apoptosis induction and turnover of damaged organelles by mitophagy. Between different cell types and experimental conditions mitochondria display a large variety of shapes. These range between "giant" spherical and "hyperfused" tubular morphologies. The various structural phenotypes likely represent different metabolic states and are important for mitochondrial adaptation to metabolic stress. Interestingly, evidence was provided that changes in mitochondrial volume and (ultra)structure directly affect the properties of (diffusion-limited) biochemical reactions within the mitochondrial matrix. This might allow cells to control the dynamics of biochemical reactions in this compartment by modifying its nanostructure. During both normal and pathological conditions mitochondria and other cellular constituents generate reactive oxygen species (ROS) which can act as signaling and/or damaging molecules.

Accumulating evidence suggests a mechanistic link between cellular and mitochondrial ROS signals and mitochondrial (ultra)structure and motility. The fact that mitochondria can be important sources of cellular ROS, especially under pathophysiological conditions, opens the intriguing possibility that mitochondrial ROS could act as autoregulatory factors of mitochondrial (and thereby cellular) function and metabolism.

Supported by the "Centres for Systems Biology Research initiative" (CSBR09/013V) of NWO (The Netherlands Organisation for Scientific Research).

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A2-04 Hormone-induced mitochondrial fission is utilized by brown adipocytes as an amplification pathway for energy expenditure.

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Adrenergic stimulation of brown adipocytes (BA) induces mitochondrial uncoupling, thereby increasing energy expenditure by shifting nutrient oxidation towards thermogenesis [1]. The brown adipocyte is a unique system to study the relationship between mitochondrial architecture and bioenergetic function. Here we describe that mitochondrial dynamics is a physiological regulator of adrenergically-induced changes in energy expenditure.

Brown pre-adipocyes were harvested from 4-week-old wild-type male C57BL6/J mice and differentiated in culture. Oxygen consumption was measured using Seahorse XF24. Mitochondrial membrane potential was measured using TMRE and Zeiss LSM 710 confocal microscope. Measurements were taken before and after activation with NE (1 uM) and FFA (palmitate or oleate, 0.4 mM).

The sympathetic neurotransmitter norepinephrine (NE) induced complete and rapid mitochondrial fragmentation in BA, characterized by Drp1 phosphorylation and Opa1 cleavage. Mechanistically, NE-mediated Drp1 phosphorylation was dependent on protein kinase-A (PKA) activity [2], whereas Opa1 cleavage required mitochondrial depolarization, mediated by FFAs released as a result of lipolysis. This change in mitochondrial architecture was observed both in primary cultures and brown adipose tissue from cold-exposed mice. Mitochondrial uncoupling, induced by NE in brown adipocytes, was reduced by inhibition of mitochondrial fission through transient Drp1 DN overexpression. Furthermore, forced mitochondrial fragmentation in BA through Mfn2 knock down increased the capacity of exogenous FFAs to increase energy expenditure.

These results suggest that, in addition to its ability to stimulate lipolysis, NE induces energy expenditure in BA by promoting mitochondrial fragmentation. Taken together these data reveal that adrenergically-induced changes of mitochondrial dynamics are required for BA thermogenic activation and for the control of energy expenditure.

Supported by T32 Cardiovascular Biology training grant.

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A2–05 Mitochondrial cristae morphology changes: from Hackenbrock to hypoxia.

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Hackenbrock's classic observation [1] distinguished orthodox cristae at a resting LEAK state and condensed cristae conformation for isolated mitochondria at the active phosphorylating OXPHOS state, which are, however, not encountered *in situ* [2]. Since it resides in MINOS complexes, joining the mitochondrial inner membrane, mtIM, and outer membrane (mtOM), the mtIM protein mitofilin contributes to cristae shapes. The aim of our study was to find out how altered metabolism at hypoxia is reflected by changes in cristae morphology.

3D dSTORM microscopy (3D immunocytochemistry) showed distinct mitofilin *foci* projected on the mtOM in HepG2 cells cultivated for 72 h at 5% oxygen (termed "hypoxic cells"), while the mtOM-projected surface density of mitofilin molecules *vs*. normoxic cells decreased by ~40% (Figure 1), accompanied by ~20% loss of mitofilin and its transcript. Cryo–electron microscopy documented intracristal space (ICS) expansion by cristae width increase, predominantly in glycolytic cells, not occurring in reduced or mtOM–detached cristae of OPA1– and mitofilin–silenced HepG2 cells, respectively. The hypoxic ICS expansion resembles Hackenbrock's classic observation of condensed cristae [1]. Moreover, we confirmed paradoxical observations of orthodox cristae in cells undoubtedly phosphorylating, which had more shrunken ICS and expanded matrix space at atmospheric oxygen. In turn, upon hypoxia, the IMS expansion reflected the established Hackenbrock condensed cristae conformation [1].

Furthermore, ATP-synthase dimers *vs*. monomers ratio and OXPHOS/LEAK respiratory control ratios were higher under normoxia.

Since these ATP-synthase dimers predominantly locate at sharp cristae edges, whereas at hypoxic ICS expansion these edges are disrupted at more round cristae, we hypothesize that for glycolytic cells the ICS expansion represents adaptation, decreasing the number of ATP-synthase dimers, serving for ATP synthesis downregulation during cell survival under hypoxia.

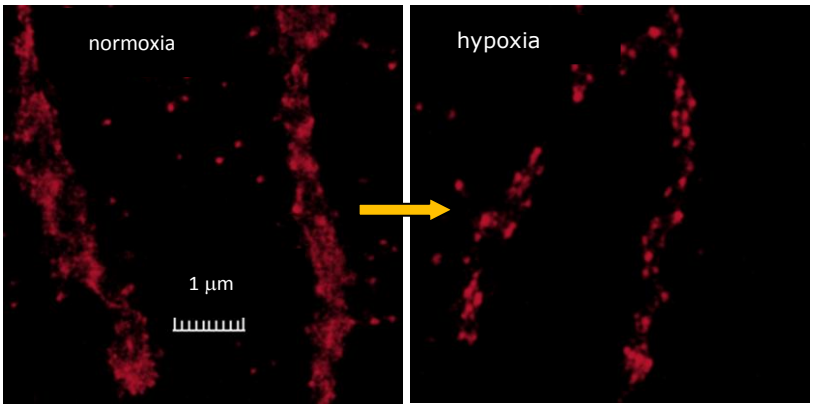

Figure 1. Mitofilin 3D immunocytochemistry.

Supported by GACR grants P302/10/0346 and 13-02033S.

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A2-06 Striatal deregulation of Cdk5 alters mitochondrial dynamics in Huntington's disease.

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Huntington's disease (HD) is characterized by motor disturbances associated with dysfunction and degeneration of the medium spiny neurons within the striatum [1,2]. The molecular mechanisms underlying striatal vulnerability are still unknown, but growing evidence suggests that mitochondrial dysfunction occurs during the pathogenesis of the disease [3]. We previously described that deregulation of cyclin-dependent kinase 5 (Cdk5), activity induced by mutant huntingtin, increases the susceptibility of striatal neurons to dopamine via D1 receptor activation [4]. Interestingly, Cdk5 has been shown to act as a mitochondrial regulator during neuronal apoptosis [5].

We investigated whether this aberrant Cdk5 signalling contributes to the striatal neurodegeneration by altering mitochondrial dynamics processes. We first observed that presence of striatal cells expressing mutant huntingtin (mHtt) increased mitochondrial fragmentation which worsens after dopaminergic stimuli. These mitochondrial defects can be widely rescued by Cdk5 inhibition with roscovitine or Cdk5 knockdown with siRNA transfection. Furthermore, we found that mHtt deregulates the levels and the subcellular distribution of fission/fusion proteins, while activation of D1 receptors promotes an increase of fission protein Drp1 levels and its translocation to the mitochondria. We demonstrated that mHtt-induced Cdk5 activation is involved in the deregulation of the Drp1 GTPase activity, since its inhibition prevents the aberrant activation of this fission protein.

In summary, our findings support the hypothesis that Cdk5 plays a crucial role in mitochondrial defects involved in the striatal neurodegeneration in HD.

Supported by Ministerio de Innovación y Ciencia SAF2012-39142, CHDI Ministerio de Sanidad y Consumo (CIBERNED CB06/05/0054), Fundación Ramón Areces.

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A2-07 Role of mitochondrial fusion and motility in distributing young mitochondrial protein and removing old protein from the mitochondrial network.

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Mitochondrial fusion and fission have been shown to allow for the equilibration of protein across the mitochondrial population as well as coordinate autophagy-mediated removal of defective mitochondria [1]. We hypothesized that mitochondrial fusion is serving a role in shuttling newly synthesized proteins from a subset of mitochondria that import them to the rest of the mitochondrial population, thereby determining distribution of young and old protein within the network.

We assessed mitochondrial protein age by targeting a time-sensitive fluorescent protein, MitoTimer, to the mitochondrial matrix. Emitted fluorescence of a newly translated timer is green and over time the emission shifts to red [2]. We fused the fluorescent timer to the mitochondrial targeting sequence of the Cox8a subunit, to form the MitoTimer construct.

Mitochondrial protein age, a result of equilibrated young and old proteins, was dependent on turnover rates as pulsed synthesis or autophagic inhibition increased the proportion of old MitoTimer protein. Mitochondrial fusion promotes the distribution of young mitochondrial protein across the mitochondrial network as cells lacking essential fusion genes Mfn1 and Mfn2, or down-regulated Opa1, displayed increased heterogeneity in mitochondrial protein age and accumulation of mitochondria with old protein. Experiments in hippocampal neurons illustrate that intracellular spatial organization also impairs distribution of young mitochondrial material and this effect was reduced by over-expression of mitochondrial transport protein, Miro1.

Collectively our data show that equilibration of young and old protein in the mitochondrial network is dependent on mitochondrial turnover, fusion, and transport.

Supported by National Science Foundation Graduate Research Fellowship.

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MiP2013: Helena Oliveira, Werner Koopman

A3 Mitochondrial response to exercise and aging

A3–01 Denervation modulates mitochondrial function when aging muscle atrophy becomes severe: implications for therapeutic intervention.

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Aging of skeletal muscle is associated with progressive atrophy, reaching clinically relevant thresholds in terms of weakness, mobility impairment and physical frailty in a significant fraction of individuals ≥80 y of age. Amongst the factors posited to be involved, mitochondrial alterations are implicated in the atrophy of aging muscle through recruitment of mitochondrial-mediated pathways of apoptosis and proteolysis. However, denervation is also known to recruit these same mitochondrial pathways. In view of the sporadic denervation that occurs in aging muscle, consideration of denervation's role in recruitment of mitochondrial atrophy pathways is essential to identify relevant therapeutic targets. As such, this presentation will review our current evidence from human skeletal muscle biopsies across a range of ages and physical activity levels, examining the impact of aging on mitochondrial function and the role played by denervation across this continuum.

As will be demonstrated, skeletal muscle mitochondrial alterations in septuagenarian subjects appears to be a primary event unrelated to denervation, where an increased susceptibility to mitochondrial permeability transition persists even in physically active subjects. In contrast, octogenarian subjects exhibit denervation-induced modulation of mitochondrial reactive oxygen species emission, suggesting failed reinnervation rather than mitochondrial dysfunction as a more appropriate therapeutic target when aging muscle atrophy becomes most clinically relevant.

A3-02 Mitochondrial adaptations to high-intensity training in young and elderly men and women.

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This study investigated how skeletal muscle mitochondria adapt to high intensity interval training (HIT) and whether adaptations differed according to age.

Two groups of healthy sedentary adults completed 18 sessions of low volume HIT (5x1 min @ ~132% of W_{max} with 1.5 min rest periods). The groups were matched for BMI and baseline V_{O2max} but differed in age (P<0.001). One group (N=9) aged 36±3 years (BMI 34±2 kg·m^{-2} and V_{O2max} 2.7±0.2 l·min^{-1}) was considered young (YOUNG), and the other group (N=4) aged 67±2 years (BMI 32±1 kg·m^{-2} and V_{O2max} 2.1±0.3 l·min^{-1}) was considered old (OLD). Mitochondrial respiration was measured using high-resolution respirometry (OROBOROS Oxygraph-2k) in permeabilized muscle fibers from the vastus lateralis, and mitochondrial ADP sensitivity was determined using Michaelis-Menten kinetics.

Following training, YOUNG increased V_{O2max} by 7% (P=0.003), with no change in OLD. Mitochondrial capacity for oxidative phosphorylation (OXPHOS) was increased in both groups (48±2 to 84±4 pmol O_2·s^{-1}·mg^{-1} in YOUNG P<0.001 and 59±9 to 71±11 pmol O_2·s^{-1}·mg^{-1} in OLD P<0.05), and maximal noncoupled respiration was increased in YOUNG

(59±4 to 92±5 pmol $O_2 \cdot s^{-1} \cdot mg^{-1}$, $P<0.05$) but not in OLD (71±9 to 80±10 pmol $O_2 \cdot s^{-1} \cdot mg^{-1}$). Mitochondrial ADP sensitivity did not change following training in either group (K_m^{app} [ADP] 0.19±0.10 to 0.16±0.04 in YOUNG, and 0.26±0.18 to 0.15±0.06 in OLD), but maximal ADP stimulated respiration (J_{max}) increased in YOUNG (11±1 to 21±2 pmol $O_2 \cdot s^{-1} \cdot mg^{-1}$, $P<0.05$) but not in OLD (13±2 to 17±2 pmol $O_2 \cdot s^{-1} \cdot mg^{-1}$).

Our results indicate that the mitochondrial adaptation to HIT in elderly men and women move in the same direction as younger subjects, although only OXPHOS capacity increased significantly in OLD. Due to a low number of subjects in OLD, inclusion of additional subjects is necessary to elucidate exactly how elderly men and women adapt to HIT, compared with younger controls.

A3-03 Exercise modality specific mitochondrial adaptations in skeletal muscle of elderly people.

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In aged subjects, mitochondrial function is impaired, characterized by diminished oxidative capacity, reduced oxidative phosphorylation [1] and mitochondrial uncoupling [2]. Exercise training has previously been indicated to increase mitochondrial content in untrained [3] and elderly subjects [4]. Despite the consensus in the literature: that high intensity training (HIT) is superior to moderate intensity training (MIT) with regards to health effects [5], it is unclear how these training modalities affect mitochondrial function, particularly in elderly people.

To address this issue, subjects (N=20), aged between 70-75 y were randomized to eight weeks, three times a week, supervised, isocalorically matched sessions of either HIT, consisting of 4x4 intervals at 90% HR_{max} with 3 min active breaks in between, MIT, consisting of 50 min continuous running on 70% HR_{max}, or a control group that remained sedentary. Muscle biopsies were taken from the *vastus lateralis* before and after the intervention and high-resolution respirometry in saponin permeabilized muscle fibers was performed. Addition of various substrates and inhibitors allowed evaluation of TCA cycle and electron transfer system function.

Results indicated that combined Complex I- and Complex II-linked oxidative phosphorylation capacity (CI&II) increased after both MIT and HIT ($P<0.05$). Complex I-linked oxidative phosphorylation (CI) increased only after HIT ($P<0.05$) and this coincided with a relative increase of CI/CI&II ($P<0.05$), suggesting qualitative improvements within CI, independent of mitochondrial density. HIT also improved mitochondrial coupling efficiency during fatty acid oxidation ($P<0.05$). There were no changes in mitochondrial function in the control group.

The findings of the current study show that both MIT and HIT improve CI&II-linked oxidative phosphorylation in elderly people. However, mitochondria seem to adapt differently to the respective training modalities. While MIT gains seemed to rely on mitochondrial density, HIT seemed to additionally improve mitochondrial quality.

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A3-04 Lessons from the master athlete: mitochondrial contributions to aging.

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Age related loss of independence and mobility and an ill health are largely associated with sarcopenia, for which a prominent explanation is mitochondrial damage. Increases in mitochondrial DNA (mtDNA) mutations and mitochondrial dysfunction have been reported in aging skeletal muscle [1,2]. Master athletes (MAs) continue training and competing well into old age and represent an prominent model of healthy muscle aging [3]. The cellular mechanisms facilitating this achievement are currently unknown. Given their remarkable aging trajectory, it is reasonable to hypothesize that MAs have superior mitochondrial function and indices of mtDNA integrity.

15 world class elite MAs and 14 age-sex matched none-athlete controls (NAC), all over the age of 75, underwent muscle MRI scans to assess muscle mass and a biopsy of the *vastus lateralis*. mtDNA integrity was assessed using the duel cytochrome *c* oxidase/succinate dehydrogenase stain to ascertain the number of fibres with a respiratory system defect (RSD) and QPCR to provide an indication of mtDNA deletions and copy number. Three aspects of mitochondrial function were assessed in permeabilized myofibres: ROS production, respiration and calcium retention capacity.

MAs had significantly more muscle mass than their sedentary counterparts, fewer myofibres with a RSD and an increased mtDNA copy number. However, there were very few differences in any of the three aspects of mitochondrial function examined. Therefore, while MAs have less RSD than NACs at the individual fibre level, this is not sufficient to result in an improvement in mitochondrial function, when studied at the whole muscle level. Thus a superior mitochondrial profile probably does not explain MAs' remarkable muscle aging trajectory. However, in the presence of age related comorbidities the increased RSD may result in an exacerbation of these conditions in NACs.

This data raises questions regarding the impact of age related mitochondrial changes on the muscle, as MAs, with the same level of mitochondrial function as controls, still displayed remarkable strength.

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A3-05 The effect of high intensity training on mitochondrial fat oxidation in skeletal muscle and subcutaneous adipose tissue.

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High intensity interval training (HIT) is known to increase mitochondrial content in a similar way as endurance training (60-90% of maximal oxygen uptake (V_{O2peak}). Whether HIT increases mitochondria's ability to oxidize lipids is currently debated. We investigated the effect of HIT on mitochondrial fat oxidation in skeletal muscle and adipose tissue.

Mitochondrial oxidative phosphorylation (OXPHOS) capacity, mitochondrial substrate sensitivity (K'_m) and mitochondrial content were measured in skeletal muscle and adipose tissue in healthy overweight subjects before and after six weeks of HIT (three times per week at 298±21 W).

HIT significantly increased V_{O2peak} from 2.9±0.2 to 3.1±0.2 l·min^{-1}. No differences were seen in maximal fat oxidation in either skeletal muscle or adipose tissue. K'_m for octanoyl carnitine or palmitoyl carnitine were similar after training in skeletal muscle and adipose tissue. Maximal OXPHOS capacity with Complex I&II-linked substrates was increased after training in skeletal muscle but not in adipose tissue.

In conclusion, six weeks of HIT increased V_{O2peak}. Mitochondrial content and mitochondrial OXPHOS capacity were increased in skeletal muscle but not in adipose tissue. Furthermore, mitochondrial fat oxidation was not improved in either skeletal muscle or adipose tissue.

A3-06 Exercise improves insulin sensitivity and muscle mitochondrial respiration following Roux-en-Y gastric bypass surgery.

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The impact of exercise on insulin sensitivity and mitochondrial respiration following Roux-en-Y Gastric Bypass (RYGB) surgery is unknown. Our aim was to examine the effects of RYGB surgery-induced weight loss, both with and without aerobic exercise, on insulin sensitivity and mitochondrial respiration.

One to three months following RYGB, participants (N=128) were randomized to six-months of either moderate structured exercise (EX) or lifestyle education control (CON). Insulin sensitivity (S_I) was determined by intravenous glucose tolerance test (IVGTT). Biopsies of the vastus lateralis were obtained, before and after the six-month interventions. Mitochondrial respiration was measured using two SUIT protocols in permeabilized muscle fiber bundles. Data were analyzed using a per-protocol (PP) approach to assess the efficacy of exercise performed (>120 min/week).

One hundred participants completed the *a priori* defined intervention protocols: (EX, N=44; CON, N=56). Weight loss was similar for both groups (Post CON vs. Post EX; 84.2±21.3 vs. 84.3±17.2 kg, P=0.23). Cardiorespiratory fitness was improved in EX but not in CON. EX provided an additive improvement in S_I (Post CON vs. Post EX; 3.80±2.1 vs. 4.86±2.6, P=0.002) and only EX improved Complex I (CI; Pre EX vs. Post EX; 141±65 vs. 195±71 pmol·s^{-1}·mg^{-1}, P=0.018) and Complex I&II (CI&II; Post CON vs. Post EX; 264±82 vs. 323±102 pmol·s^{-1}·mg^{-1}, P=0.02) supported OXPHOS capacity (state P). Interestingly, there was a robust time effect for increased phosphorylation system control ratio (P/E) (P=0.006) and increase in the CI/CI&II$_P$ substrate control ratio (P=0.007), suggesting surgery-induced weight loss specific effects on mitochondrial respiration.

Aerobic exercise performed following RYGB surgery elicits further improvement in insulin sensitivity, which may be due to enhanced mitochondrial respiration. We also observed unique weight loss specific (time) effects on mitochondrial respiratory performance.

Trial Registration. clinicaltrials.gov identifier: NCT00692367. Funded by the National Institutes of Health/National Institute of Diabetes and Digestive and Kidney Diseases (R01 DK078192).

A4 Coupling efficiency and mitochondrial respiratory control

A4-01 Assessing ATP production and oxygen consumption simultaneously in permeabilized fibers: ATP/O.

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The biochemical efficiency of oxidative phosphorylation (OXPHOS), quantified as the amount of ATP produced per oxygen atom consumed (ATP/O or ~P/O), is vital to maintaining proper myocyte energetics. However, despite its central importance, it is difficult to experimentally determine the ~P/O ratio as a function of metabolic demand, and therefore, the relationship between OXPHOS efficiency and metabolic demand is poorly understood. O_2 consumption (high-resolution respirometry) and ATP production (determined fluorometrically using a 2-deoxyglucose – hexokinase – glucose-6-phosphate dehydrogenase – $NADP^+$ respiratory clamp system [1]), rates were measured simultaneously in permeabilized mouse oxidative and glycolytic skeletal muscle fiber bundles using a customized OROBOROS Oxygraph-2k.

With pyruvate and malate (5+2 mM) as substrate, at low [ADP] (5-20 µM), the ~P/O ratio increased as a function of [ADP], independent of an increase in O_2 consumption. Maximal ~P/O peaked at ~2.0 and was not different between oxidative and glycolytic muscle. Pharmacological inhibition of adenylate kinase decreased ATP production rate but did not alter ADP-dependent increases in OXPHOS efficiency.

These findings suggest that mitochondria respond to low levels of metabolic demand by initially increasing OXPHOS efficiency.

Supported by National Institute of Health R01 DK096907 (USA).

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A4-02 Cell ergometry: OXPHOS and ETS coupling efficiency.

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Analogous to ergometric measurement of V_{O2max} or V_{O2peak} on a cycle or treadmill, cell ergometry is based on OXPHOS analysis to determine OXPHOS capacity, $J_{O2P}=P$ [pmol $O_2 \cdot s^{-1} \cdot mg^{-1}$], at the cellular and mitochondrial level. V_{O2peak} and J_{O2P} provide reference values for a subject's or a cell's aerobic or mitochondrial competence. Aerobic catabolic flux (1 ml $O_2 \cdot min^{-1} \cdot kg^{-1}$ = 0.744 µmol$\cdot s^{-1} \cdot kg^{-1}$) is multiplied by the corresponding Gibbs force ($\Delta_k G_{O2} = \partial G / \partial_k \xi_{O2}$; typically -470 kJ/mol or -0.47 J/µmol O_2) to obtain the mass-specific aerobic input power [$W \cdot kg^{-1}$]. The corresponding mechanical output power, P_{peak} [$W \cdot kg^{-1}$], in cycle *ergo*metry results in *ergo*dynamic efficiencies [1] of about 0.25,

$$\varepsilon_{peak} = P_{peak} / -(J_{O2peak} \cdot \Delta_k G_{O2}) = (P_{peak} / J_{O2peak}) / -\Delta_k G_{O2} \quad (1)$$

In OXPHOS analysis the output power is mitochondrial ATP production, $J_{\sim P} = \sim P$, times the Gibbs force of phosphorylation ($\Delta_p G_{\sim P} = \partial G / \partial_p \xi_{\sim P}$), which is typically 48 to 62 kJ/mol ~P [1]. Ergodynamic efficiency is a power ratio, partitioned into a flux ratio (the famous ~P/O_2 ratio; ATP yield per oxygen consumed, $Y_{\sim P/O2} = J_{\sim P} / J_{O2P} = \sim P/P$) and force ratio,

$$\varepsilon_P = (J_{\sim P} \cdot \Delta_p G_{\sim P}) / -(J_{O2P} \cdot \Delta_k G_{O2}) = \sim P/P \cdot \Delta_p G_{\sim P} / -\Delta_k G_{O2} = j_{\approx P} \cdot f_{\approx P} \quad (2)$$

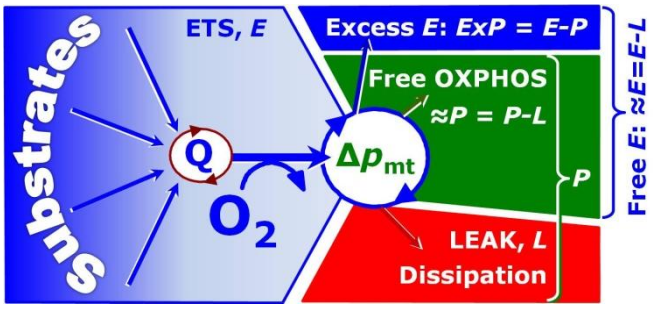

Figure 1. Capacities for ETS, OXPHOS and LEAK respiration (*E, P, L*). Free devided by total OXPHOS capacity, *≈P/P*, is the OXPHOS coupling efficiency; free divided by total ETS capacity, *≈E/E*, is the ETS coupling efficiency [2]. The ETS excess capacity, *ExP*, is available for coupled processes other than phosphorylation.

The upper limit of *~P/P* is the mechanistic ~P:O_2 ratio or stoichiometric number, $v_{\sim P/O2}$. The free respiratory OXPHOS capacity, *≈P=P-L*, is potentially available to drive phosphorylation, *~P* (Figure 1). Quantitatively justified in cases [3] but better adjusted to the protonmotive force, Δp_{mt}, the dissipative LEAK *component*, *L*, in the OXPHOS state *P* can be assessed by respiration, *L*, measured in the LEAK state,

$$v_{\sim P/O2} = \sim P_{limit}/P = \sim P/(P\text{-}L) = \sim P/\approx P \quad (3)$$

~P/P divided by *~P/≈P* defines the OXPHOS coupling efficiency, $j_{\approx P}$, as a normalized flux ratio, which is a hyperbolic function of RCR (Figure 2),

$$j_{\approx P} = \approx P/P = (L\text{-}P)/P = 1\text{-}L/P = 1\text{-}RCR^{-1} \quad (4)$$

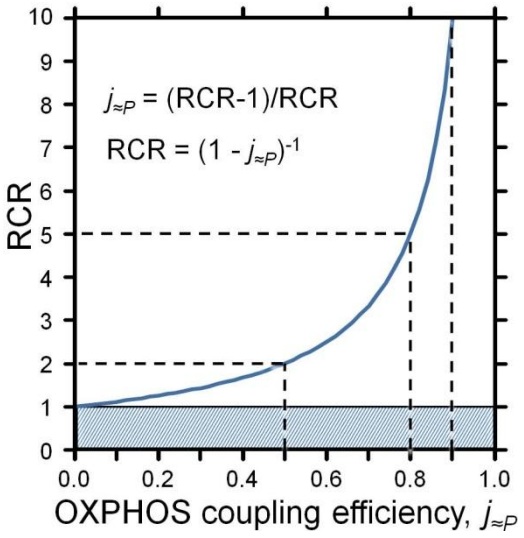

Figure 2. Respiratory acceptor control ratio as a function of OXPHOS coupling efficiency, $j_{\approx P}$. RCR is the State 3/State 4 flux ratio [4], equal to *P/L* if State 3 is at saturating [ADP] and [P_i]. RCR from 1.0 to infinity is highly non-linear in the typical experimental range of RCR 3 to 10: when $j_{\approx P}$ increases from 0.8 to 0.9, RCR doubles from 5 to 10. RCR increases to infinity at the limit of $j_{\approx P}$=1.0. Statistical analyses of RCR±SD require linearization by transformation to $j_{\approx P}$.

OXPHOS coupling efficiency in Equation (4) is determined by respirometric OXPHOS analysis. At the limit of maximum $j_{\approx P}$=1.0 the dissipative LEAK processes, *L*, are zero. Ergodynamic efficiency, *ε*, not only depends on mechanistic coupling but also on the force ratio or force efficiency. At ergodynamic equilibrium, *ε*=1.0, fluxes vanish to zero when $j_{\approx P} = f_{\approx P} = 1$ (Equation 2).

The OXPHOS state can be established experimentally in cells or tissues by selective permeabilization of plasma membranes, with ADP and P_i at kinetic saturation and fuel substrate combinations which reconstitute physiological TCA cycle function (Figure 1). The free OXPHOS capacity may be kinetically limited by the phosphorylation system to utilize Δp_{mt}. Then ETS capacity is in excess of OXPHOS capacity by the factor j_{ExP}=(*E-P*)/*E*. Such kinetic limitation diminishes the effective $j_{\approx P}$ independent of coupling control. Therefore, the ETS coupling efficiency is defined as $j_{\approx E}$=(*E-L*)/*E* (compare Eq. 4) and related to $j_{\approx P}$ by taking into account the apparent ETS excess capacity (Figure 1),

$$j_{\approx E} = j_{\approx P}\cdot(1\text{-}j_{ExP}) + j_{ExP} \quad (5)$$

Flux control factors and coupling efficiencies were derived from principles of thermodynamics rather than arbitrarily introduced as jargon of a specialized discipline.

Supported by K-Regio project *MitoCom Tyrol.*

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A4–03 Analysis of mitochondrial function in multiple brain regions through *in situ* permeabilization.

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Mitochondria interact with their environment in a tissue-specific manner, and in the brain, the regulation of mitochondrial function is regionally unique. Analysis of mitochondrial respiration within the brain is traditionally assessed through utilization of isolated mitochondria, an approach with high tissue requirements that limit the applicability of the method in investigating function in smaller brain regions. This tissue limitation impedes the study of neurodegenerative diseases, which are often attributed to mitochondrial dysregulation within smaller specific brain regions. In addition, isolating mitochondria interrupts the native reticular structure of the mitochondria and also yields a final suspension composed of mitochondria from multiple sub-populations from various cell types, making physiological interpretations challenging. Therefore, the current methodology does not appear adequate for investigating the source of changes in brain mitochondrial function.

As a result, we established a method for determining mitochondrial function *in situ* through permeabilization of small brain samples of approximately 2 mg. The method was validated through electron transfer system complex-specific stimulation and inhibition, and imaging confirmed conservation of mitochondrial morphology. We applied the permeabilized brain tissue preparation to investigating regional variation in mitochondrial function in the mouse brain with both acute and chronic perturbations and compared our results with the traditional isolated mitochondria preparation.

The permeabilization of brain tissue *in situ* circumvents the large tissue requirements of isolated mitochondria while leaving the native mitochondrial intact, allowing for analysis of mitochondrial respiration in multiple regions in a single mouse brain.

A4-04 Region-specific differences in Complex I- and Complex II-linked respiration in the mouse brain.

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Mitochondrial dysfunction appears to be a common factor in neurodegenerative diseases. However, such diseases differ markedly in the nervous tissue affected. To test potential differences in mitochondrial respiratory capacity of different brain tissues under physiological or pathological conditions, we established a SUIT protocol for the analysis of oxidative phosphorylation (OXPHOS) and electron transfer system capacity (ETS) of small amounts of defined brain-tissues of mice. This protocol enables us to measure, independently, Complex I-, II- and IV-linked (CI, CII, and CIV, respectively) respiration, as well as the combined CI&II-linked OXPHOS- and electron transfer system (ETS) capacity in a single run from as little as 2 mg tissue applying the OROBOROS high-resolution respirometry system [1].

The reproducibility within one experiment (two replica from the same tissue sample) and between experiments was very high. We observed significantly higher CI-linked oxygen fluxes in the motorcortex and CII-linked respiration in the striatum, when comparing motorcortex, striatum, hippocampus and brainstem obtained from young, healthy, adult, male C57BL6/J mice. No differences were found for CI&II-linked ETS capacity and CIV activity expressed as oxygen consumption per tissue mass or as CIV/CI&II$_E$ flux control ratio. The *P/E* coupling control ratio (CI&II), an index of the limitation of OXPHOS capacity by the phorphorylation system, was significantly different between motorcortex and hippocampus.

The established protocol allows detailed analysis of mitochondrial function from small amounts of specific tissues. It thus enables comparison of different brain tissues implicated in neurodegenerative diseases of the healthy mouse and disease models while leaving sufficient amounts of sample for additional analysis of the tissues.

Supported by FWF W1206-B05 (CS) and K-Regio project *MitoCom Tyrol* (EG).

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A4-05 OXPHOS analysis in small prostate biopsies.

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In 2012, adenocarcinoma of the prostate was the most prevalent male cancer type in Western civilized countries, accounting for 9.5% of all cancer related deaths in Europe [1,2].

Numerous studies have addressed the metabolic characteristics and molecular pathways of the disease, primarily applying prostate cancer related cell lines as models. In contrast, very little is known about the metabolic properties of fresh prostate cancer tissue in terms of mitochondrial oxidative phosphorylation (OXPHOS) and ATP production. This lack of data is mainly due to the limited access to fresh prostate tissue biopsies and their small sample size. Therefore, in order to overcome this limitation, we developed a method which, for the first time, enables OXPHOS analysis of very small benign and malignant prostate tissue biopsies (2-5 mg wet weight per individual measurement).

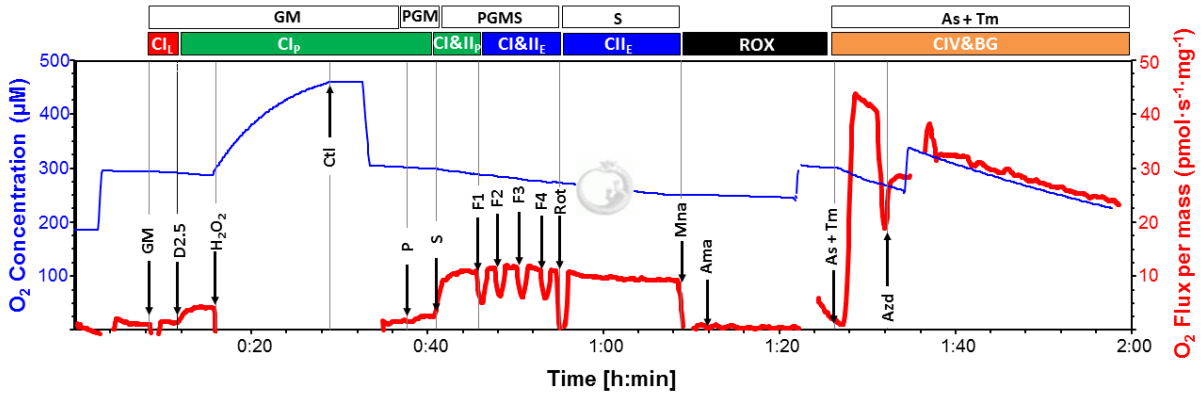

Figure 1. SUIT protocol with permeabilized prostate tissue biopsies (4 mg W_w per chamber, MiR05Cr, 37 °C). Initial oxygenation to reach O_2 concentration of 300 µM to avoid oxygen dependent effects during the experiment; LEAK respiration with glutamate and malate (GM), CI_L; addition of 2.5 mM ADP (D2.5) for measurement of CI-linked OXPHOS capacity, CI_P; H_2O_2 incubation (500 µM, 15 min) to induce oxidative stress; termination of oxidative stress by addition of catalase (Ctl) and opening of chamber to restore O_2 concentration of 300 µM; after flux stabilization addition of pyruvate (P); titration of succinate (S) to quantify CI&II-linked OXPHOS capacity, $CI\&II_P$; stepwise uncoupling with FCCP (F1-F4) to obtain CI&II-linked ETS capacity, $CI\&II_E$; inhibition of CI with Rotenone (Rot) allows evaluation of CII-linked ETS capacity (CII_E) and addition of malonate followed by antimycin A enables assessment of residual oxygen consumption (ROX) remaining after inhibition of CII and CIII; addition of Ascorbate and TMPD (AsTm) for the measurement of CIV activity and chemical background (CIV + BG); after CIV inhibition by azide (Azd) a correction for the chemical side reactions of As and Tm can be performed.

Samples were harvested immediately after radical prostatectomy and examined using high-resolution-respirometry (OROBOROS Oxygraph-2k) and a sophisticated substrate-uncoupler-inhibitor titration (SUIT) protocol. For this purpose, prostate tissue was

mechanically permeabilized which, as previously reported, represents a very good mitochondrial preparation alternative to isolated mitochondria, both reducing the amount of required sample material and largely preserving structural integrity of the cell [3]. An artificial incubation with H_2O_2 was used to assess the mitochondrial response to cellular stress exerted by enhanced ROS production [4]. In addition, an easy and fast assay for determination of CIV activity was applied adding ascorbate and the CIV substrate *N,N,N′,N′*-Tetramethyl-p-phenylenediamine dihydrochloride (TMPD) followed by inhibition with sodium azide to correct for chemical background reactions. The SUIT protocol, including different substance combinations, allows measurement of flux control variables in different substrate and coupling states (Figure 1).

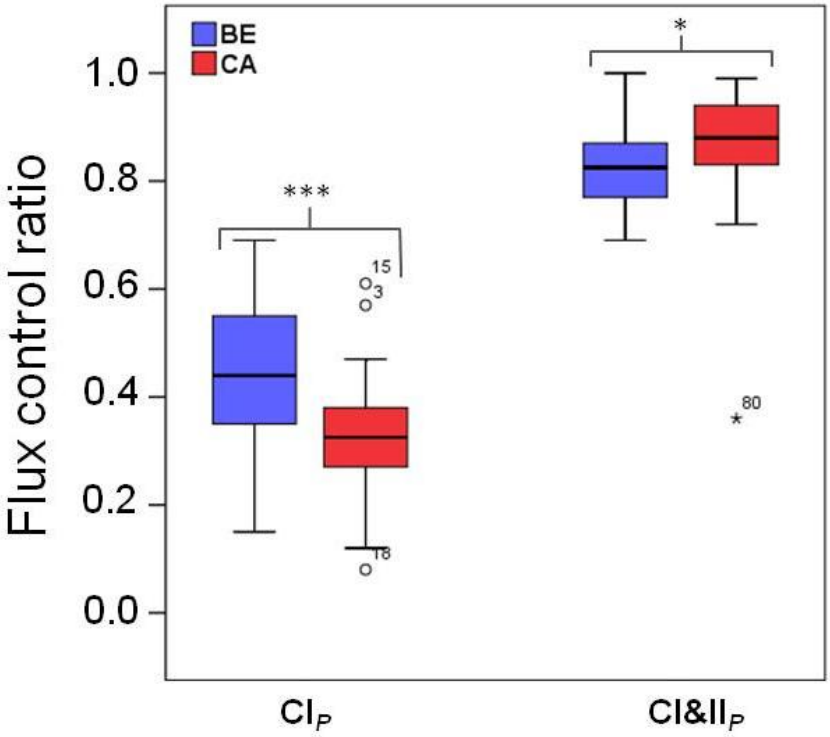

Figure 2. Comparison of benign (BE) vs malignant (CA) tissue in different substrate and coupling states (N=42). CI$_P$: CI-linked OXPHOS capacity with malate and glutamate before H_2O_2 incubation. CI&II$_P$: CI&II-linked OXPHOS capacity with malate, glutamate, pyruvate and succinate after H_2O_2 incubation. Data are means ± SD; P<0.001 (Bonferroni–Holm sequential corrected P-values).

Any sample biopsy (app. 6–10 mg of wet weight) was divided into two subsamples and mechanically permeabilized using two pairs of extra-sharp forceps [5]. Each subsample was placed into one of the four chambers of two OROBOROS Oxygraph-2k operated in parallel. Measurements were performed in MiR05 with creatine (MiR05Cr) at 37 °C. Parallel analyses of a paired sample consisting of one benign and one malign biopsy from a single patient could be conducted within little more than two hours, yielding high quality respirometry data while preserving tissue structure for subsequent tissue analysis and DNA extraction.

The first data evaluations revealed an overall decreased CI-linked OXPHOS capacity before H_2O_2 treatment, a higher vulnerability toward cellular stress mediated by H_2O_2 but a similar CI&II-linked OXPHOS capacity after H_2O_2 treatment in cancer compared to benign tissue (Figure 2).

Supported by Oncotyrol[2-4] and K-Regio project *MitoCom Tyrol*[1,5].

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A4-06 Mitochondrial function in white adipose tissue: palmitoleic acid (C16:1n7) treatment enhances white adipocyte oxygen consumption.

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White adipose tissue (WAT) has a crucial role in the development of obesity and related diseases, and the relevance of WAT mitochondrial function has been highlighted in the literature during the last decade [1,2,4]. Mitochondrial parameters, such as reactive oxygen species, biogenesis, fatty acid oxidation, respiration and uncoupling have been implicated in white adipocyte proliferation, adipogenesis, transdifferentiation, lipolysis and lipogenesis [1,2,4]. Therefore, WAT mitochondria function regulation is a promising target for the development of therapies tackling insulin resistance, obesity and related diseases.

Palmitoleic acid is a monounsaturated n-7 fatty acid (16:1n7), produced and released by adipocytes, that has been shown to enhance whole body glucose disposal, to attenuate high-fat-fed mice hepatic steatosis, to protect pancreatic beta-cells from palmitic acid-induced death and to improve circulating lipid profile in both rodents and humans [3]. Our group has recently found strong evidence that palmitoleic acid is an important positive modulator of white adipocyte lipolysis and the content of the major lipases ATGL and HSL through a PPAR alpha-dependent mechanism *in vitro* and *in vivo*. Acute and chronic palmitoleic treatment led to enhanced lipolysis and inhibited lipogenesis [3].

To study the correlation of the previously described effects of palmitoleic acid in WAT with mitochondrial function, we performed oxygen consumption experiments using the OROBOROS Oxygraph-2k. Our results show that both acute and chronic treatments with palmitoleic acid enhanced ROUTINE oxygen consumption in 3T3-L1 adipocytes by 7.6% and 12.8%, respectively. Experiments were carried out to test whether lipolysis and respiration enhancement by palmitoleic acid are linked to improved mitochondrial fatty acid oxidation and/or uncoupling.

Supported by FAPESP, CAPES. Acknowledgement: Prof. Alicia Kowaltowski and group (Univ Sao Paulo, Brazil).

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A4–07 Severity of polymicrobial sepsis modulates mitochondrial function in rat liver.

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Microcirculatory and mitochondrial dysfunction are considered as the main pathophysiological mechanisms in septic shock and multiorgan failure [1]. However, results concerning mitochondrial function in the liver are controversial [2-4]. One possible reason for this heterogeneity could be a wide spectrum of septic models used, with varying degrees of sepsis severity. The aim of the present study was to analyze

hepatic mitochondrial function during polymicrobial sepsis with abdominal focus, depending on severity of the septic process.

31 Wistar rats were divided into four groups: control, sham (laparotomy only) and two septic groups (colon ascendens stent peritonitis, CASP, using 16G or 14G stent). 24 h after sham or CASP operation, liver mitochondria were isolated. Mitochondrial oxygen consumption was determined using a Clark type electrode in the presence of glutamate and malate (GM) or succinate (S), to examine Complex I- or Complex II- (CI- or CII-) linked respiration, respectively. LEAK (L_N, presence of substrates, no adenylates) and OXPHOS (ADP stimulated) respiration were assessed. The respiratory acceptor control ratio (RCR, OXPHOS/LEAK) and ADP/O ratio (ADP added/oxygen consumed) for both substrate states were calculated. Data are presented as means±SD, 1-way ANOVA followed by Tukey's post hoc-test.

RCR (CII) was higher after laparotomy compared to control. In septic animals with the smaller stent (16G) RCR (CI and CII) was higher compared to controls. In contrast, in septic animals with a larger stent size (14G) RCR was similar to controls. ADP/O ratios were comparable in all groups:

Mean±SD	RCR (GM)	RCR (S)	ADP/O (GM)	ADP/O (S)
Control	8.7±3.0	6.2±2.0	3.3±1.1	2.7±1.3
SHAM	12.5±2.7	8.4±1.0*	3.4±1.0	2.6±0.4
16G	15.2±2.9*	9.6±1.0*	3.5±0.7	2.8±0.6
14G	11.2±2.9$^{\#}$	7.8±0.9	3.3±0.5	2.4±0.3

*P<0.05 vs control, $^{\#}P$<0.05 vs 16G.

Operative stress induced by laparotomy and, to a greater extent, moderate sepsis (CASP 16G) lead to a higher energy coupling without affecting the efficacy of oxidative phosphorylation. More severe sepsis (CASP 14G) does not affect hepatic mitochondrial function. Thus, the severity of sepsis might explain the heterogeneity of mitochondrial function reported in the literature.

Performed with approval of the local animal care and use committee.

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B1 Experimental regimes in functional studies of mitochondrial preparations

B1-01 Evaluation of critical experimental parameters for assessing mitochondrial bioenergetics in permeabilized myofibres.

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Respirometric and fluorometric approaches for assessing the regulation of submaximal and maximal ('capacity') oxidative phosphorylation and mitochondrial oxidant emission respectively, in permeabilized myofibres, have been documented extensively [1-3 and other]. Optimizing such bioenergetic measurements requires control of critical experimental parameters including partial pressure of oxygen (p_{O2}), temperature and assay media composition (ionic balance, oxidant scavenging capacity, etc.) [1-4]. Recently, myofibre contraction, analogous to ADP-induced rigor, has also been reported to alter respiratory sensitivity to ADP (K'_m) and maximal respiration in a temperature-sensitive manner which can be prevented with myosin-ATPase inhibitors [3,4].

The purpose of this presentation is to stimulate debate regarding the need for standardizing specific parameters throughout the field vs the value of customizing certain parameters to each hypothesis. An emphasis will be placed on the manner by which each factor affects mitochondrial bioenergetics in permeabilized myofibres.

Supported by National Institute of Health (USA) and National Science and Engineering Research Council (Canada).

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B1-02 NAMPT remodels substrate metabolism in skeletal muscle.

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In mammals, nicotinamide phosphoribosyltransferase (NAMPT) is responsible for the first and rate-limiting step in the "salvage pathway" that converts nicotinamide to nicotinamide adenine dinucleotide (NAD^+). We previously showed that exercise increased skeletal muscle NAMPT expression which correlates with mitochondrial content in humans [1]. Caloric restriction-induced beneficial effects in oxidative stress, mitochondrial biogenesis and metabolic adaptation in mice require NAMPT [2]. The mechanisms responsible for metabolic adaption are unclear. We hypothesized that muscle-specific overexpression of NAMPT in mice (*Nampt*Tg) would improve muscle respiratory capacity and control and would be additive with endurance exercise training in these mice.

*Nampt*Tg mice express 10-fold more skeletal muscle NAMPT protein compared to wild type (WT) mice based on western blotting studies. A concomitant elevation of NMN (5-

fold) and NAD^+ (3-fold) in *Nampt*Tg skeletal muscle revealed increased NAMPT enzymatic activity. WT and *Nampt*Tg mice were housed with ('trained') or without ('sedentary') running wheels to elicit voluntary exercise for a period of 7 weeks (WT Sedentary; WT Trained; *Nampt*Tg Sedentary; *Nampt*Tg Trained). No differences were observed in the proclivity of *Nampt*Tg mice to engage in voluntary exercise. Mice were fasted for 5 hours prior to sacrifice. Quadriceps muscles were used for the respiration measurements. Quadriceps muscles were used for the respiration measurements. SUIT protocols were performed in permeabilized fiber bundles using Complex I and II-linked substrates (malate, glutamate, succinate) in combination with a fatty acid oxidation (FAO, palmitoyl-carnitine) substrate (CI&II&FAO; Figure 1).

NAMPT TG mice had an increased fatty acid oxidative (CI&II&FAO) respiration after training ($P<0.05$; Figure 1A). Notably, this training-induced improvement in CI&II&FAO in the NAMPT TG mice trended to be higher than the trivial increase in CI&II&FAO flux with training in WT mice ($P=0.0595$; Figure 1A). Increased NAMPT levels were sufficient to dramatically improve mitochondrial coupling efficiency, as reflected by the significant reduction in the ratio of proton leak (*L*) to OXPHOS capacity (*P*) in the NAMPT TG mice compared with WT mice at baseline ($P<0.05$; Figure 1B). While training improved coupling efficiency in the WT mice ($P<0.05$, Figure 1B), it tended to further improve coupling efficiency in the NAMPT TG mice as well ($P=0.107$; Figure 1B).

Taken together, decreased leak/OXPHOS capacity (i.e. improved coupling efficiency) and an increased capacity to burn CI&II&FAO-linked substrates indicate a synergistic improvement in muscle mitochondrial function via NAMPT and endurance exercise whereby an increased amount of NAMPT is sufficient to enhance the exercise-induced improvements in substrate metabolism.

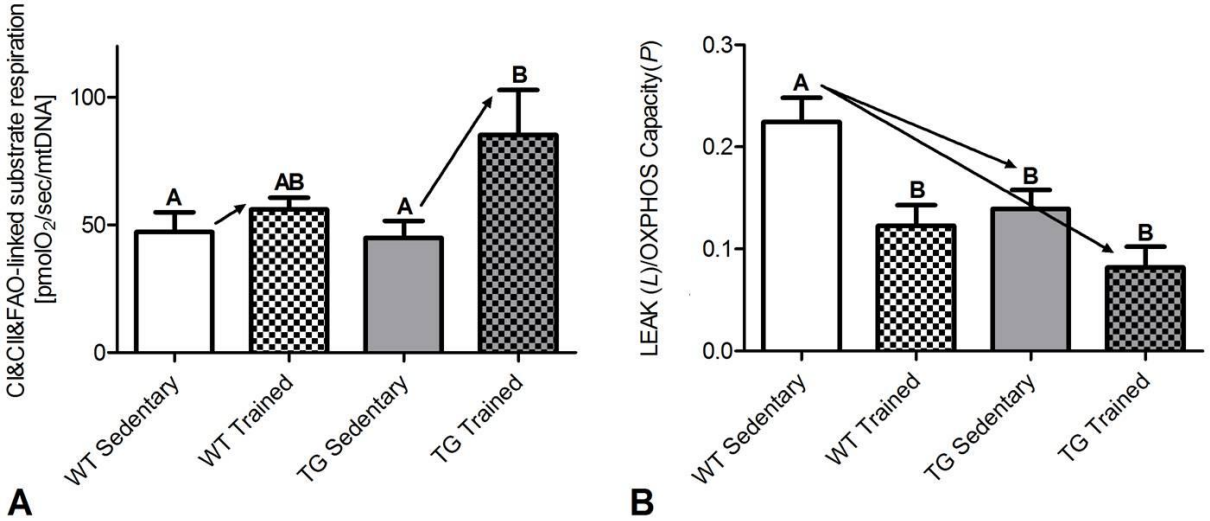

Figure 1. Overexpression of NAMPT improves mitochondrial function after training in skeletal muscle. Respiration was measured in permeabilized muscle fiber bundles using a SUIT protocol with combined CI&II&FAO-linked substrates, adding a fatty acid substrate (palmitoyl-carnitine) and CI&II-linked substrates simultaneously. **A**: OXPHOS (*P*) capacities in the presence of adenylates (ADP) are presented as respiration per mtDNA copy number. **B**: LEAK (*L*) respiration was measured in the absence of adenylates (ADP) and coupling control ratios are presented as LEAK divided by OXPHOS capacity. Bars represent mean±SEM for wild type (WT) sedentary and trained mice and for NAMPT Transgenic (TG) sedentary and trained mice. Letters different from each other indicate statistically significant differences ($P<0.05$; Tukey's HSD). $N=6$ (WT sedentary); $N=7$ (WT Trained); $N=6$ (TG sedentary); $N=5$ (TG trained).

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B1-03 The mitochondrial enzyme 4-hydroxy-2-oxoglutarate aldolase (HOGA) – involvement in the TCA cycle.

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4-hydroxy-2-oxoglutarate aldolase (HOGA) is a bi-functional mitochondrial enzyme, expressed predominantly in liver and kidney. HOGA is involved in the hydroxyproline degradation pathway (HOG→glyoxylate+pyruvate), and mutations in HOGA result in primary Hyperoxaluria Type III, characterized by excessive oxalate production and kidney stone deposition [1]. We hypothesized that HOGA may also be involved in the TCA cycle as an oxaloacetate decarboxylase (oxaloacetate→pyruvate; Figure 1), which may allow the TCA cycle to turnover in the absence of pyruvate and/or excess oxaloacetate.

The kinetics of HOGA with substrates HOG and oxaloacetate were investigated by measuring the K'_m and k_{cat} of recombinant human HOGA, using an LDH-coupled microplate assay. The role of HOGA in the TCA cycle was investigated using mitochondria, isolated from rat liver and kidney, where HOGA is highly expressed, and brain and heart, where expression is lower. ADP-stimulated malate respiration was expressed relative to ADP-stimulated respiration with malate and pyruvate (M:PM), using oxygraphy (OROBOROS Oxygraph-2k). Note malate was used as oxaloacetate cannot cross the inner mitochondrial membrane.

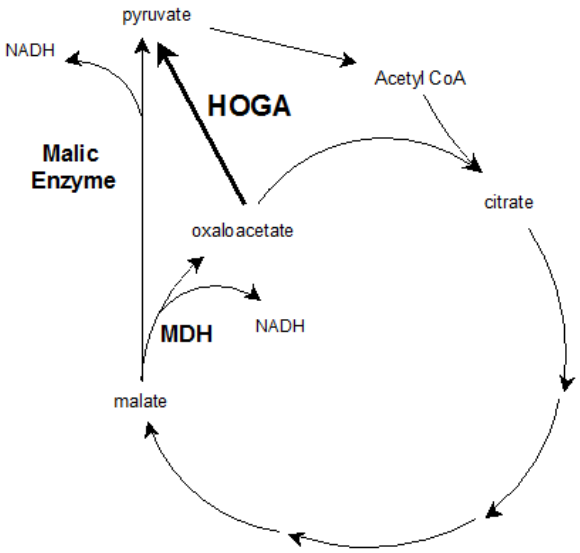

Figure 1. Potential role of HOGA in the TCA cycle as an oxaloacetate decarboxylase (arrow in bold). This activity may allow turnover of the TCA cycle in the absence of incoming pyruvate and/or excess oxaloacetate. Malic enzyme can also form pyruvate from within the TCA cycle. MDH=malate dehydrogenase.

While HOGA was 75% less efficient at cleaving oxaloacetate than its other substrate, HOG (K'_m/k_{cat}), the K'_m for oxaloacetate was within range of that estimated for TCA intermediates ($K'_{m,ox}$=129±8 μM, $k_{cat,ox}$=0.52±0.01 s^{-1}; $K'_{m,HOG}$=55±5 μM, $k_{cat,HOG}$=1.01±0.03 s^{-1}). Overall, HOGA appears to use the same catalytic mechanism to cleave both HOG and oxaloacetate substrates. Interestingly, the TCA cycle intermediate α-ketoglutarate was found to be a competitive inhibitor of HOGA oxaloacetate decarboxylase activity (K_i=2.8 mM). Mitochondria from rat liver had the highest M:PM respiration relative to all other organs (0.46±0.05, P<0.05). Though kidney had a higher M:PM respiration than heart (0.27±0.02 vs 0.15±0.02, P<0.05 in kidney and heart, respectively), brain respired as well as kidney (0.33±0.04).

In summary, HOGA cleaves oxaloacetate and HOG using the same catalytic mechanism but was less efficient with oxaloacetate. Liver and kidney have high HOGA expression, and mitochondria from both respire significantly better on malate relative to PM than heart mitochondria. The brain respires just as well with malate compared to kidney, and this may be due to high expression of malic enzyme, which can convert malate directly to pyruvate (Figure 1). Malate supported respiration in HOGA overexpressing cells will confirm the direct role of HOGA in the TCA cycle.

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B1-04 Methylenblue, an enhancer of mitochondrial substrate-level phosphorylation.

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Neurodegenerative disorders are associated with mitochondrial disfunction. In Alzheimer's and Parkinson's disease defects of respiratory system components have been described. Methylenblue (MB), a potential neuroprotective agent, is efficient in various Alzheimer and Parkinson models. The mitochondrial effects of MB are explained by the so-called alternative electron transfer model.

In the present study the effect of MB on mitochondrial substrate-level phosphorylation (SLP) was investigated. SLP in mitochondria is attributed to succinyl-CoA ligase catalyzing the formation of ATP and succinate from succinyl-CoA, ADP and P_i, in the citric acid cycle. This reaction can be considered an alternative way of ATP synthesis, because it is partially independent from the electron transfer system (ETS) and from the mitochondrial protonmotive force. Therefore, it has a great significance in hypoxia or when the ETS is impaired. Besides that, SLP plays an important role during thermogenesis, when the mitochondria are uncoupled by the activation of thermogenin. MB can transfer electrons from Complex I to cytochrome *c*, bypassing the intermediate components of the ETS. In case of Complex I inhibition it avoids NADH accumulation and thus inhibition of mitochondrial dehydrogenases, particulary α-ketoglutarate dehydrogenase (α-KGDH). Therefore, in the presence of MB the α-KGDH reaction can proceed and provide succinyl-CoA for matrix SLP.

Our measurements were carried out on isolated mitochondria prepared from guinea-pig brain cortex. In mitochondria, ATP can be generated by oxidative phosphorylation, substrate-level phosphorylation and the adenylate kinase reaction catalyzing the formation of AMP and ATP from 2 mol ADP. The latter reaction was inhibited by AP5 during our measurements. ATP synthesis was measured spectrophotometrically with a coupled enzyme assay. The mitochondrial membrane potential was detected by safranine fluorescence and oxygen consumption was measured by high-resolution respirometry (OROBOROS Oxygraph-2k).

In the presence of ETS inhibitors, mitochondrial ATP synthesis was inhibited. In the presence of oligomycin, OXPHOS was inhibited but a low level of SLP could be detected. In the presence of ETS inhibitors, addition of MB partially restores $\Delta\Psi_{mt}$ and electron flow, resulting in ATP synthesis from OXPHOS and SLP. In the presence of oligomycin, addition of MB resulted in increase of SLP, which could be further stimulated by uncouplers. In the simultaneous presence of ETS inhibitors and oligomycin administration of MB stimulates SLP and the formed ATP can contribute to the alleviation of energetic insufficiency. The effects of MB on SLP, however, are substrate dependent. α-ketoglutarate supports MB's effect on SLP, but addition of succinate does not stimulate SLP.

We conclude that MB mediated stimulation of SLP can be an important factor to maintain energetic competence of mitochondria.

Supported by OTKA (NK 81983), TÁMOP (4.2.2./B-09/1), MTA (MTA TKI 2013), National Brain Research Program (KTIA_13_NAP-A-III/6).

MiP2013: Sir John Walker

B1-05 Optimization of malate concentration for high-resolution respirometry: mitochondria from rat liver and brain.

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Complex substrate-uncoupler-inhibitor titration (SUIT) [1] protocols, used in high-resolution respirometry, are designed to determine respiration with CI-linked and CII-linked substrates in a single experiment. Observations on multiple types of mitochondria revealed that malate, a substrate used to fuel CI-linked respiration, inhibits CII-linked respiration (succinate in the presence of rotenone, Rot; coupled and noncoupled) at 2 mM concentration. Mutual interference between succinate and malate was already described by Harris and Manger [2], and the effect was attributed to accumulation of oxaloacetate, an inhibitor of succinate dehydrogenase (SDH) and fumarate [1,3].

In this study, we examined the effect of various concentrations of malate on CI-linked and CII-linked respiration, as well as on the involvement of SDH as a constitutive part of Krebs cycle, in the respiration with CI-linked substrate combinations, in mitochondria from rat liver and brain.

In both liver and brain mitochondria, 0.5 mM malate, added in combination with 5 mM pyruvate and 10 mM glutamate, supported >90% of maximal CI-linked respiration observed with saturating 2 mM malate. Conversely, when malate was added to noncoupled mitochondria fueled by CII-linked substrate succinate(Rot), it inhibited ETS capacity by 6% and 8% at 0.5 mM, 22% and 25% at 2 mM, and 33% and 37% at 5 mM malate concentration, in mitochondria from liver and brain, respectively. A similar degree of inhibition of noncoupled CII-linked respiration by malate was observed when mitochondria were previously exposed to CI&II–linked substrates followed by inhibition of CI with rotenone. Assuming that this effect is caused by an indirect impact of malate on SDH, lower inhibition of respiration at lower malate concentration would be suggestive of a higher involvement of SDH in respiration with CI-linked substrate combinations. Indeed, this involvement, as determined by inhibition of SDH with 5 mM malonate after ADP-stimulated respiration with pyruvate, glutamate and malate, amounted to more than 50% without malate, ~35% with 0.5 mM, ~20% with 2 mM, and ~15% with 5 mM malate concentration in both types of mitochondria.

In summary, our observations showed that despite tremendous physiological differences between liver and brain mitochondria, malate affected their respiratory patterns in a similar manner, suggesting that this may be a more general phenomenon. Therefore, we recommend that when malate is used in complex SUIT protocols, a concentration of 0.5 mM should be used rather than previously applied higher concentrations (2 mM), balancing its stimulatory and inhibitory effects on mitochondrial respiration.

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B1-06 Multipotent stromal cells injection improves rat liver regeneration through activation of mitochondrial functions after subtotal hepatectomy.

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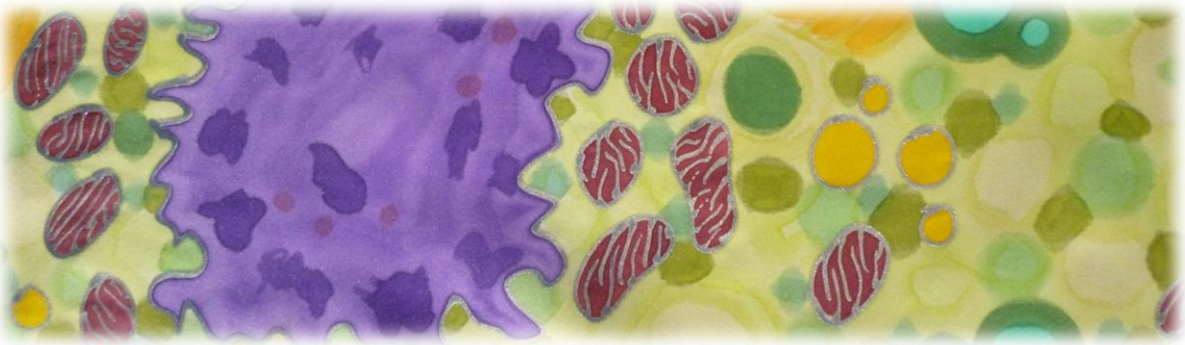
Mammalian liver has unique regenerative capacity, which makes liver resection useful research on regeneration and ways to accelerate regenerative processes. The aim of our work was to study mitochondrial function in rat liver tissue after subtotal hepatectomy and to investigate effects of multipotent stromal cells (MSCs) injection on liver regeneration.

On day 0 we performed a 80% hepatectomy on adult white male rats. Liver mitochondria were isolated from dissected liver tissue. On day 7 we performed total hepatectomy and isolated liver mitochondria under the same experimental conditions; after that animals were sacrificed. Rats were divided into two groups: control (N=12); MSCs (N=9). On day 0 animals of MSCs group were subjected to intrasplenic injection of 10^6 MSC in saline. Rats of the control group were injected with the same volume of saline. MSCs were isolated from rats' umbilical cord by enzyme digestion, cultured in appropriate growth medium and rats' phenotype characterized in accordance with a standard protocol. Mitochondrial respiratory parameters were measured using an oxygraph (Hansatech, UK).

At the end of the experiment, seven and six rats had survived in control and MSCs groups, respectively. Only mitochondria isolated from these rats were taken into account for the final analysis. A decreased mitochondrial protein amount to liver tissue weight ratio was observed on day 7, compared to day 0, in both groups. In the MSCs group the decrease of mitochondrial protein concentration was significantly less than in the control group (MSCs: 1.5±0.2 fold; control: 2.1±0.3 fold). At the same time the ~P/O ratio did not change in the control group (day 0: 3.0±0.4; day 7: 3.0±0.3) but slightly (P<0.1) increased in the MSCs group (day 0: 2.7±0.3; day 7: 3.2±0.1). Mitochondrial oxygen flux (nmol $O_2 \cdot min^{-1} \cdot mg^{-1}$ protein) in the control group significantly increased on day 7 compared to day 0 in the presence of glutamate and malate (CI_L: 5.2±0.4 vs 3.0±0.2), +rotenone, succinate and ADP (CII_P: 53.9±1.7 vs 34.7±5.2), +oligomycin (CII_L: 9.9±0.7 vs 7.2±0.9), and +FCCP (CII_E: 53.7±4.5 vs 43.0±3.7). No increase in respiration was found in the MSCs group. *L/E* and *P/E* coupling control ratios for CII-linked respiration were unchanged in both groups.

In summary, we can assume that MSCs injection accelerates not only proliferation and liver growth but also differentiation of progenitors to different cell types, through normalization of liver mitochondrial function in terms of correlation between respiration, $\Delta\Psi_{mt}$ and ROS production, in rat liver tissue after 80% liver resection.

Supported by grant RFBR № 14-04-01224 A.



B2 Are mitochondria sources or sinks of reactive oxygen species?

B2-01- Mitochondria are sources, rather than sinks, of reactive oxygen species. Effects of mitochondria-targeted antioxidants.

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Assuming that mitochondria are sources of reactive oxygen species (ROS), causing a number of pathologies, predicts that mitochondria-targeted antioxidants should decrease intracellular ROS and cure humans suffering from various ROS-linked diseases much stronger than non-targeted antioxidants or antioxidants targeted to compartments other than mitochondria. The first observation of this kind was done by Murphy's group, where mitochondria-targeted CoQ derivative MitoQ was found to inhibit ROS-induced apoptosis of cell cultures at $5 \cdot 10^2$ times lower concentration than non-targeted CoQ [1]. Later, Chernyak's group in our laboratory showed an even larger difference between mitochondria-targeted plastoquinones (SkQ1 or SkQR1) and non-targeted N-acetyl cysteine (NAC) and trolox [2-4]. The stronger effect of SkQs, compared to MitoQ, was mainly due to a much larger window between anti- and prooxidant activities of these quinones. A 10^6 difference between doses of SkQ1 and NAC was shown in our group by Kopnin and coworkers, who studied an increase in lifespan of $p53^{-/-}$ mice who died due to lymphoma [5]. Such a great advantage of SkQ1 over NAC could be predicted if one takes into account that (1) the antioxidant effect of SkQ1 results in a prevention of the chain reaction of cardiolipin peroxidation, localized in the inner mitochondrial membrane; and (2) extracellular SkQ1, in contrast to NAC, electrophoretically accumulates by a factor of 10 in cytosol, 10^3 in the mitochondrial matrix and 10^4 in the membrane, because of a high octanol/water distribution coefficient. As a result, SkQ1 concentration in the inner mitochondrial membrane can be 10^8 ($10 \cdot 10^3 \cdot 10^4$) times higher than extramitochondrial [SkQ1] [6,7]. Large differences between acting concentrations of SkQ1 and those of vitamin E or NAC were revealed by Kolosova and coworkers when studying progeric OXYS rats (age-dependent development of cataract, retinopathy and an IGF-1 decrease were investigated) [5-8]. Rabinovich and his colleagues succeeded in an *in vivo* targeting of catalase to mitochondria [9-12]. In particular, an antiprogeric effect was observed in "mutator" mice defective in the proof-reading domain of mitochondrial DNA polymerase [12]. Such mice were shown to have an elevated content of mitochondrial H_2O_2 [13]. Targeting of catalase to nucleus or peroxisomes proved to be much less effective than to mitochondria [9].

The final aim of ROS studies is certainly the treatment of ROS-induced pathologies in humans. There is already a precedent when a mitochondria-targeted antioxidant - eye drops Visomitin containing 250 nM SkQ1, which is an efficient treatment of the previously incurable disease "dry eye syndrome" [14,15] - were officially recommended as a medicine and became available in pharmacies. Clinical trials of this drug showed that it is also beneficial in two other age-related diseases, i.e. cataract and glaucoma. Again, SkQ1 proved to be much more efficient than thymolol, a non-targeted antioxidant.

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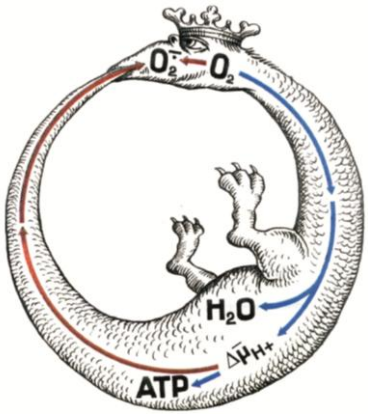

B2-02 Mitochondria as H_2O_2 buffering system.

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For decades, mitochondria have been considered a major intracellular source of reactive oxygen species (ROS). Mitochondrial ROS have been viewed as a negative factor, contributing to the damage of cellular components. More recent data point to a more physiological role of mitochondrial-produced ROS in mitochondria-cell communication and intra- and extracellular signaling [1].

The eventual unraveling of the complex and powerful mitochondrial ROS detoxication system resulted in a concept that mitochondria are more a sink than a source of ROS [2,3]. However, our research indicates that mitochondria are a ROS buffering system that can maintain the intracellular level of ROS in relation to the metabolic circumstances.

In this presentation, I will briefly summarize the state of knowledge on the sources and regulation of ROS production and scavenging in mitochondria and present experimental data on mitochondrial ROS-buffering properties.

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B2-03 Acute diet-induced insulin resistance is associated with increased adipose tissue mitochondrial ROS emissions.

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While it is widely accepted that consuming a high-fat diet (HFD) increases the risk of developing type II diabetes mellitus and obesity, little is known about the initiating factor causing insulin resistance. A recent study suggests that white adipose tissue (WAT) insulin sensitivity is drastically attenuated as early as one week on a HFD, however the underlying cause of this tissue specific insulin resistance is unknown as inflammation occurs many weeks later [1]. Since increased mitochondrial reactive oxygen species (ROS) and oxidative stress are associated with an insulin resistant state, we propose that elevated mitochondrial ROS emissions may be a causal factor in WAT insulin resistance. The aim of the current study was to determine mitochondrial respiration and ROS emission rates in WAT of mice under control and HFD conditions.

Adult male mice were placed on a standard chow (10% kcal fat) or a HFD (60% kcal fat) for one week. Mitochondrial respiration was measured by high-resolution respirometry in permeabilized adipose tissue. In addition, we optimized and validated a method to measure WAT mitochondrial ROS emissions.

One week on a HFD reduced glucose homeostasis, as WT HFD mice displayed increased plasma insulin levels as well as whole body insulin resistance determined by an intraperitoneal insulin tolerance test. While skeletal muscle insulin signaling was preserved, visceral WAT insulin signaling was reduced in WT HFD mice, confirming previous reports of acute HFD [1]. Moreover, we observed lipid-supported increased mtROS emissions in visceral WAT from WT HFD mice with no alterations in mitochondrial content or adipocyte cell size after one week of high-fat feeding, suggesting an early association between WAT mtROS emissions and insulin resistance.

In conclusion, we propose that elevated mtROS emission observed after one week of a HFD is a causal factor of WAT insulin resistance which contributes to altered whole body glucose homeostasis reported with an acute HFD.

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B2–04 The effect of bioactive phenolics on chronic mitochondrial stress associated with the onset of diabetes.

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There is sufficient evidence to suggest that mitochondrial (mt) dysfunction plays a pivotal role in the onset of type II diabetes mellitus and that dietary (poly)phenols may help alleviate this [1-4]. Colonic microbial metabolites of (poly)phenols are of particular interest, as they can be present in blood *in vivo* at high concentration in peripheral tissues [5]. Furthermore, cells are chronically exposed to these compounds for long periods of time which implies that they could have a lasting effect on mt function.

To study these potential effects, human hepatocyte cells were cultured in high glucose in the presence of (poly)phenols and relevant biomarkers investigated. Mitochondria were isolated and characterized. Spectrophotometric enzymic assays were used to assess the activity of the protein complexes of the electron transfer system (ETS), and reactive oxygen species (ROS) generation was studied using a fluorescence assay. In addition, high-resolution respirometry on corresponding intact cells enabled the measurement of mt respiration and analysis of oxidative phosphorylation. We find that high glucose

concentration significantly decreases mt ETS Complex I NADH oxidase activity (Figure 1), and the ability of biologically active molecules to affect this modulation will be presented.

Supported by European Research Council Advanced Grant *POLYTRUE? (322467)*.

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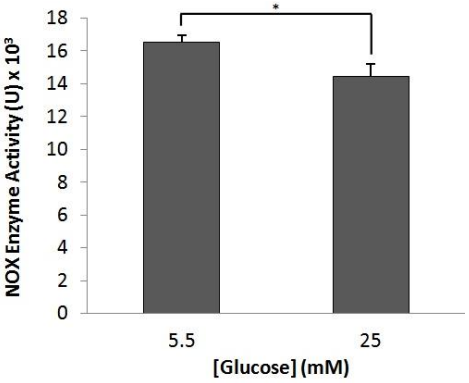
Figure 1: Electron transfer Complex I NADH oxidase (CI) activity in mitochondria isolated from human HepG2 cells, maintained in MEM Eagle supplemented with 10% FBS, in the presence of 5.5 or 25 mM glucose, for 24 h. Mt preparations were assayed in 150 µM NADH and absorbance at 340 nm monitored for 1 h. Enzyme activity (U) is equivalent to µmol NADH oxidised $min^{-1} \cdot mg^{-1}$ total protein. Bars represent mean+SD; *N*=3 (**P*<0.05).



B2-05 The role of phospholipase A2γ in the regulation of mitochondrial uncoupling protein 2–dependent antioxidant function.

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Mitochondrial uncoupling protein 2 (UCP2) has been suggested to participate in the attenuation of the reactive oxygen species production, but the mechanism of action and the physiological significance of UCP2 activity remain controversial. The protonophoretic function of recombinant reconstituted UCP2 is essentially dependent on non-esterified fatty acids [1], and we showed that mitochondrial phospholipase A2γ participates in the regulation of UCP2 function [2,3].

Because UCP2 plays an antioxidant role in pancreatic β–cells [4], we also tested our hypothesis of iPLA2γ–dependent regulation of UCP2, using the model of INS–1E insulinoma cells. High-resolution respirometry and parallel fluorometric detection of membrane potential and mitochondrial superoxide formation revealed pro-oxidant–induced increase in respiration, decrease in mitochondrial membrane potential and decrease in mitochondrial superoxide formation in non-targeting shRNA INS–1E controls (ntgINS–1E) but not in UCP2–silenced and iPLA2γ–silenced cells. In addition, we observed identical glucose–stimulated insulin secretion in ntgINS–1E controls, UCP2–silenced and iPLA2γ–silenced cells in the absence of a pro-oxidant insult. Addition of the pro-oxidant *tert*-butyl hydroperoxide results in markedly elevated insulin release in both UCP2–silenced and iPLA2γ–silenced cells but not in ntgINS–1E controls.

Fatty acids are important for normal function of pancreatic β-cells, but elevated levels of free fatty acids are associated with increased production of reactive oxygen species and augmented glucose-stimulated insulin secretion [4]. Therefore, we tested whether the UCP2–mediated, iPLA2γ–dependent antioxidant action protects pancreatic β–cells from acute cytotoxic effects of saturated fatty acids. We exposed the INS-1E insulinoma cells to various concentrations of palmitate and measured the kinetics of insulin secretion and the rate of superoxide production in the mitochondrial matrix. Reasonably low concentrations of palmitate (10–30 $nmol \cdot 10^{-6}$ cells) cause elevated insulin secretion in ntgINS–1E controls but markedly inhibit insulin secretion in both UCP2–silenced and iPLA2γ–silenced cells. Corresponding concentrations of palmitate also lead to attenuation

of mitochondrial superoxide formation in ntgINS–1E controls but not in UCP2–silenced or iPLA$_2$γ–silenced cells.

These results contribute to the understanding of UCP2–dependent regulation of mitochondrial superoxide production and insulin secretion in pancreatic β-cells and to the understanding of free fatty acid–mediated antioxidant function provided by synergic actions of iPLA$_2$γ and UCP2. Our observations further indicate that UCP2 and iPLA2γ protect β–cells against toxicity associated with acute moderate fatty acid intake.

Supported by Grant Agency of the Czech Republic, grant No. P302/10/034, P305/12/1247, and P304/10/P204.

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B2-06 Investigation of the mitochondrial functions to understand p66Shc and reactive oxygen species interplay in tumor cells.

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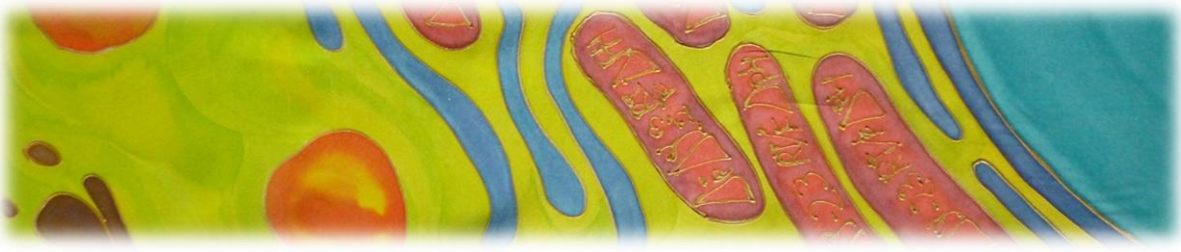
Alternations of pivotal mitochondrial function – oxidative phosphorylation as well as abnormal cellular ROS production - can potentially be responsible for pathogenesis of cancer. In the last years, implications of p66Shc adaptor protein in the cellular response to oxidative stress have been discovered. Involvement of this protein in cell death is related to oxidative stress. Phosphorylation of p66Shc at Ser36 can be activated by extracellular or intracellular reactive oxygen species (ROS), and an initiated cascade of events is finally involved in the amplification of mitochondrial ROS production.

The available literature does not contain a lot of data concerning the role of p66shc and its Ser36 phosphorylation in tumorigenesis and cancer growth. Therefore, we studied the relationship between ROS production, antioxidant defense systems and the level of p66Shc as well as p66Shc phosphorylation in murine cancer cell lines, derived from ectoderm (B16-F10, B78, MmB16, EMT6, 4T1), mesoderm (Renca) and endoderm (CT26.WT, Hepa1-6, LLC, Panc02).

The cancer cells exhibited various levels of p66Shc and its Ser36 phosphorylation, which simultaneously is negatively correlated with the level of superoxide dismutase 2 in some of the investigated cancer cell lines.

ROS can mediate opposing cellular functions like cell proliferation and apoptosis. In turn, p66Shc Ser36 phosphorylation pathway is involved in regulation of mitochondrial metabolism and is responsible for elevated intracellular ROS levels. Moreover, p66Shc seems to play an important role in cancer metastasis and cancer cell adhesion. This emphasizes the importance of understanding the mechanisms and sites of ROS formation in cancer cells, the role of p66Shc in this process and the effect on tumor physiology.

Supported by Statutory Founding from Nencki Institute of Experimental Biology and Polish Ministry of Science and Higher Education grant W100/HFSC/2011.



B3 Hypoxia, respiration and fermentation - mitochondrial physiology in cellular energetics

B3-01 Bioenergetic responses to cyclic hypoxia reveal mitochondrial mechanisms of hypoxia tolerance in marine bivalves.

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Marine organisms are exposed to periodical oxygen deficiency (hypoxia) in estuarine and coastal zones, due to the tidal cycles and/or seasonal formation of the benthic "dead zones". In sessile organisms, such as bivalves, which cannot escape hypoxic exposures, the ability to survive hypoxia is critically dependent on the physiological and cellular tolerance mechanisms that allow coping with oxygen deficiency and quickly recovering upon reoxygenation. Energy limitation and damage due to reactive oxygen species are major stressors during hypoxia and post-hypoxic recovery, and it is not well known how mitochondria of hypoxia-tolerant marine organisms cope with these challenges.

We studied mitochondrial and cellular responses to hypoxia and post-hypoxic recovery in two common bivalves: a hypoxia-tolerant intertidal hard clam *Mercenaria mercenaria* and a hypoxia-sensitive subtidal bay scallop *Argopecten irradians*. Respiration, mt-membrane potential ($\Delta\Psi_{mt}$), $\Delta\Psi_{mt}$-dependent kinetics of three major mitochondrial subsystems (substrate oxidation, proton leak and phosphorylation), as well as energy reserves and expression of phosphorylated AMPK (pAMPK) and elongation factor (eEF) were measured in clams and scallops exposed to normoxia or short-term hypoxia (17 h at <1% O_2), followed by a 1 h period of normoxic recovery. Mitochondrial and cellular responses to hypoxia and reoxygenation dramatically differed in the two studied species.

In scallops, hypoxia suppressed the capacity of all three mitochondrial subsystems, especially the phosphorylation subsystem. Mitochondrial condition further deteriorated during reoxygenation, with strong depolarization of mitochondria and a decrease in the flux capacity of the substrate oxidation and phosphorylation subsystems. In contrast, in clams, hypoxia increased the $\Delta\Psi_{mt}$-dependent capacity of the substrate oxidation subsystem and had weak inhibitory effects on the flux through the phosphorylation and proton leak subsystems. During reoxygenation, the substrate oxidation capacity of clam mitochondria further increased and the capacity of the phosphorylation subsystem returned to normal. Lipid levels increased during hypoxia in clams and scallops, possibly due to the inhibition of mitochondrial catabolism of fats; during reoxygenation, the lipid levels rapidly declined in clams but continued to increase in scallops. Glycogen reserves decreased during hypoxia and reoxygenation in scallops indicating high dependence on glycolysis. Protein levels of phosphorylated eEF increased in clams but not in scallops during hypoxia, indicating suppression of the protein synthesis in the more hypoxia-tolerant species. Levels of pAMPK increased during reoxygenation in scallops indicating energy stress but remained stable throughout hypoxia and reoxygenation in clams. No oxidative damage of the mitochondrial membrane lipids was detected in either species.

Decreased protein synthesis provides an energy-saving mechanism during hypoxia in clams while upregulation of the substrate oxidation capacity poise them for a quick recovery upon reoxygenation. Scallops do not possess these mechanisms and suffer from mitochondrial deterioration and energy deficiency, limiting their ability to survive and recover from hypoxia.

Supported by UNC Charlotte.

B3–02 Substrate utilisation in the hypoxic heart: a glycolysis/glucose oxidation mismatch?

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Hypoxia presents a physiological challenge to cardiac mitochondrial ATP generation [1]. Limiting atmospheric oxygen in both short-term chamber studies and longer term sojourns to altitude has been associated with decreased cardiac energetics in humans [2]. However, the metabolic mechanisms underlying these alterations are yet to be fully understood. Here, we set out to investigate the nature and timing of hypoxia-induced changes in cardiac substrate utilisation and electron transfer system (ETS) capacity, using high-resolution respirometry in permeabilized muscle fibres and enzyme activity assays.

Male Wistar rats were exposed to 10% environmental oxygen for either two or 14 days, with rats maintained in normoxia as controls. Despite a 12% loss in body mass (P<0.0001), 2 d hypoxia did not induce any metabolic changes. However, after 14 d hypoxia, tissue-mass specific OXPHOS capacity, driven by octanoyl carnitine (OctM) and pyruvate (PM), dropped by 30% (P<0.05) and 21% (n.s.), respectively. The ratio of Oct to pyruvate-driven OXPHOS was 12% lower after 14 d hypoxia compared with controls (P<0.01), indicative of a switch in substrate preference. The loss of fatty acid oxidation capacity could not be explained by a loss of 3-hydroxyacyl-CoA dehydrogenase (HOAD) activity, as this was unaffected by hypoxia. Hexokinase activity, however, was augmented by 33%, following hypoxic exposure, when expressed relative to protein mass (P=0.02), and 39% when expressed relative to pyruvate-driven OXPHOS capacity (P=0.002), possibly indicating worsened coupling of glycolysis to glucose oxidation. In agreement with previous studies, 14 d hypoxia induced a 22% drop in Complex I-linked OXPHOS capacity (P<0.05). LEAK to OXPHOS ratio, a measure of mitochondrial uncoupling, was unaffected by hypoxia.

Paradigms of a hypoxia-induced metabolic substrate switch and the development of a diminished Complex I capacity are emerging, and these are supported by the present study. Although the molecular basis of the short-term loss in cardiac energetics is yet to be fully elucidated, the present data supports the hypothesis that the worsened energetic profile brought about by sustained hypoxia is propagated by a mismatch between glycolysis and glucose oxidation. The resulting proton production may have implications for mechanical function in the intact heart.

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B3-03 Effects of perinatal asphyxia in mitochondria of brain during development of the central nervous system.

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Hypoxia-ischemia is a major cause of fetal and perinatal brain damage. In Brazil, perinatal asphyxia is an important cause of death and also the most important cause of encephalopathy and permanent brain damage in children. Here we used a model of perinatal asphyxia in rats to study short- and long-term effects caused by the perinatal asphyxia in the development of the cerebral cortex.

Pregnant females were monitored between the 20th and the 22nd day of gestation. At the beginning of labor, the fetus was submitted to an acute period of complete asphyxia

by submersion of the one uterine horn into a PBS bath for 15 min at 37 °C (asphyxiated group). In parallel, pups from the other uterine horn were rapidly removed, cleaned and directed to a foster mother (control animals).

In the group exposed to asphyxia, the survival rate was 67%. There was no difference in body weight gain between the groups until 21 days postnatal (P21), although reduction of weight brain of asphyxia animals was detected at P7. Perinatal asphyxia caused permeabilization of the blood-brain barrier, but no raise in the level of inflammatory cytokines was detected after birth. Oxygen consumption was increased at P0 and P7, and resistance against formation of mitochondrial membrane permeability transition (MPT) was increased, as shown by monitoring the mitochondrial membrane potential (mtMP) by safranine O. After two weeks, we observed a normalization of mitochondrial oxygen consumption with a concomitant increase in susceptibility to the formation of the MPT in asphyxiated animals and, consequently, an increased sensitivity to cell death.

Taken together, our results reinforce the hypothesis that brain development is altered by perinatal asphyxia, in particular neuronal biochemistry. These changes may contribute to neurological disorders found in children exposed to perinatal asphyxia.

B3-04 The metabolic panorama during tumor progression towards malignancy.

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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 melan-a (ma), non-tumorigenic cell line 4C (obtained after four cycles of adherence abrogation), non-metastatic 4C11- and metastatic 4C11+ melanoma cell lines. 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 [1].

Our results show that only metastatic cell line (4C11+) released the highest amounts of lactate and exhibited high LDH activity related to 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 succinate oxidation (Complex II) and fatty-acid oxidation. Additional results suggest that lipid droplets may function as an extra source of fatty acids for mitochondrial β-oxidation.

These results suggest that mitochondria of tumor cells could function as energy- and redox sensors to maintain metastases. We hypothesize that the oxidative metabolism of

tumor cells in connection with the inactivation of anoikis may have been co-opted through a non-adaptive evolutionary process [2]. Detailed analysis of patterns in this and other models of tumor progression may reveal whether the modulation of the oxidative metabolism is a feature of the metastatic process.

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B3-05 Bioenergetics' traits of tongue cancer metastatic cells.

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Buccal cancer affects 3% of all cases of cancer in the world. The most common malignant tumors of the oral cavity are of the spinocellular type and are characterized by invasive growth, frequently perineural. Although the main etiological factors, tobacco and alcohol, are known, at this point the biochemical features associated with the progression of the disease are largely fragmentary.

We investigated the bioenergetics of oral tongue squamous cell carcinoma (OTSCC) metastatic spread. An orthotopic model was developed by the implantation of SCC-9 ZsGreen cells into the tongue of BALB/c nude mice. The animals were sacrificed 60 days later and the axillary lymph nodes collected. Fragments of positive lymph nodes were used for explants cultures, from which the LN-1 ZsGreen cell line was isolated. The retransplantation generated LN-2 ZsGreen cell lines. LN1 and LN2 present degrees of aggressiveness as measured by their metastatic behavior [1].

When compared for ATP content, SCC9, LN1 and LN2 cells displayed similar profiles. All three were strongly sensitive to glycolytic inhibitors as 2-DOG and iodocetamide, but were quasi-insensitive to respiratory the inhibitors rotenone and antimycin A. These results indicate that SCC9, LN1 and LN2 primarily depend on glycolysis for ATP synthesis, although their mitochondria were not dysfunctional. Our results with high-resolution respirometry (HRR) show that mitochondrial function is similar in all three cell lines. Interestingly, oxygen consumption of digitonin permeabilized cells displayed a decreased activity of Complex I- but not of Complex II-linked respiration. Furthermore, mitochondrial content was decreased. Interestingly, the content of Complex III increased linearly with the metastatic behavior of the cells. Since many types of cancer involve defects in respiratory complexes, it is possible that the observed reduced activity of Complex I is compensated by an enhanced activity of Complex III. Alternatively, in the more aggressive cells electrons bypassing Complex I may increase the speed of the entire electron transfer system, therefore facilitating ROS production, migration and invasion. To investigate whether tongue cancer cells used specific energy substrates in a differential manner, palmitoyl CoA was added to the incubation medium. Stimulation of OXPHOS capacity by SCC9 and LN1 cells, and, less so, by the more aggressive LN2 cells was observed only with palmitoyl CoA and malate. Additionally, inhibition of beta-oxidation using etomoxir induced a decrease in ATP levels in LN1 and LN2 cells. These results suggest that mitochondria of tongue metastatic cells can select specific energy substrates such as amino acids and/or fatty acids to maintain redox balance and ATP balance during invasion.

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B3-06 Regulation of energy metabolism in human colorectal cancer cells *in situ*.

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Human colorectal cancer (HCC) is an important cause of cancer-associated mortality worldwide. For this study we used saponin-treated, post-operational tissue samples of HCC in combination with high-resolution respirometry to estimate and characterize altered metabolism of mitochondria in HCC. Mitochondrial respiration of tissue samples was measured at 25 °C using the OROBOROS Oxygraph-2k. For immunocytochemistry we used standard Abcam protocol for formalin fixed paraffin-embedded tissue sections. Confocal images were collected using Olympus FV10i-W microscope, RNA isolation and RT-PCR were performed using Kits from QIAGEN sciences and Applied Biosystems and enzymatic activities were assessed spectrophotometrically at 25 °C [1].

The apparent Michaelis-Menten constant (K'_m) for ADP and maximal tissue-mass specific respiratory flux (J_{max}) were calculated for the characterization of the affinity of mitochondria for exogenous ADP: normal colon tissue displays low affinity (K'_m=260±55 μM) whereas in tumor tissue, affinity was significantly higher (K'_m=126±17 μM). Despite the increased permeability of the outer mitochondrial membrane, OXPHOS capacity (J_{max}) of the tumor samples was 60-80% higher than that in control tissue. These results show that HCC is a non-hypoxic oxidative tumor with significantly increased mitochondrial respiration and is sensitive to its inhibitors.

We found that the localization and function of β-tubulin isotypes varies in different malignant cells. The absence of βII-tubulin in cancer cells allows binding of HK-2 to VDAC, mediating thus the initiation of the Warburg effect. According to Pedersen's model [2], the voltage dependent anion channel (VDAC), located within the mitochondrial outer membrane (mtOM) and linked with HK-2, usually shows increased activity in tumor cells and is one of the main pathways mediating the "Warburg effect" in cancer. Our RT-PCR and immunocytochemistry (ICH) studies have revealed that HCC cells express genes encoding HK-1 and HK-2. Studies using confocal microscopy and immunostaining showed the colocalization of the HK-2 isoenzyme with VDAC and a possible binding of HK-2 to mitochondria in these malignant cells. Consequently, in HCC cells the expression of HK-2 and HK-1 is associated with binding either one or both isoforms with VDAC in the mtOM.

Using spectrophotometric assessment of enzymatic activities in HCC showed that during carcinogenesis the total activity of hexokinase (HK) does not change in comparison with normal tissue. Furthermore, only minor alterations in the expression of HK-1 and HK-2 isoforms were observed, and similar results were obtained measuring the total activity of CK. In contrast, total adenylate kinase (AK) activity is upregulated in HCC cells, and there could be a functional coupling between oxidative phosphorylation and the AK system in contrast to normal colon tissue. The structure-function relationship in bioenergetic regulation in comparison to tumor and normal tissue of HCC requires further investigation.

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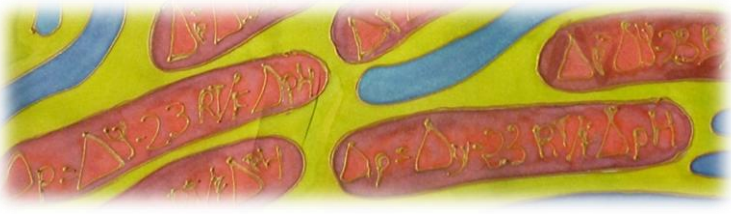

B3-07 Myotube formation does not require mitochondrial ATP production but is inhibited by rotenone via the Rho-associated, coiled-coil containing protein kinase II.

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Increased expression of the mitochondrial oxidative phosphorylation (OXPHOS) system and elevated cellular O_2 consumption were observed during myoblast differentiation and their fusion into myotubes [1,2]. These processes were inhibited by high concentrations of rotenone (Rot; 10 µM), a widely used inhibitor of OXPHOS Complex I (CI), suggesting that activity of this complex is required for myotube formation [3]. However, Rot can also display off-target effects on microtubules and Rho-GTPases [4,5]. In this study we aimed at determining whether Rot-induced inhibition of myotube formation is an on- or off-target effect.

Myoblasts were isolated from mouse extensor digitorum longus (EDL) muscle of wild-type (WT) and CI-deficient (*NDUFS4*$^{-/-}$) mice and differentiated for three days. In parallel, WT myoblasts were differentiated in the presence of two CI inhibitors (Rot or piericidin A, PA) or in the presence of the CIII inhibitor antimycin A (Ama). For all inhibitors, the lowest concentration that fully inhibited myoblast O_2 consumption (100 nM) was used. Cell cultures were then analyzed for myotube formation using immunocytochemistry.

Our results demonstrate that genetic CI deficiency (*NDUFS4*$^{-/-}$ myoblasts) as well as CI and CIII inhibition by PA and Ama do not affect myotube formation *in vitro*. In sharp contrast, Rot treatment significantly inhibited this process. Hydroethidine (HEt) oxidation was similarly increased in Rot- and PA-treated cells, suggesting that HEt-oxidizing reactive oxygen species (ROS) are not responsible for the differential effect on myotube formation. The inhibitory effects of Rot on myoblast fusion were partially reversed by Y27632, a specific inhibitor of the Rho-associated, coiled-coil containing protein kinase (ROCK). Isoform specific knockdown studies revealed that Rot action was mediated by ROCK2 but not by ROCK1 or RhoA.

We conclude that formation of primary myotubes by myoblast fusion *in vitro* does not require active mitochondrial ATP production and is inhibited by Rot via a ROCK2-dependent mechanism. This means that Rot is not an appropriate tool to study CI and/or OXPHOS dysfunction in murine myoblasts and myotubes.

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B3-08 The function of mitochondrial pyruvate carriers in the adaptation of respiration capacity and stress tolerance in yeast.

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Yeast cells adapt mitochondrial morphology, biomass and activity to changing environmental conditions in a dynamic manner. Here we investigate the mechanisms of mitochondrial adaptation to different respiration rates, oxidative and salt stress.

Specifically, we identifed the *MPC* gene family as particularly regulated upon changes from fermentative to respiratory growth or upon stress. Mpc proteins are highly conserved from yeast to humans and are necessary for the uptake of pyruvate into mitochondria [1,2], which is used for leucine and valine biosynthesis and as a fuel for respiration.

The highly similar Mpc2 and Mpc3 proteins are regulated in an antagonistic manner: Mpc2 is most abundant under fermentative non-stress conditions and important for amino acid biosynthesis, while Mpc3 is most abundant upon salt stress or respiratory growth. Overexpression experiments demonstrate that Mpc3 stimulates respiration and oxidative stress tolerance, while Mpc2 inhibits respiration and oxidative stress tolerance. Therefore, the regulated mitochondrial pyruvate uptake via different Mpc proteins might be an important determinant of respiration rate and stress resistance [3]. We additionally analyzed the degradation rate of different respiratory complex subunits in response to higher respiration rates and mitochondrial damage.

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MiP2013: Antonio Galina, Nivea Amoêdo

B4 Instrumental platforms: developments and applications in mitochondrial physiology

B4-01 Assessing mitochondrial dysfunction in fibroblast cells – a comparison between O2k and multiwell respirometry.

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Different platforms for the measurement of respiratory fluxes are available. The OROBOROS Oxygraph-2k (O2k) is a two-chamber respirometer based on high-resolution polarographic oxygen sensors. The Seahorse XFe96 extracellular flux analyzer (XFe96) is a fluorescence-based, 96-well sensor cartridge approach. In this study, we aimed to compare both platforms by measuring human skin fibroblasts (HSF) and HeLa cells, which are both characterized by low respiratory fluxes.

We analyzed intact cell respiration in Dulbecco's Modified Eagle Medium containing pyruvate/ glutamate using a classical phosphorylation control protocol (using oligomycin, CCCP, and rotenone/antimycinA).

Instrumental background of the calibrated O2k was 2.9±0.4 pmol $O_2 \cdot s^{-1} \cdot ml^{-1}$ at air saturation. XFe96 background oxygen consumption varied interexperimentally between ±3 to ±14 $pmol \cdot min^{-1}$. ROUTINE respiration of $2 \cdot 10^6$ HSF or HeLa per 2-ml O2k chamber, was in the range of 40 pmol $O_2 \cdot s^{-1} \cdot ml^{-1}$. Consistent monolayers of HSF ($2.5 \cdot 10^4$ cells per well) and HeLa ($4 \cdot 10^4$ cells per well) were in the range of 50 and 100 $pmol \cdot min^{-1}$, respectively. ROUTINE coupling control ratio (ROUTINE flux over electron transfer system capacity, ETS) was similar in HSF between both platforms (0.45), whereas the ratio was 1.0 (XFe96) or 0.6 (O2k) in HeLa cells. This difference in HeLa might be influenced by the fact that respiratory flux is assessed in monolayers (XFe96) or suspension (O2k). When converted to comparable SI units, ROUTINE oxygen flow was 40 (O2k: HSF and HeLa) versus 33 and 42 (XFe: HSF and HeLa) $pmol \cdot s^{-1} \cdot 10^{-6}$ cells; ETS was 89 and 67 (O2k: HSF and HeLa) versus 74 and 42 (XFe: HSF and HeLa) $pmol \cdot s^{-1} \cdot 10^{-6}$ cells, respectively. ETS capacity in HSF and HeLa was approximately 80% and 60% in the XFe compared to the O2k.

In conclusion, O2k and XFe96 are both platforms that are suitable to assess mitochondrial respiration of human fibroblast and HeLa cells. Instrumental background and interexperimental variability is lower in O2k experiments, whereas amount of sample is smaller and throughput of multiple conditions is higher using XFe96 respirometry.

B4-02 Resveratrol as a double edged sword on mitochondrial function.

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Resveratrol (Resv), a polyphenolic compound, impacts the function of isolated mitochondria, but its modulation of mitochondria in whole cells remains poorly defined. In experiments with isolated mitochondria, Resv inhibits the activity of several complexes in the electron transfer system (ETS) [1,2]. In experiments during which animals were exposed to Resv, an increase in mitochondrial activity by Resv was observed [3,4]. Therefore, the aim of the present study was to study the effect of Resv on the mitochondrial activity in HeLa cells, noninvasively.

A Beckman Coulter Z2 Cell and Particle Counter as well as an ICELLigence system were used to analyze cell number, proliferation and size. Fluorescence-activated cell sorting FACS was used to characterize each cell in regard to relative fluorescence intensity. A Seahorse XF-24 analyzer was used to test effects of Resv on mitochondrial

activity. Quantitative PCR was used to determine relative amounts of a known sequence of key genes related to mitochondrial function.

Our results show that Resv decreases the cell number dose-dependently in both HeLa WT and HeLa Rho 0 (depleted of mtDNA), but show no effect on cell proliferation with regard to functional mitochondria. A significant increase in cell diameter was observed in HeLa WT but not in HeL Rho 0; hence functional mitochondria seem to be a prerequisite for the cell enlargement effect by Resv. An overall increase in mitochondrial number, membrane potential and reactive oxygen species was observed in HeLa Rho 0 compared to HeLa WT. Exposure to Resv induced only small (statistically insignificant) differences. The oxygen consumption rate was dose-dependently up-regulated by Resv, which also is observed on extracellular acidification rate. To further evaluate if mitochondria of HeLa WT and HeLa Rho 0 cells were affected by Resv treatment, the expression levels of several key genes related to mitochondrial function were measured, leading to results consistent with previous reports [5].

Our results indicate that in HeLa cells Resv upregulates mitochondrial respiration and cellular glycolysis.

Supported in part by the Danish Council for Strategic Research.

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B4–03 Oxygen-consuming and ROS-producing activities in synaptosomes.

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Although originally defined as harmful byproducts of aerobic metabolism, reactive oxygen species (ROS) are currently believed to play a critical role in downstream signaling, which regulates protein kinases, phosphatases, transcription factors and ion transport channels. However, mechanisms by which ROS is responsively produced, sensed and translated in cellular domains, especially neurons, remain elusive. Recently, NADPH oxidase (NOX), which is a multimeric enzyme that catalyzes the production of superoxide ($O_2^{\bullet-}$) from O_2 and NADPH and was originally identified in neutrophils as essential for the host response respiratory burst, has been shown to localize in the brain. The unexpected presence of NOX in neurons has led to the idea that NOX-induced ROS are important in non-host defense contexts; e.g. intracellular and intercellular redox signaling. In previous works, we showed that NOX is actively producing $O_2^{\bullet-}$ in the brain and might therefore be an important element that influences redox homeostasis in health, disease, and aging. Questions on specific connections between NOX activation and neuronal dysfunctions remain open for exploration by unconventional experimental approaches capable of probing the implications of *in vivo* NOX assembly and activation.

Here, we studied oxygen-consuming, superoxide-producing NOX basal as well as induced activities in synaptosomes. Isolated synaptosomes (severed nerve terminals) are studied because they contain all necessary components of a functional neuronal environment including ion channels, receptors, and mitochondria. We demonstrate the ability of the OROBOROS Oxygraph-2k, in parallel with spin-trapping/labeling electron paramagnetic resonance (EPR) techniques, to study sources and dynamics of ROS in synaptosomes. To the best of our knowledge, this is the first time that the OROBOROS system has been employed to quantify NOX activity in synaptosomes.

B4-04 Simultaneous high-resolution measurement of mitochondrial respiration and hydrogen peroxide production.

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Mitochondrial respiration is associated with the formation of reactive oxygen species, primarily in the form of superoxide ($O_2^{\bullet-}$) and particularly hydrogen peroxide (H_2O_2). Since H_2O_2 plays important roles in physiology and pathology, measurement of hydrogen peroxide has received considerable attention over many years. Here we describe how the well-established Amplex Red assay can be used to detect H_2O_2 production in combination with the simultaneous assessment of mitochondrial bioenergetics by high-resolution respirometry. Fundamental instrumental and methodological parameters were optimized for analysis of the effects of various substrate, uncoupler and inhibitor titrations (SUIT [1]) on respiration versus H_2O_2 production. The sensitivity of the H_2O_2 assay was strongly influenced by compounds present in different mitochondrial respiration media, which exerted significant effects on chemical background fluorescence changes. Near-linearity of the fluorescence signal was restricted to narrow ranges of accumulating resorufin concentrations independent of the nature of mitochondrial respiration media. Finally, we show an application example using mouse brain mitochondria as an experimental model for the simultaneous measurement of mitochondrial respiration and H_2O_2 production in SUIT protocols.

Supported by K-Regio project *MitoCom Tyrol*.

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B4–05 Mitochondrial inefficiencies and anoxic ATP hydrolysis capacities in diabetic rat heart.

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As approximately 80% of diabetics die from heart failure, understanding diabetic cardiomyopathy is crucial. Mitochondria occupy 35-40% of the mammalian cardiomyocyte volume, supply 95% of the hearts' ATP, and diabetic heart mitochondria show impaired structure, arrangement and function. We predict that bioenergetic inefficiencies are present in diabetic heart mitochondria; therefore, we explored mitochondrial proton and electron handling by linking oxygen flux within streptozotocin (STZ)-induced-diabetic Sprague-Dawley rat heart tissues, to steady-state ATP synthesis, reactive oxygen species (ROS) production and mitochondrial membrane potential ($\Delta\Psi_{mt}$). By coupling high-resolution respirometers with purpose-built fluorometers, we followed Magnesium Green (ATP synthesis), Amplex Ultra Red (ROS production) and safranin-O ($\Delta\Psi_{mt}$). Relative to control rats, the tissue-mass specific respiration of STZ-diabetic hearts was depressed in oxidative phosphorylating (OXPHOS) states. Steady-state ATP synthesis capacity was almost a third lower in STZ-diabetic heart and relative to O_2 flux, this equates to an estimated 12% depression in OXPHOS efficiency. However, with anoxic transition, STZ-diabetic and control heart tissues showed similar ATP hydrolysis capacities through reversal of the F_1/F_0 ATP synthase. STZ-diabetic cardiac mitochondria also produced more net ROS, relative to oxygen flux (ROS/O) in the OXPHOS state. While $\Delta\Psi_{mt}$ did not differ between groups, the time to develop $\Delta\Psi_{mt}$ with the onset of OXPHOS was protracted in STZ-diabetic mitochondria. ROS/O is higher in life-like OXPHOS states, and potential delays in the time to develop

$\Delta\Psi_{mt}$ may delay ATP synthesis with inter-beat fluctuations in ADP concentrations. Whereas diabetic cardiac mitochondria produce less ATP in normoxia, they consume as much ATP in anoxic infarct-like states.

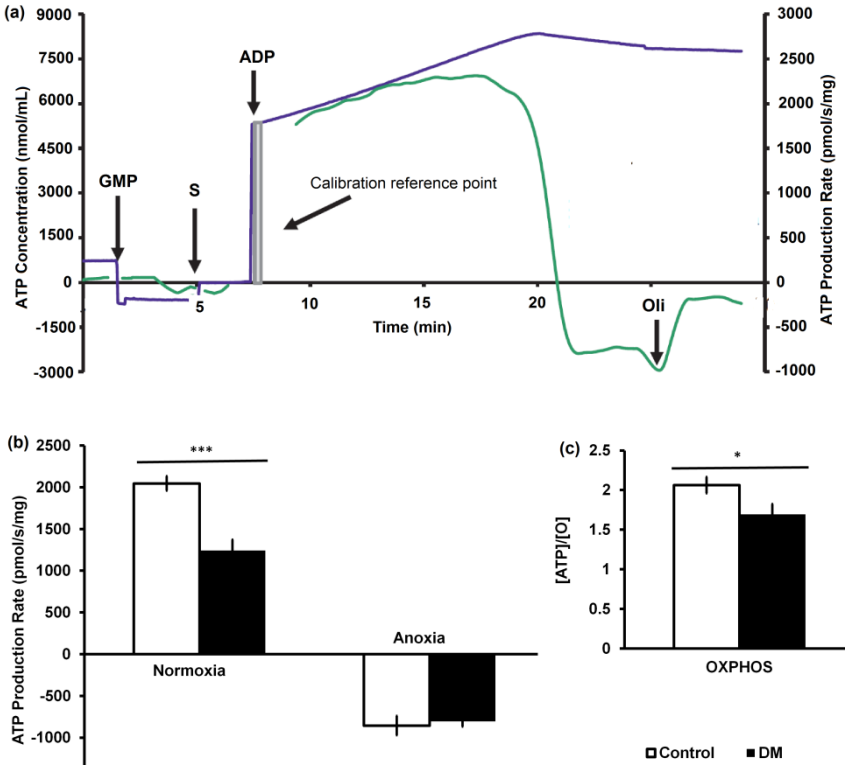

Figure 1. ATP production capacities (mg^{-1}) of diabetic (black) and age matched control hearts (open). (a) Simultaneous measurement of mitochondrial O_2 flux (not shown) and ATP production. Respiratory flux was followed in the presence of oxygen and a CI&II-linked substrate combination (inset a, GMP: glutamate, malate and pyruvate, S: succinate), and then Mg^{2+}-free ADP was added to initiate ATP synthesis (OXPHOS). Respiration was allowed to run into anoxia after which oligomycin (Omy) was added and the background ATP hydrolysis signal subtracted as background. (b) ATP production per mass of tissue ($pmol \cdot s^{-1} \cdot mg^{-1}$) in normoxia and anoxia. (c) The active or steady-state ~P/O ratio was then determined from the rate of ATP synthesised relative to the flux of molecular O in OXPHOS. (*P<0.05, **P<0.01, ***P<0.005, N=12 per group 8-week diabetic rat hearts and their age-matched controls).

B4–06 The relationship between cytochrome redox state and oxygen consumption in isolated mouse and beef heart mitochondria during hypoxia.

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This study describes a low-noise, rapid spectrophotometric system using visible light (440-605 nm) for the measurement of cytochrome redox state combined with high-resolution respirometry. The system was tested in an investigation using beef and mouse heart isolated mitochondria (BHImt, MHImt) in order to determine the relationship between respiratory rate and cytochrome redox state at steady-state levels of hypoxia. Monophasic hyperbolic relations were observed between respiratory rate, j (OXPHOS with glutamate and malate and saturating ADP concentrations), and oxygen partial pressure, p_{O_2}, in the range <1.1 kPa for both BHImt and MHImt with $p_{50,j}$ (p_{O_2} at j=0.5 J_{max}) of 0.015 and 0.021 kPa, respectively [1]. The oxidation fractions of cytochromes aa_3 and c were biphasic hyperbolic functions of p_{O_2}. The relationships between cytochrome oxidation states and j were more complex with an initial steep decrease in the oxidation fraction of cytochrome c to a value of j of approximately 0.7 followed by a plateaux and a

further steep decrease at j<0.2 (Figure 1). This relationship was less apparent with cytochrome *b* redox state. Using these functions, it was possible to create a model that successfully described the measured relationship between cytochrome oxidation state and oxygen consumption.

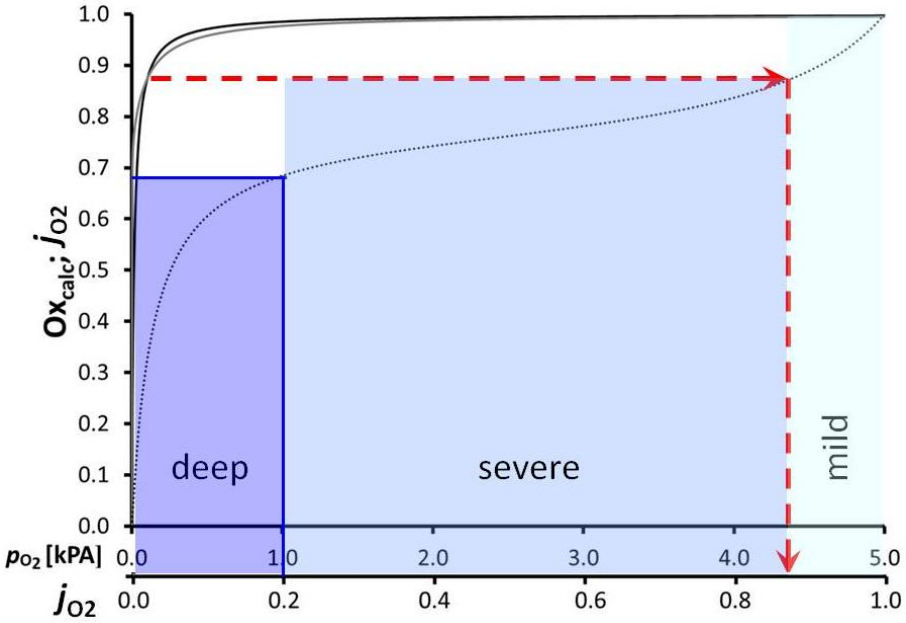

Figure 1. Cytochrome aa_3 oxidation (Ox, calculated as a biphasic hyperbolic fit; grey full line) and respiratory rate (j_{O2}, monophasic hyperbolic; black full line) as a function of p_{O2}. Superimposed is Ox_{calc} in relation to j_{O2} (dotted line). The cross-over between respiration and redox state occurred at Ox_{calc} (=j_{O2}) near 0.9 and separates mild from severe hypoxia at the level of the mitochondria (dashed arrows). A 'cushioning effect' is seen at the onset of mild hypoxia. In the region of severe hypoxia redox state is maintained at relatively constant levels despite a severe decline in oxygen flux. Deep hypoxia is marked by the steep decline of redox state when flux declines from 0.2 to zero in the narrow oxygen range of 0.003 to zero kPa.

Supported by K-Regio project *MitoCom*.

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MiP2013: Anthony Hickey

C1 Blood cells and cultured cells as models for diagnosis of mitochondrial pathologies

C1-01 Manifestation of mitochondrial disorders of nuclear origin in lymphocytes.

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Mitochondrial diseases belong to most severe inherited metabolic diseases affecting the pediatric population. Diagnostics of a substantial part of mitochondrial diseases with unknown genetic cause relies on clinical symptoms and biochemical analysis of energetic function and content of individual mitochondrial proteins in patient tissues – mainly in bioptic samples of skeletal muscle and cell cultures of skin fibroblasts. However, due to their invasive nature, the biopsies are often refused by the patient's parents. Therefore, we tested the diagnostic suitability of easily obtainable patient material – lymphocytes isolated from peripheral blood.

High-resolution respirometry enables sensitive analysis of the mitochondrial oxidative phosphorylation system in isolated lymphocytes. Substrate-inhibitor measurements in digitonin-permeabilized lymphocytes provide a complex evaluation of individual respiratory complexes, coupled ATP synthesis and kinetic parameters of mitochondrial respiratory enzymes. We employed this approach in a large cohort of 48 children including mostly subjects with suspected mitochondrial disease, previously diagnosed patients with OXPHOS disorders, and controls. In combination with cytofluorometric detection of mitochondrial membrane potential and protein analysis by SDS and native electrophoreses, it was possible to diagnose specific defects of Complexes I, IV and V using small amounts of peripheral blood within 1-2 days. Importantly, functional manifestations of mitochondrial disorders caused by *SURF1* [1] and *TMEM70* [2] mutations in lymphocytes recapitulate previous findings in fibroblasts. Moreover, in contrast to cultured fibroblasts Complex IV deficiency was also manifested in lymphocytes harbouring *SCO2* mutations.

The noninvasiveness, reliability and speed of such an approach demonstrate the high potential of isolated lymphocytes for diagnostics of oxidative phosphorylation disorders of nuclear origin in patients with suspected mitochondrial disease.

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C1–02 Validation of oxygen consumption measurements in muscle and fibroblasts from patients with mitochondrial diseases.

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Diagnosis of mitochondrial disorders is mainly based on the analysis of OXPHOS complexes in muscle biopsies. However, normal enzyme activities do not rule out the presence of a mitochondrial disorder. Therefore, analysis of the integrated mitochondrial energy generating system by oxygen consumption is frequently used.

We compared the diagnostic value of respiratory versus enzymatic OXPHOS analysis in fibroblasts and muscle biopsies from patients with a genetically confirmed mitochondrial disease. A standardized substrate-uncoupler-inhibitor-titration (SUIT) protocol [1,2] was used for measuring respiration of permeabilized fibroblasts and single muscle fibers. OXPHOS enzyme activities were determined spectrophotometrically according to standard protocols.

Only the combination of both measurements enables us to identify a mitochondrial disorder in all of the present patients:

Fibroblasts	Positives	Negatives	Total	**Muscle**	Positives	Negatives	Total
Enzyme activity measurements	11	4	15		6	0	6
Oxygen consumption	14	1	15		5	1	6

Moreover, specific flux control ratios showed higher diagnostic sensitivity than complex specific O_2 fluxes.

The established SUIT protocol is an important tool in the diagnostic process. Therefore, we recommend oxygen consumption measurements in addition to enzymatic OXPHOS analysis, to increase the number of identified patients.

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C1–03 Adaptation to nutrient availability in human fibroblasts with mitochondrial dysfunction: the role of sirtuins.

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Defects in the mitochondrial oxidative phosphorylation system (OXPHOS) lead to an extremely heterogeneous group of disorders with an incidence of ≈1 in 5,000 live births [1]. Diagnosis of mitochondrial disorders is still challenging and requires extensive clinical and laboratory evaluation. One screening test for detection of defects in the OXPHOS is the incubation of primary patient fibroblasts cell lines (FBs) in galactose based medium, where some FBs with OXPHOS defects fail to survive [2,3]. The adaptation to galactose medium in FBs is not well understood. It is hypothesized that metabolic sensors may play a role in this process. The sirtuins seem to fulfil the role of metabolic sensors during conditions of caloric restriction or change from glucose to galactose based medium [4,5]. Sirtuins are a family of NAD^+-dependent protein deacetylases (SIRT1 - SIRT7). SIRT1 is located in the nucleus/cytoplasm, whereas SIRT 3 - 5 are mainly located in the mitochondria. SIRT1 and SIRT3 are deacetylases, in contrast to SIRT4 (ADP-ribosyltransferase activity) and SIRT5 (demalonylase, desuccinylase and weak deacetylase activity) [6].

For this study six FBs were selected (one control and five with a mitochondrial defect). The basis for the experiments was a 20 h incubation time in galactose based medium and, as control, an incubation in glucose medium. After 20 h the change in the expression level of acetylated proteins, as well as the expression level of SIRT1 and SIRT3 - 5, in the mitochondrial and cytosolic fraction was analyzed by Western Blot. The process of adaptation to galactose and the role of glutamine was further investigated with an XF24 extracellular flux analyzer (Seahorse Bioscience) measuring cellular oxygen consumption rates (OCR) and extracellular acidification rates (ECAR; an indirect estimate of glycolysis).

We observed a general decrease of acetylated proteins in the mitochondrial fraction in all cell lines after incubation in galactose. The extent of this decrease could not be

correlated with respiratory defects. Because SIRT3 is the main-deacetylase in mitochondria, we suspected this deacetylation process to be the result of a higher SIRT3 activity in galactose [4,7]. This could be either because of an altered SIRT3 expression at the protein level, which was not the case in these experiments, or an altered SIRT3 activity due to a changed mitochondrial NAD^+ concentration, which would require further testing.

Mitochondrial SIRT4 was decreased at the protein level in all FB after galactose incubation. This uniform down-regulation suggests that SIRT4 is involved in the adaption to galactose, unaffected by respiratory function. Under standard conditions, the FBs of all patients with an OXPHOS defect had a decreased cytosolic expression of SIRT1, SIRT3 and SIRT5 compared to the control. In glucose (+Gln), OCR differed in the FBs, whereas the control FBs and a Complex I deficient FB had the highest values. ECAR values were generally decreased in galactose medium in all FBs (± Gln). Two FBs with Complex I deficiency showed higher OCR in galactose than in glucose. This may reflect a compensatory increase in OXPHOS, induced by galactose. The OCR with glucose was dependent on glutamine in several FBs, whereas glutamine had no effect on OCR in galactose-based medium.

In conclusion: (1) all FBs show lower levels of acetylated proteins in mitochondria in galactose-based media. This deacetylation cannot be attributed to changing sirtuin expression; (2) the lower cytosolic SIRT1, 4 and 5 protein expression under normal conditions in fibroblasts with a mitochondrial defect needs to be further investigated as a potential marker; (3) in contrast to galactose-based medium, OCR was dependent on glutamine in glucose-based medium.

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C1–04 High-resolution respirometry and bioenergetics evaluation in fibrobalsts derived from patients with *TP53* germline mutations.

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Li-Fraumeni Syndrome (LFS) and Li-Fraumeni-like Syndrome (LFL) are inherited disorders, associated to TP53 germline mutations and characterized by increased predisposition to multiple early-onset cancers [1]. Studies in families from Southern and Southeastern Brazil with LFS/LFL phenotype have identified a germline founder mutation in the TP53 gene, the p.R337H mutation (c.1010G>A), in a high population prevalence (~0.3%) [2]. Unlike the majority of the mutations in TP53, which are missense mutations located in the DNA-binding domain (DBD) of the protein (exons 5-8), the TP53 p.R337H (c.1010G>A) is located in exon 10, corresponding to the oligomerization domain (OD). The p53 nuclear phosphoprotein is known for its functions in the DNA damage response and apoptosis. Recently, this protein has been shown to regulate many aspects

of energy metabolism as well as enzymes that are involved in cell responses to oxidative stress, manly through TIGAR activation [3].

In a previous work, we analyzed the levels of several markers of oxidative stress responses in blood samples of p.R337H mutation carries and non-carries. We observed oxidative damage in lipids and proteins. Moreover, there was increased erythrocyte GPx activity, as well as increased total antioxidant status in the p.R337H mutation carries [4]. Therefore, our study was able to establish the relationship of oxidative stress with the loss of function of p53.

The aim of this work was to evaluate the association between TP53 germline mutations with deregulation of cell bioenergetics. For this purpose, we performed high-resolution respirometry (HHR) of intact human fibroblast cells, derived from patients. Preliminary results showed a distinct pattern of HHR in different TP53 germline mutations genotypes. Fibroblasts from carriers of DBD mutations and wt/p.R337H showed higher ROUTINE, total and extramitochodrial respiration, as well as LEAK respiration, compared to p.R377H/p.R337H mutants and WT/WT cells. In agreement with HHR results, cells with DBD mutation showed increased ROS (reactive oxygen species) by DCF assay. On the other hand an unexpectedly high production was found of ROS by p.R377H/p.R337H. These data were correlated with antimetabolic drug sensitivity, mitochondrial membrane potential and cellular doubling time to better evaluate the potential role of these findings for the increased predisposition to multiple early-onset cancers presented by Li-Fraumeni patients.

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C1-05 Characterization of the effects of pro-inflammatory cytokines on energy metabolism in human myoblasts.

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Ageing is frequently associated with sarcopenia, which has been attributed to low grade inflammation, suppressed regenerative potential of muscle precursor cells and homeostatic changes in the niches of satellite cells of old persons [1,2]. The aim of this study was to investigate mitochondrial function in primary cell cultures, derived from biopsies taken from young and old individuals.

Primary muscle cell culture myoblasts, obtained from biopsies of vastus lateralis in young (19-29 y) and old (70-80 y) subjects, were purified with CD56 antibody microbeads on MACS and cultured in the presence of HGF. The cultures were stimulated with differentiation media supplement, insulin-transferrin-sodium selenite (ITS), for 6 days with one of cytokines IL1, IL6 or TNF-α. The function of respiratory complexes (OXPHOS) was assessed by high-resolution respirometry.

The myoblasts cultivated from old individuals differentiated into myotubes markedly slower than myoblasts from young individuals in ITS medium ($P<0.0001$). The effect of IL-6 depended on donor age, as its effect on myoblast differentiation decreased with age. Treatment of human myoblasts with TNF-α and IL-1β increased the proliferation and blocked differentiation in the presence of ITS. The inhibitory effect of TNF-α and IL-1β on myotubes formation was mediated by down-regulation of mRNA levels of myogenin and muscle-specific isoforms of CK (CKM and CKMT2). The data on mitochondrial respiration revealed that IL-1β caused a significant decrease in mitochondrial Complex I- and II-linked respiration, normalized on cell protein content both in the myotubes of old and

young individuals. This action of IL1-β was not seen when the respiratory results were normalized on citrate synthase activity, revealing the role of a decrease in mitochondrial content in these cells. TNF-α, on the contrary, caused a significant increase in mitochondrial Complex I- and II-linked respiration, normalized on protein in myotubes of old and young subjects. This action of TNF-α remained significant when respiration was normalized on citrate synthase activity. The mode of action of these pro-inflammatory cytokines on OXPHOS of muscle cell cultures was the same in both groups, young and old persons.

Our data suggest that the myoblasts cultivated from biopsies of old individuals differentiate into myotubes slower than those from young individuals. The actions of pro-inflammatory cytokines on OXPHOS level of these cell cultures are different: IL-1β decreased, TNF-α stimulated but IL-6 exerted no alteration on OXPHOS activity, both in old or young individuals. The OXPHOS capacity in myogenic cell culture depends more on the mode of action of cytokine than the donor's age.

Supported by EU-MYOAGE project FP7 223576 and grants of Estonian Science Foundation 7823 and 8736.

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C1-06 Investigation of the energy metabolism of microglial cells.

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Microglial cells play a key role in the pathomechanism of neurodegenerative disorders. These cells can enter metabolically different compartments in the CNS. We investigated, which of the compounds available in the extracellular space can serve as metabolic fuels for these cells.

Cellular oxygen consumption was measured with the OROBOROS Oxygraph-2k, and extracellular acidification rate (ECAR) was measured on primary microglia and on the BV-2 microglial cell-line with the Seahorse Extracellular Flux Analyzer. ECAR was considered a parameter of glycolytic activity. Cells were incubated in Artificial Cerebrospinal Fluid (ACSF) supplemented with substrates available to the cells in the CSF: glutamine, glucose, lactate, keton bodies or pyruvate. ATP and ADP levels were measured using a luciferin/luciferase bioluminescent method. Viability was detected with annexin/calcein fluorescent staining and MTT spectrophotometric assay.

All of the substrates applied supported the metabolism of the cells and none of them influenced their viability negatively. In the presence of glutamine and pyruvate ROUTINE respiration was increased; furthermore, glutamine increased the scope of uncoupler-stimulated respiration above ROUTINE activity levels of the cells. However, in the presence of glucose, the respiration was decreased and the ECAR raised, indicating that glucose, added to microglial cells, stimulated glycolysis but inhibited oxidative metabolism (Crabtree effect). Addition of a lactate dehydrogenase inhibitor after glucose reversed this effect. In the presence of glucose, adding a mitochondrial fatty acid transporter inhibitor further increased the ECAR.

We conclude that microglial cells show high metabolic plasticity and use a wide range of substrates. Interpreting the ECAR results, we claim that these cells show high glycolytic capacity. Furthermore, we found that, besides glucose, glutamine was the most preferred substrate for microglial cells.

Supported by OTKA (NK 81983), TAMOP (4.2.2./B-09/1), MTA (MTA TKI 2013), Hungarian Brain Research Program (KTIA_13_NAP-A-III/6).

C1-07 Complex I inhibition in microglia and inflammatory neuronal loss.

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Brain inflammation may contribute to neuronal loss in infectious, ischemic, traumatic and neurodegenerative brain pathologies. We and others have shown that: a) brain inflammation induces the expression in microglia and astrocytes of inducible nitric oxide synthase (iNOS), which produces high levels of NO, b) NO derivatives peroxynitrite and S-nitrosothiols inactivate mitochondrial Complex I, resulting in a stimulation of oxidant production by mitochondria, c) oxidant production by microglia contributes to their inflammatory activation, and d) activated microglia can cause neuronal loss by eating them alive [1-4]. Thus, we were interested in whether activated microglia may inhibit their mitochondrial Complex I, resulting in sustained activation and phagocytosis of live neurons.

There is evidence that in Parkinson's disease and general brain aging mitochondrial Complex I is inhibited in affected parts of the brain. Rotenone is an environmental toxin and Complex I inhibitor that can cause activation of microglia and a Parkinson's like pathology in rodents. We, therefore, tested whether it could cause microglia to phagocytose live neurons.

We found that low nanomolar levels of rotenone could indeed activate microglial phagocytosis and cause neurons to phagocytose co-cultured neurons [5]. Removal of microglia or inhibition of phagocytic signalling prevented rotenone-induced neuronal loss, leaving viable neurons [5].

Low levels of brain inflammation during ageing may cause partial inhibition of Complex I, resulting in oxidant production which sustains inflammation, and induces microglia to phagocytose synapses and cell bodies of live neurons. This process may be exacerbated in Parkinson's disease and prevented by blocking inflammation or phagocytic signalling.

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MiP2013: Vilma Borutaite

MiP*summer* 2012, Cambridge, UK: Guy Brown

C2 Integrity of mitochondrial preparations and experimental quality control

C2-01 Controversies regarding the cytochrome *c* test for mitochondrial membrane integrity.

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Cytochrome *c* is a small soluble protein which has two main functions: transfer of electrons within the mitochondrial respiratory system and formation of apoptosomes when released into cytosol. Under normal conditions, the outer mitochondrial (mt) membrane is not permeable to cytochrome *c*. Therefore, stimulation of mitochondrial respiration by exogenous cytochrome *c* is widely used as a test for outer mt-membrane damage.

Using this test, we and others have shown that heart ischemia causes rapid permeabilization of the outer mt-membrane, resulting in loss of cytochrome *c* from mitochondria and subsequent inhibition of mitochondrial respiration. In contrast, addition of exogenous cytochrome *c* to mitochondria isolated from ischemia-damaged brains does not stimulate mitochondrial respiration, suggesting that during brain ischemia the outer mt-membrane remains intact. We also found that in the presence of certain polyphenolic plant compounds (anthocyanins), addition of cytochrome *c* to isolated mitochondria results in acute stimulation of mitochondrial respiration. This effect is not linked to the permeabilization of the outer mt-membrane, as these anthocyanins can directly reduce cytochrome *c*, thus facilitating electron transfer to cytochrome *c* oxidase.

Certain anthocyanins, such as delphinidin-3-O-glucoside or cyaniding-3-O-glucoside, can serve as electron acceptors at Complex I of the mitochondrial respiratory system and, therefore, in pathological conditions related to inhibition of Complex I, facilitate an alternative electron transfer from Complex I to cytochrome *c* and cytochrome *c* oxidase.

Supported by European Social Fund under the Global grant measure; project VP1-3.1-SMM-07-K-01-130.

C2-02 Assessing mitochondrial lactate oxidation in permeabilized skeletal muscle fibers highlights important experimental considerations: malate concentration and the cytochrome *c* effect.

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Lactate serves as an important metabolic intermediate for many tissues including skeletal and cardiac muscles, liver and brain. It is thought that a primary purpose of cytosolic lactate production from pyruvate by lactate dehydrogenase (LDH) is to regenerate NAD^+ for continued glycolytic ATP production [1]. Considered in isolation, the NAD^+ recycling afforded by cytosolic lactate production does not reconcile with the traditional view of aerobic glycolysis in which pyruvate, generated by glycolysis, enters the mitochondria directly for subsequent oxidation in the TCA cycle. The intracellular lactate shuttle hypothesis posits that lactate, generated in the cytosol, is oxidized by mitochondrial LDH of the same cell [2]. The details of the shuttle, however, are not entirely clear.

Evidence is presented which supports that in skeletal muscle, extra-matrix LDH is strategically positioned within the cell to functionally interact with mitochondria [3]. A model incorporating mitochondrial lactate oxidation makes sense of aerobic glycolysis by permitting, among other things, cytosolic NAD^+ regeneration, locally. However, experimental support requires attention to methodological detail. Important experimental conditions for assessing mitochondrial lactate oxidation in permeabilized fibers are

discussed. Proper malate concentration [4] is necessary for robust NAD^+-dependent lactate oxidation, suggesting that a functional malate-aspartate shuttle is essential to the assay. The cytochrome *c* test, a convenient means of confirming the integrity of the mitochondrial outer membrane [5], may not accurately reflect the integrity of mitochondrial preparations when assessing lactate oxidation. Indeed, parallel experiments in high-resolution respirometry reveal that in permeabilized rat skeletal muscle fibers, exogenous cytochrome *c* stimulates respiration with lactate but not with pyruvate as substrate.

These findings highlight the importance of optimizing seemingly trivial experimental variables.

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C2-03 Cytochrome *c* flux control factor as a quality criterion in respiratory OXPHOS analysis in canine permeabilized fibres.

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Mitochondrial (mt) preparations (isolated mitochondria, permeabilized cells and tissues, tissue homogenates) provide a fundamental basis for comprehensive OXPHOS analysis for the study of substrate and coupling control of mitochondrial respiration [1]. Plasma membrane permeabilization with mechanical separation of muscle fibre bundles and chemical permeabilization with mild detergents may influence the integrity of the outer mt-membrane and thus induce partial release of cytochrome *c* (*c*). In mitochondria isolated from healthy skeletal muscle, CI&II-linked OXPHOS capacity decreases linearly with cytochrome *c* loss during isolation [2]. The cytochrome *c* effect is expressed as the flux control factor FCF_c, which is the increase of OXPHOS capacity after addition of 10 µM *c* normalized for *c*-stimulated respiration [1-3]. There is no consensus as to the threshold of FCF_c applied as a quantitative exclusion criterion in permeabilized fibres obtained from healthy muscle tissue.

We aimed at establishing a reference method for the application of a cytochrome *c* threshold as exclusion criterion in mitochondrial OXPHOS analyses. Our study involved Alaskan sled dogs (*N*=6) studied 72 to 120 h after finishing a competitive 1,000 mile race in nine days. Permeabilized fibres (wet weight per chamber of 0.81-1.28 mg ± 0.12 SD) were prepared from needle biopsies and immediately studied by high-resolution respirometry [4] using 12 chambers in parallel (OROBOROS Oxygraph-2k). Compared to human skeletal muscle fibres, the canine samples were more trexturally supple and sticky, requiring delicate fiber separation under light microscope, and disintegrating to various degrees during substrate-uncoupler-inhibitor titration (SUIT) protocols. This was reflected in variable and sometimes extremely high cytochrome *c* effects. However, there was no loss of CI- or CI&II-linked OXPHOS and ETS capacity with increasing FCF_c (Figure 1).

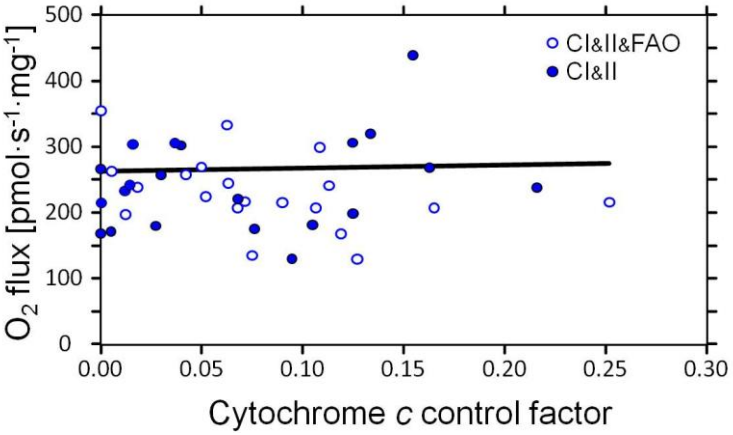

Figure 1. Independence of O_2 flux (ETS capacity in the presence of cytochrome *c*) of the cytochrome *c* control factor,

$$FCF_c = (J_{CHOc}-J_{CHO})/J_{CHOc}$$

ETS capacity was 238±64 pmol·s^{-1}·mg^{-1} W_w independent of the CHO substrate combination supporting CI&II-linked electron flow in the presence or absence of 0.2 mM octanoyl carnitine (FAO).

Apparently, the damage caused by mt-preparation even in cases with FCF_c up to 0.25 could be rescued by addition of 10 µM *c* and thus restore capacities comparable with samples of negligible FCF_c. In contrast, multiple defects associated with increasing FCF_c in human muscle fibres cannot be compensated fully by addition of cytochrome *c* [2,5]. Cytochrome *c* was applied early in the two SUIT protocols, in the CI-linked or CI&FAO-linked OXPHOS state. This allowed consistent analysis of subsequent respiratory states which were all supported by the externally added cytochrome *c* (Figure 1).

OXPHOS and ETS capacities with FAO- and CI&II-linked substrates were higher than in muscle from competitive horses and humans [5,6]. The present approach (Figure 1) allows evaluation of the FCF_c threshold as a potential exclusion criterion in healthy controls.

Supported by K-Regio project *MitoCom Tyrol*.

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C2-04 Bioenergetic differences between permeabilized red and white fibers of a non-obese model of type 2 diabetes.

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The cause and effect relationship between mitochondrial dysfunction and insulin resistance (IR) is still under debate. It appears that lipid accumulation is the principal cause of IR, while the mechanisms leading to fat accumulation in skeletal muscle are not clear yet [4]. The reduced mitochondrial content, β-oxidation impairment or fat overload could contribute to the lipid imbalance in the obese population [1,2,4]. Furthermore, evidence has been provided for metabolic alterations in the skeletal muscle of insulin resistant non-obese patients. In particular, *in vivo* ATP synthesis in the skeletal muscle of young, lean, insulin resistant offspring of type 2 diabetes mellitus (T2DM) patients was 30% lower than that in control subjects [5]. Both T2DM and physical inactivity cause functional adaptations in skeletal muscle, in which IR is reflected in higher ratios of

glycolytic to oxidative capacities [7]. Thus, a study on fructose-induced insulin resistant rats revealed differences in energy metabolism between oxidative and glycolytic fibers; mitochondrial function is preserved in oxidative fibers (e.g. Type I) whereas β-oxidation is impaired in glycolytic fibers (e.g. Type II) [8].

We used a spontaneous non-obese model of T2DM, the Goto Kakizaki (GK) rat, to study the relationship between IR and mitochondrial bioenergetics. Age-matched (18 weeks) Wistar (W) rats were used as control. We investigated skeletal muscle mitochondrial bioenergetics using permeabilized fibers of soleus and white gastrocnemius muscle. The permeabilization and respirometric measurements of the fiber were performed on fresh muscle samples according to the previously developed protocol [3]. Mitochondria content was determined by citrate synthase (CS) and succinate dehydrogenase (SDH) activities [6].

Respiration of soleus muscle fibers was similar in both W and GK rats for Complex I- and Complex II-linked substrates (Figure 1). In contrast, respiration of white gastrocnemius fibers was lower in GK rats than in W rats, in the presence of Complex II substrate (Succinate, S). In both fiber types respiration in the presence of Complex IV substrate was lower in GK rats than in W rats. In soleus muscle, CS and SDH activities of GK rats (CS: 42.5±3.6, SDH 3.5±0.4 $mU \cdot mg^{-1}$) tended to be lower than in W rats (CS: 46.3±1.9, SDH 4.3±0.4 $mU \cdot mg^{-1}$, $P<0.1$). Similarly, in white gastrocnemius muscle, CS and SDH activities of GK rats (CS: 14.2±0.6, SDH 0.9±0.2 $mU \cdot mg^{-1}$) were lower than those in W rats (CS: 17±1.9, SDH 1.3±0.3 $mU \cdot mg^{-1}$, $P<0.05$).

Our results indicate that in absence of obesity, insulin resistance in skeletal muscle is related to a reduced mitochondrial content in both fibers types and to metabolic alterations only in type II fibers. Further studies are needed to investigate the relationship between IR and mitochondria biogenesis as well as the fibers contribution to substrate utilization during the progression of T2DM.

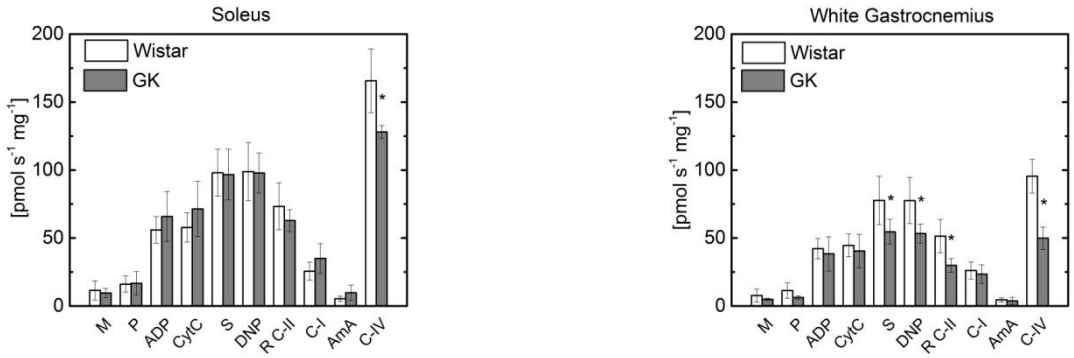

Figure 1. Oxygen consumption rates in permeabilized soleus and white gastrocnemius muscles of W and GK rats (*$P<0.05$).

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C2-05 Improved isolation of high quality subsarcolemmal and interfibrillar mitochondria from skeletal muscle.

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The subsarcolemmal (SSM) and interfibrillar (IFM) mitochondria in cardiac and skeletal muscle exhibit distinct biochemical and structural properties affecting energy metabolism in health and disease states. The method of isotating mitochondria affects the quality and quantity of the SSM and IFM [1] separated by subcellular fractionation techniques. Previous rat skeletal muscle studies reported lower yield and respiratory control ratios (RCR) of isolated SSM and IFM [2,3] than those isolated from heart [4]. In these animal studies the functional and structural properties of the mitochondrial subpopulations were not comprehensively investigated. A more recent dog skeletal muscle study [5] used a new isolation protocol for SSM and IFM in which RCR was higher than in rats; the mitochondrial yield was slightly increased only for the IFM population in comparison to the rat studies.

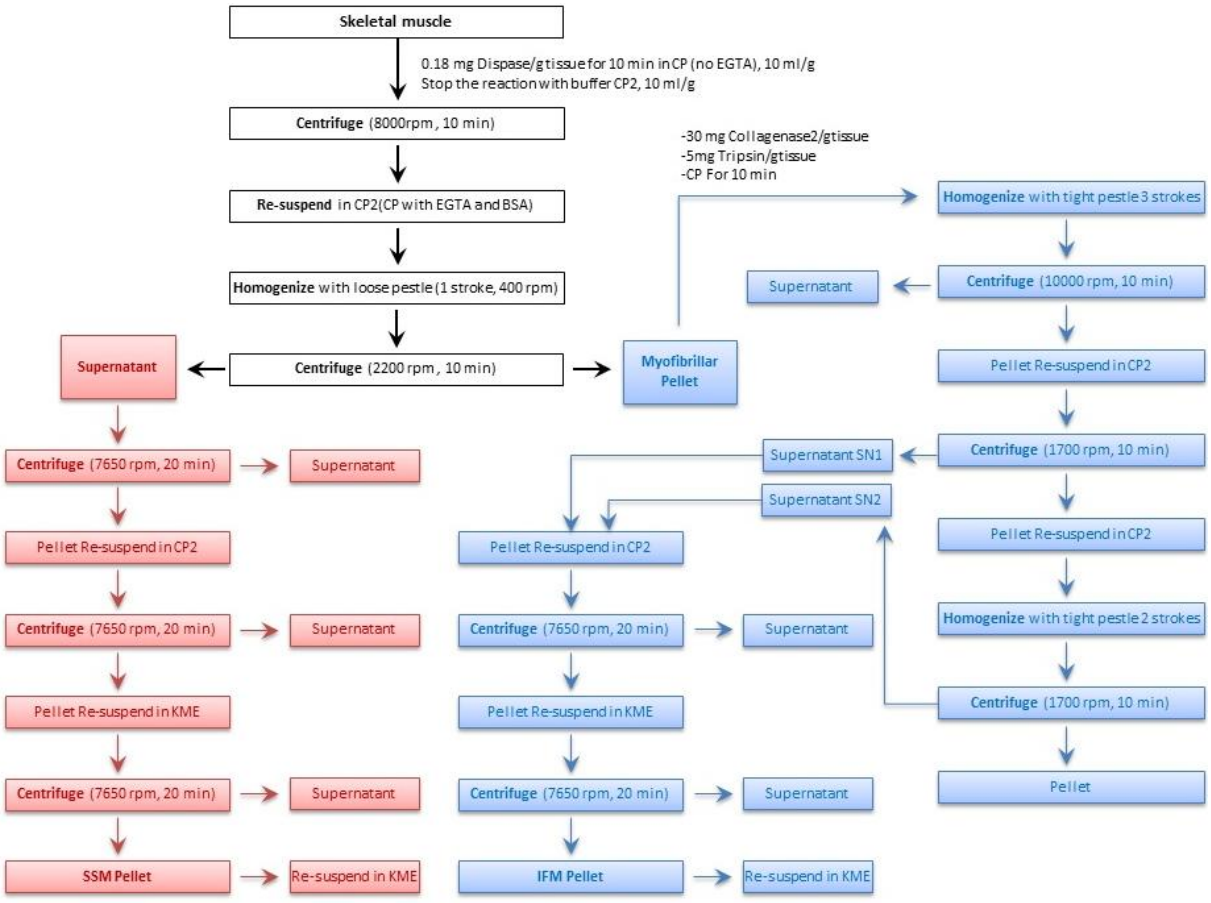

Figure 1. Preparation and Separation of subsarcolemma (SSM) and interfibrillar (IFM) mitochondria.

We modified our protocol, based on subcellular fractionation [5] to improve the isolation of SSM and IFM from rat skeletal muscle. Oxidative phosphorylation, enzymatic, and morphological assays were used to relate functional and structural properties of mitochondria. The major step of the isolation protocol includes skeletal muscle mincing, homogenization, enzymatic treatment and differential centrifugation (Figure 1). Neutral protease was used to disperse the myofibrils and facilitate the release of SSM during homogenization. The myofibrillar pellet was treated with trypsin and collgenase type II to extract the IFM population during homogenization.

The yields of SSM and IFM (3.5±0.5; 8.6±1.5 mg·g^{-1}) from rat skeletal muscle were higher than those previously obtained with rats [2,3] and dog skeletal muscle [5]. Mitochondrial respiration was measured in the presence of glutamate as substrate for SSM and IFM with sequential additions of ADP at non-saturating (0.2 mM; State 3) and saturating (2 mM; OXPHOS) concentrations, and of the uncoupler dinitrophenol (DNP; 0.2 mM). State 3 (SSM 2.6±0.1; IFM 3.0±0.2 nmol·s^{-1}·mg^{-1}) and RCR (SSM 19.6±4.2; IFM 18.8±4) were higher than those previously reported in rat skeletal muscle [2,3]. OXPHOS capacity at saturating ADP was 3.3±0.2 and 4.7±0.4 nmol·s^{-1}·mg^{-1} for SSM and IFM, respectively. The electron transfer system capacity measured after uncoupling with DNP was 3.7±0.3 and 5.0±0.5 nmol·s^{-1}·mg^{-1} for SSM and IFM, respectively. Citrate synthase and succinate dehydrogenase activities were measured to quantify the mitochondrial distribution in the subcellular fractions. Integrated mitochondrial function, measured as oxidative phosphorylation, was used with different substrates to probe oxidation and phosphorylation systems. The activity of respiratory enzyme complexes was measured to quantify the biochemical capacity of ETS components [5]. Electron microscopy images of the subpopulations of mitochondria confirmed that the procedure preserved the structure of SSM and IFM.

The improved method allowed isolation of high quality subpopulations of skeletal muscle mitochondria, comparable to those from heart [3]. This is a valuable approach to study the relationship between function and structure of skeletal muscle mitochondria in disease conditions.

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C2-06 Methodological aspects of assessing mitochondrial function in small intestinal mucosal samples of rats and guinea pigs.

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Although the renaissance of functional mitochondrial studies targets many tissues and organs, small intestinal mitochondria are less frequently examined. Nevertheless, the integrity of small intestinal mucosa is potentially threatened in various local and systemic pathologies, such as ischemia-reperfusion, septic shock or inflammatory bowel diseases. Mitochondrial dysfunction is critically involved in the pathomechanism of these diseases. Furthermore, timely restitution of adequate mitochondrial function can be a key step towards potentially effective therapeutic strategies.

Our goal was to provide firm experimental data for functional mitochondrial investigations, and as a first step, we aimed to test and validate the available methods used for isolation of small intestinal mitochondria in rodents [1,2]. Whole thickness small intestinal mucosal samples of rats and guinea pigs were used, and mitochondria were isolated according to published protocols using chelating agents and differential centrifugation. The assessment of the functional state of isolated mitochondria was performed by means of high-resolution respirometry (OROBOROS Oxygraph-2k). The integrity of the outer mt-membrane was tested with cytochrome *c* addition and photometric assay for mitochondrial swelling, while membrane potential changes were monitored by safranin fluorescence measurements.

In case of rat samples, low respiratory control rates and extremely high cytochrome *c* responses were found, and swelling of mitochondria indicated serious damage of the outer mt-membrane. In contrast, guinea pig mitochondria were presented with good respiratory control, low cytochrome *c* response and baseline swelling parameters.

Based on high-resolution respirometry we conclude that using the accessible, published methodologies, only functionally impaired mitochondria can be isolated from the rat. However, high quantities of intact and well coupled mitochondria can be obtained from the guinea pig. These mitochondria are suitable for further focused studies. Further methodological investigations, possible modifications or even new protocols are needed in order to clarify the cause of this significant interspecies difference.

Supported by grants OTKA K104656, TAMOP-4.2.2A-11/1-KONV-2012-0035, TAMOP-4.2.2A-11/1/KONV-2012-0073, TAMOP-4.2.4.A/2-11/1-2012-0001 'National Excellence Program'.

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C2-07 Mitochondrial respiration and calcium transport in rat tissues: conversely from skeletal muscle, heart and brain, isolated liver mitochondria exhibit gender dimorphism in respiratory activity.

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Previous data provided evidence that isolated liver mitochondria from female rats present higher rates of resting (LEAK) and ADP-stimulated (OXPHOS) O_2 consumption than from male ones [1]. Furthermore, estrogens are cytoprotective, drive mitochondrial biogenesis and may modulate mitochondrial calcium homeostasis in brain and heart in *in vitro* models [2,3]. It is unknown whether such gender dimorphism occurs for other mitochondrial functions or in other tissues. Therefore, we aimed to study whether mitochondrial respiration and mitochondrial calcium influx and efflux rates exhibit tissue-specific gender dimorphism.

Liver, skeletal muscle, heart and brain mitochondria were isolated from female and male Wistar rats by differential centrifugation. Mitochondrial respiratory states were evaluated by high-resolution respirometry. Mitochondrial calcium transport (ruthenium red-sensitive initial influx at 25 µM external free calcium and sodium-dependent, at 15 mM sodium, and -independent efflux) was assessed by following external free calcium levels with the fluorescent probe CaGreen-5N under suitable conditions and in the presence of inhibitors of mitochondrial permeability transition. In isolated male mitochondria O_2 fluxes (mean±SD nmol·min^{-1}·mg^{-1}) for OXPHOS and LEAK respectively, were: 37.6±9.8 and 8.6±2.6 in liver, 106.1±24.7 and 12.8±3.4 in skeletal muscle, 137.1±42.1 and 25.9±4.7 in heart, and 31.1±8.2 and 4.8±1.3 in brain. Calcium influx and sodium-dependent efflux respectively, were (mean±SD nmol·min^{-1}·mg^{-1}): 228.6±77.1 and 0.69±0.39 in liver, 56.12±20.5 and 3.31±0.67 in skeletal muscle, 39.4±18.6 and 8.25±1.42 in heart, and 54.6±23.9 and 2.25±0.89 in brain from males. Among the assessed respiratory and calcium transport variables, the only statistically significant (P<0.05) difference between genders occurred for liver mitochondrial OXPHOS capacity, which was 15% higher in female than in male rats.

We conclude that gender dimorphisms for the mitochondrial functions evaluated here is tissue-specific and is confined to higher maximal ADP-stimulated respiration in isolated liver mitochondria from female rats.

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C3 Physiological and pharmacological intervention with mitochondrial function

C3-01 The cardiolipin-targeting peptide Bendavia preserves post-ischemic mitochondrial energetics by sustaining respiratory supercomplexes.

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Bendavia is a cytoprotective mitochondria-targeting peptide [1-4], currently being tested in the EMBRACE-STEMI trial for reducing injury during acute coronary syndromes. We previously showed that the cardioprotective effects of Bendavia involved improving cardiolipin-dependent mitochondrial membrane fluidity. As membrane fluidity influences the ability of proteins to assemble, we hypothesized that a consequence of augmented membrane fluidity would be higher expression of mitochondrial respiratory supercomplexes.

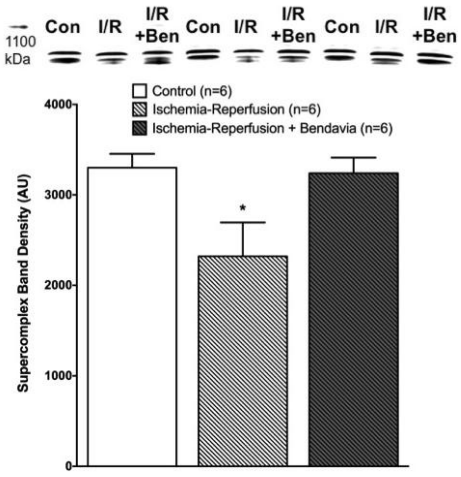

Figure 1: Maintenance of mitochondrial supercomplexes after ischemia/reperfusion (I/R) with the mitochondria-targeting peptide Bendavia.

Rat hearts (*N*=42) were subjected to ischemia-reperfusion (I/R) with our without 1 nM Bendavia perfusion, beginning at the onset of reperfusion. Left ventricular tissue was split into one of two study arms: 1. Supercomplex expression using blue-native gel electrophoresis (BN-PAGE), or 2. High-resolution respirometry using permeabilized ventricular fibers. For BN-PAGE studies, respiratory supercomplex bands were decreased with I/R, and restored with Bendavia (Figure 1). High-resolution respirometry studies indicated suppressed Complex I-dependent respiration after I/R (208±19 v 42±9 pmol $O_2 \cdot s^{-1} \cdot mg^{-1}$) in control v I/R, respectively; $P<0.05$. Complex II-dependent respiration was also lower (753±41 v 168±13 pmol $\cdot s^{-1} \cdot mg^{-1}$ in control versus I/R; $P<0.05$). Perfusion with Bendavia during reperfusion significantly increased Complex I- (100±13 pmol $O_2 \cdot s^{-1}$ mg^{-1}) and CII-dependent (334±63 pmol $O_2 \cdot s^{-1} \cdot mg^{-1}$) respiration ($P<0.05$ versus untreated IR for both).

Taken together, these data suggest that Bendavia's protective mechanism of action involves preserving supercomplex-dependent mitochondrial function during cardiac reperfusion.

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C3-02 Experimental considerations for evaluating bioenergetic effects of a mitochondria-targeted compound, TPP-IOA.

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Triphenylphosphonium-conjugated imidazole-substituted oleic acid (TPP-IOA) is an inhibitor of cytochrome *c* peroxidase activity that appears to initiate apoptotic cell death via cytochrome *c* release. As a molecule that interacts directly with cytochrome *c*, TPP-IOA may also affect mitochondrial respiration. We are evaluating the dose-effect relationships of TPP-IOA's interactions with mitochondria.

Although original data came from cells maintained in glucose media in culture, the interpretation of these data is limited by the fact that under these conditions the cells derive little ATP from oxidative phosphorylation and have low mitochondrial densities. We are revisiting the effects of TPP-IOA on mitochondrial respiration and apoptotic cell death in glucose-free media, in which energy is derived from galactose and glutamine and cells are highly reliant on oxidative phosphorylation. Cells growing under these conditions are highly sensitive to drugs that may perturb oxidative phosphorylation. We are also evaluating the effects of 3-hydroxypropyl-TPP (TPP), the TPP moiety of TPP-IOA, on mitochondrial function in this system and in isolated mitochondria.

At concentrations that effectively inhibit mitochondrial-mediated cell death in cells, TPP-IOA exerted significant effects on oxidative phosphorylation, e.g. reducing the respiratory control ratio. Conjugation to TPP is frequently used to promote accumulation of molecules in mitochondria. However, since TPP and linker molecules can themselves have effects on mitochondrial function it is necessary to evaluate these independently, an experimental control that is often not performed. We find similar effects of TPP and TPP-IOA on mitochondrial OXPHOS, suggesting that it is the TPP moiety that interferes with normal mitochondrial function in this assay.

Taken together, these preliminary data indicate the importance of (i) choosing appropriate cell culture conditions that promote reliance on oxidative phosphorylation to evaluate the effects of molecules that are targeted to mitochondria, and (ii) including experimental controls of TPP, since this moiety and linker group can have significant effects on mitochondrial function.

C3–03 Cytotoxicity of grape pomace extract (*Vitis vinifera* L.) from Brazilian wine industry in human hepatocarcinoma HepG2 cells.

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Wine industry produces large quantities of by-products, rich in bioactive compounds from grapes, with potential use for nutraceutical purposes. Grape pomace represents an environmental problem due to its high quantity and its pollutant characteristics. In Brazil, the majority of grape pomace produced is discarded, while part of it is used as fertilizer and as animal ration by small agricultural producers [1]. Since grape pomace is a rich source of polyphenols including phenolic acids, stilbenes, flavonoids and tannins, the use of this by-product for these purposes is very limited, due to their potent anti-microbial and potential anti-nutrient properties (original). Therefore, other applications are required to decrease environmental impact of grape pomace and, concomitantly, add value to this residue. In this study we evaluated the cytotoxicity of a grape pomace extract, obtained from Brazilian wine industry, in human hepatocarcinoma cells (HepG2).

Hydro-alcoholic extracts were obtained from red grape (*Pinot noir*) pomace, derived from white wine vinification and concentrated by reverse osmosis. Grape pomace extract was characterized with respect to its bioactive compounds and antioxidant capacity. *In*

vitro bioactivity was assessed on HepG2 cells as a function of viability, cellular respiration and lactate production under short, medium and long-term incubation periods.

Grape pomace extract had high contents of polyphenol compounds and antioxidant capacity, compared to previously published data [2]. Total phenolics, flavonoids and anthocyanins where (mean±SE) 34,060±1,490 mg galic acid Eq/100 g, 2,146±191 mg catechin Eq/100 g and 258.69±1.66 mg cyanidin3-glycoside/100 g, respectively. The main anthocyanins found in grape pomace extract were 3-*O*-glucosides. Pomace extract antioxidant capacity was (mean±SE, mmol Trolox Eq/g) 101.43±0.12 and 16.71±1.78, by TEAC and ORAC assays, respectively. HepG2 viability reduced in a time- and concentration-dependent manner. Short-term incubation had no effect whereas medium- and long-term incubation induced a maximum of 30% and of 70% reduction in viability, respectively. Cell respiration and lactate production were assessed in short-term incubations, in order to evaluate grape pomace polyphenols bioactivity on HepG2 bioenergetics irrespective of effects on cell viability. Treated cells presented a significant 60% increase in ROUTINE respiration. Additionally, the ROUTINE flux control ratio (*R/E*) was higher in treated (0.52) when compared to control (0.40) cells. Moreover, the fraction of oxygen consumption utilized for ATP synthesis, (*R-L*)/*E*, significantly increased from 0.16 in control to 0.27 in treated HepG2 cells. These results indicate that treated cells present a lower respiratory reserve capacity and also suggest an increased requirement for ATP. Interestingly, lactate production decreased in treated cells, indicating a decreased utilization of glucose and, possibly, a compensatory effect due to increased mitochondrial respiration.

The increase in respiration, related to phosphorylation in treated HepG2 cells, seems to be an early sign of bioenergetic alterations due to polyphenol compounds in grape pomace extract, which are possibly involved in cytotoxicity observed at longer incubations. The mechanisms underlying these effects are to be determined and might be related to decreased glucose utilization by HepG2 cells. Therefore, we reasoned that pomace produced from white wine vinification from Brazilian winemaking presents important pharmacological properties possibly related to potential anticancer effects on HepG2 cells.

Supported by CAPES, CNPq and FAPERJ (Brazil).

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C3–04 Defining the role of mitochondrial electron transfer Complex I on the modulation of Ca^{2+} homeostasis: effects of metformin on cancer cell metabolism.

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Energy metabolism (generation of ATP) is driven by aerobic oxidative phosphorylation (OXPHOS), which occurs in the mitochondria, and by anaerobic glycolysis taking place in the cytoplasm. In most cancer cells, energy generation switches to glycolysis even in the presence of oxygen, a phenomenon known as the Warburg effect, which has preoccupied most cancer research while neglecting mitochondria. However, recent studies have shown that mitochondrial tricarboxylic acid (TCA) cycle, which produces NADH that is reduced by Complex I delivering the electrons for OXPHOS, is both functional and essential for tumor growth. The TCA cycle plays a pivotal role in the synthesis of macromolecules, including proteins, lipids and nucleotides. The TCA cycle is finely regulated by Ca^{2+}, which enters the mitochondria through MCU [1] driven by a considerable voltage across the inner mitochondrial membrane ($\Delta\Psi_{mt}$), generated in part by protons being pumped by respiratory electron flow. Metformin, which has been widely

used for over 40 years to treat diabetes, mimics caloric restriction acting on cell metabolism at various levels. It primarily inhibits Complex I and activates AMPK, effects also observed during inhibition of mitochondrial Ca^{2+} uptake. Interestingly, metformin and other inhibitors of Complex I can selectively cause cell death in cancer cells through a mechanism that is still unclear [2,3].

Here, using extracellular flux analyzer technology, we show that incubation with 5 mM metformin for 24 h decreased both ROUTINE respiration and uncoupled oxygen consumption (electron transfer system capacity) in MCF7 cells, recapitulating the effects observed by inhibition of Ca^{2+} uptake by MCU. These effects were accompanied by a perturbation of cytosolic Ca^{2+} concentration, a robust activation of AMPK and autophagy induction. Additionally, we demonstrated that chronic (24 h) exposure to 10 mM metformin induced inhibition of cell growth and more than 50% cell death in MCF7 cells. This effect was visible only when cells were forced to rely bioenergetically on mitochondria (in media with low glucose), reinforcing the concept that metformin has antitumor properties that probably inhibit Complex I and causing a bioenergetic collapse of the cells.

Financed by postdoctoral fellowship FONDECYT 3140458 (FJ) and regular grant FONDECYT 1120443 (CC).

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C3-05 Effect of ketogenic diet in mouse model of the mitochondrial hepatopathy GRACILE syndrome.

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GRACILE syndrome is a mitochondrial hepatopathy caused by mutated *BCS1L* (homozygous c.232A>G), a gene coding for a respiratory Complex III chaperone [1,2]. *BCS1L* mutations compromise Rieske iron-sulfur protein incorporation into CIII, which leads to electron transfer system deficiency. In a knock-in mouse model of GRACILE syndrome, the homozygotes are born healthy but display growth failure and progressive liver disorder from the fourth week of life, tubulopathy, loss of adipose tissue and a short life span [3]. Liver pathology is characterized by microvesicular steatosis with progression to fibrosis. Ketogenic diet (KD) is a high-fat and low-carbohydrate diet that has proven beneficial in some forms of epilepsy and in some models of mitochondrial disorders.

We hypothesize that, in homozygotes, KD would increase mitochondrial biogenesis and reduce dependence on glucose and thus alleviate disease progression. To test this hypothesis we fed ketogenic and control diets to groups of homozygous and wild-type mice, starting from one week of age (*N*=10 per group). The mice were sacrificed at P40-P45 and samples collected for analysis.

The KD had no effect on weight in homozygotes or wild-type mice. All homozygotes had early stage hepatopathy with no differences in standard scoring between diet groups, although subtle differences in liver histology were oberved. Wild-type mice on KD accumulated fat in the liver, whereas KD had no effect on liver fat content in homozygotes. At transcript level several markers of mitochondrial biogenesis (*Pgc1a*, citrate synthase and subunits of Complexes III and IV) were up-regulated in livers of mutant mice compared to wild-type. mtDNA copy number was not different between

groups and there were no signs of increased mitochondrial biogenesis in liver by KD. mRNA levels of genes related to fatty acid and glucose metabolism indicated different responses towards KD in mutant and wild type mice.

The effect of KD on the course of GRACILE hepatopathy and on survival is being investigated in groups with longer follow-up.

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C3–06 Insulin increases mitochondrial calcium levels regulating both mitochondrial function and intracellular signaling in muscle; this effect is disrupted in fibers from short-term high fat diet fed mice.

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Mitochondrial function rapidly responds to high energy food supply in order to deal with the supply, but the role of mitochondrial Ca^{2+} in these processes is not yet understood [1]. High-fat fed mice quickly gain weight while concomitantly developing insulin resistance in skeletal muscle [2,3]. For obesity, inappropriate lipid deposition in human skeletal muscle and a concomitant reduction in the ability of cells to completely oxidize lipids have been described [4].

Male C57BL/6J mice were fed either a normal chow diet (NCD) or a high fat diet (HFD) for one or eight weeks. Insulin resistance was evaluated by IPGTT, fasting glucose and insulin, and HOMA-IR. GLUT4*myc*-eGFP was electroporated in the *Flexor digitorum brevis* muscle (FDB). Insulin induces an increase in cytoplasmic and mitochondrial Ca^{2+} in adult fibers. The uncoupler FCCP releases Ca^{2+} from mitochondria to cytoplasm. Insulin-dependent mitochondrial Ca^{2+} uptake is decreased in fibers from short-term HFD fed mice, while insulin-dependent cytoplasmic Ca^{2+} increase appear to be faster and stronger. In fibers from NCD fed mice, insulin-dependent mitochondrial Ca^{2+} uptake was inhibited by xestospongin B, a specific inhibitor of inositol-1,4,5-trisphosphate receptor. Using $TMRE^+$ in the non-quenching mode (5 nM), we found that $\Delta\Psi_{mt}$ was larger in fibers from short-term HFD fed mice in comparison to NCD derived fibers. Using $TMRE^+$ in quenching mode (100 nM), we found that $TMRE^+$, released after uncoupler stimuli, was higher in fibers from short-term HFD fed mice than in NCD fed mice. The glucose analogue (2-NBDG) uptake and the redistribution of GLUT4*myc*eGFP, induced by insulin, were decreased in the presence of xestospongin or ruthenium red (MCU inhibitor). This effect suggests a retrograde regulation of insulin signaling by mitochondrial Ca^{2+} uptake.

Financed by FONDECYT 11130267, ACT1111.

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C3-07 Differential expression of mitochondrial transporter proteins leads to incomplete fatty acid oxidation and insulin resistance in palmitate-treated muscle cells.

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Mitochondrial dysfunction leads to reduced fatty acid oxidation, which is also found in obesity associated insulin resistance [1]. Further, lipid deposition in skeletal muscle results in insulin resistance by a poorly defined mechanism called 'lipotoxicity'. Uptake and oxidation of long chain fatty acids by mitochondria are reportedly impaired in skeletal muscles of obese diabetic individuals having insulin resistance [2].

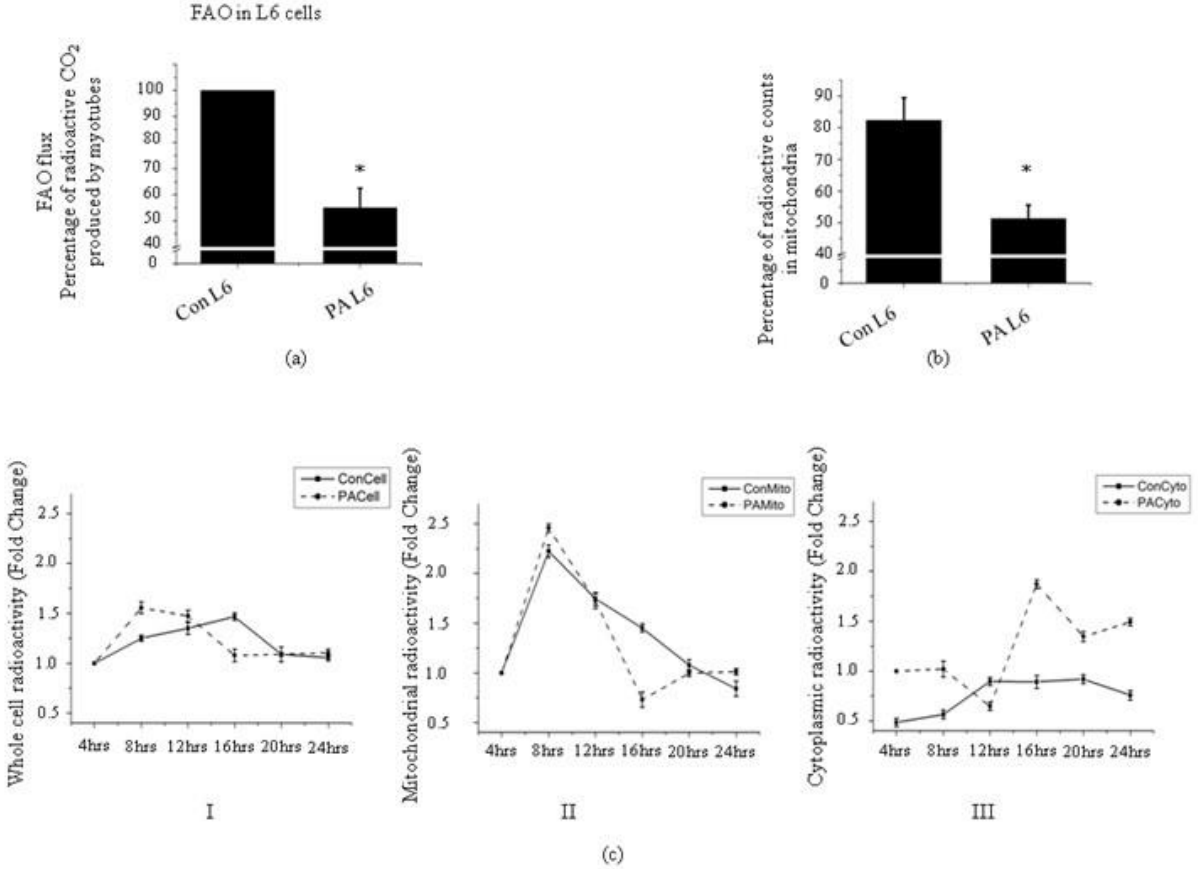

Figure 1. PA-mediated inhibition of FAO rate in muscle cells is shown in (a). Myotubes were incubated with or without PA in media containing trace amounts of radiolabelled [1-^{14}C]-PA. Incubation of cells with 1 mM PA for 18 h reduced the FAO rate by about 40%. Excess PA restricts its own entry into mitochondria prior to FAO (b). Mitochondrial entry of radioactive metabolites in control and PA-treated muscle cells were measured after 18 h of incubation in presence of [1-^{14}C]-PA. The cells were treated in presence or absence of 1 mM PA and radioactive count was measured from mitochondria and whole cell extract. (c) Time kinetics study of PA entry and its distribution in whole cells, mitochondria and cytosolic fractions. The cells incorporated more PA during 8 h of PA-treatment, but after that the, rate of PA uptake was reduced rapidly whereas control cell continues uptaking PA at a fairly constant rate (I). Mitochondrial fraction from control and PA-treated cells accumulate radioactive fatty acid derivatives almost at the same rate up to 12 h, then it decreases in PA-treated set, i.e. entry of PA in mitochondria is restricted after 12 h (II). The cytosolic fraction of PA-treated cells accumulate more radioactivity than that of control cytoplasm during 12-18 h of incubation (*P<0.05).

The resulting intramuscular fatty acids and their metabolite deposition lead to oxidative stress and activation of multiple stress-responsive signalling leading to insulin signalling defects [3]. However, the molecular mechanisms of insulin resistance in insulin target tissues are yet to be understood well.

The impact of lipotoxicity on the genes regulating fatty acid oxidation in insulin resistant skeletal muscles was studied in our laboratory. Realtime-PCR based plate array has been used for this purpose, and we have identified many genes that are important for mitochondrial function and are significantly up- or down-regulated in rat skeletal muscle after high fat diet feeding. Importantly, several mitochondrial transporter proteins were included in this list of genes.

We report for the first time that high concentration of palmitic acid differentially expresses the carnitine palmitoyl transferase isoforms associated with fatty acid-transport and thus alters their ratio in muscle cells leading to incomplete fatty acid oxidation (Figure 1). The carnitine palmitoyl transferase isoforms are a very important mitochondrial fatty acyl transporter that subsequently maintains the rate of fatty acid oxidation. Consequently, the accumulation of ceramides increases, which is a known factor for insulin resistance. PPARa agonist reinstated the ratio of carnitine palmitoyltransferase (CPT) isoforms in PA-treated muscle cells and thus normalized the rate of fatty acid oxidation (FAO) as well as the insulin-mediated glucose uptake.

Taken together, palmitate-induced differential expression of mitochondrial CPT isoforms results in incomplete FAO, which promotes insulin resistance [4]. Modulators of mitochondrial FAO may emerge as potentially useful agents to treat lipid-induced insulin resistance.

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C3-08 Effects of long-chain acyl-CoA synthetase 6 knockdown in primary skeletal muscle cells metabolism.

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When long-chain fatty acids enter the cells, long-chain acyl-CoA synthases (ACSLs) convert them to acyl-CoAs in an ATP-dependent reaction. The resulting acyl-CoAs have numerous metabolic routes within cells, including incorporation into triacylglycerol (TAG) and membrane phospholipids. Acyl-CoAs are used as substrates for beta-oxidation and protein acylation and function as ligands for transcription factors. However, the function of ACSL6 in skeletal muscle cells has not been described. The aim of this study was to investigate the effects of ACSL6 knockdown on mitochondrial metabolism in skeletal muscle cells.

Isolation of primary rat skeletal muscle cells from the lower limb was performed by collagenase II digestion [1]. Knockdown of ACSL6 was made by siRNA specific transfection. After the knockdown, the cells were collected for the following experiments: mRNA expression (RT-PCR), MS-MS lipid analyzes, cell viability (flow cytometry), oxygen consumption (OROBOROS Oxygraph-2k) [2] and reactive oxygen species (ROS) production (Amplex UltraRed).

ACSL6 siRNA transfection (20 nM) reduced the expression of ACSL6 mRNA by 70±8%. ACSL6 knockdown increased the free fatty acids C16:0 and C18:0 by 32±3% and 35±3%, respectively. siRNA transfection did not affect cell viability measured by propide

iodate. ACSL6 genic silencing increased mitochondrial respiration in all states [pmol $O_2 \cdot s^{-1} \cdot 10^{-6}$ cells]: ROUTINE respiration (297±30 vs 368±28), LEAK with oligomycin (91±5 vs 96±4) and noncoupled ETS (610±45 vs 703±41), and decreased ROS production (P<0.05). ACSL6 genic silencing increased mRNA expression of oxidative genes PGC1α (~50%), UCP2 (~3 fold) and UCP3 (~5 fold), decreased mRNA expression of ACSL3 and had no effect on ACSL1 and β-hydroxyacyl-CoA dehydrogenase (β-HAD).

ACSL6 knockdown increased the availability of free fatty acids, which are major regulators of UCP's. This may reflect the action of signaling pathways which remodel the oxidative program of skeletal muscle cells, increasing mitochondrial respiration. These mechanisms may contribute to control metabolic diseases, such as insulin resistance and obesity.

Supported by FAPESP.

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C3-09 Impairment of bioenergetic parameters induced by acute carnosine administration in skeletal muscle of young rats.

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Serum carnosinase deficiency is an inherited disorder that leads to an accumulation of carnosine in central and peripheral tissues of affected patients [1]. Considering that patients report severe episodes of dystonia and lethargy [2] and that the pathophysiological mechanisms involved in serum carnosinase deficiency remain poorly understood, we investigated the *in vivo* effects of carnosine on bioenergetic parameters, namely respiratory complexes (CI–III, CII, and CII–III) [3], malate dehydrogenase [4], succinate dehydrogenase [3], and creatine kinase [5] activities, and the expression of mitochondrial-specific transcription factors (*NRF-1*, *PGC-1α*, and *TFAM*) in skeletal muscle of young Wistar rats. We observed a significant decrease of CI–III and CII activities in animals receiving carnosine acutely, as compared to the control group. However, no significant alteration in respiratory complexes, citric acid cycle enzymes, and creatine kinase activities were found between rats receiving carnosine chronically and control group animals. Compared to the control group, mRNA levels of *NRF-1*, *PGC-1α* and *TFAM* were unchanged. The present findings indicate that energy dysfunction occurs in skeletal muscle of rats receiving carnosine acutely, which suggests that a putative mechanism might be responsible for the muscle damage observed in serum carnosinase-deficient patients.

Supported by UNESC, CNPq and NENASC.

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C3–10 Declining follicular reserve triggers mitochondrial pathway of granulosa cell apoptosis and accelerates the rate of follicular decay.

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The ovary receives a finite pool of follicles during fetal life. The follicular reserve declines at an exponential rate leading to an accelerated rate of decay during the years preceding menopause [1]. The present investigation examined if diminished follicle reserve, which characterizes ovarian aging, impacts the attrition process.

Premature ovarian aging was induced in rats by intra-embryonic injection (3 µl) of 100 µg of galactosyltransferase-antibody (GalTase-Ab) per embryo on D10 of pregnancy. The size of the follicle pool of post-natal D35 female rats was subsequently modulated by transplantation of either a wedge of fat (sham control) or an ovary from 25-day old control rats under the ovarian bursa. The ovaries were dissected out on post-natal D55. Follicular growth and atresia and ovarian microenvironment were evaluated by real-time RT-PCR analysis of growth differentiation factor-9 (GDF-9), bone morphogenetic protein 15 (BMP15), kit ligand (KL), hepatocyte growth factor (HGF), and keratinocyte growth factor (KGF); biochemical evaluation of ovarian lipid peroxidation, superoxide dismutase (SOD) and catalase activity; analysis of mitochondrial membrane potential by JC1 staining; detection of granulosa cell apoptosis by TUNEL assay, Hoechst staining and annexin V binding; and Western blot analysis of pro- and anti-apoptotic signaling molecules including p53, bax, bcl2, caspase 3 and cytochrome *c*.

The follicle-deficient ovary of the sham-operated group demonstrated highly triggered mitochondrial pathways of granulosa cell apoptosis and accelerated follicular atresia. The follicle-deficient ovary of the ovary-transplanted group, by contrast, exhibited stimulated follicle growth with increased expression of GDF-9, BMP15, KL, HGF, KGF, and bcl2 and downregulated expression of p53, bax, caspase 3 and cytochrome c. Both the host and transplanted ovaries also had significantly lower rates of lipid peroxidation with increased SOD and catalase activity.

The present results suggest that the balance between the pro-survival and pro-apoptotic factors involved in maintaining optimum intra-follicular communication between germ cell and somatic cells [2-4] is, perhaps, under the upstream regulation of an as-yet unidentified ovarian milieu that is maintained by inter-follicular communications. The declining follicular reserve is possibly the immediate thrust that increases the rate of follicle depletion when the follicle reserve wanes below a certain threshold size.

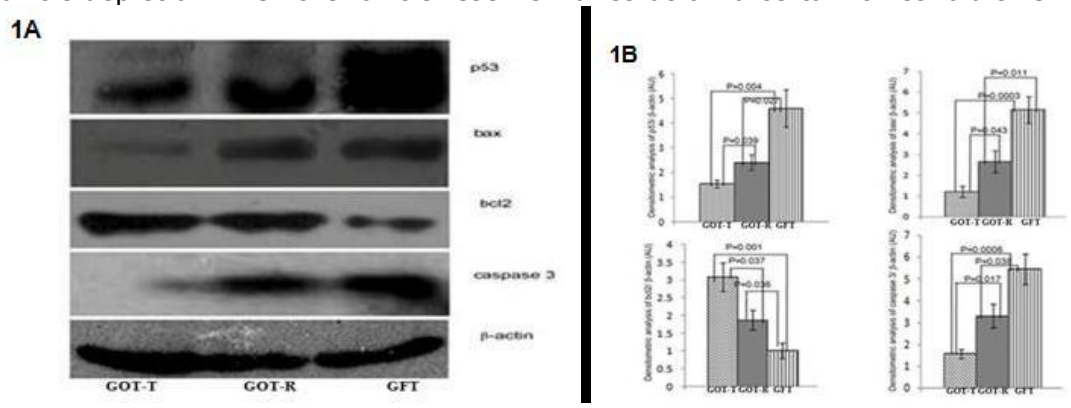

Figure 1. Immunoblot and densitometric analysis of p^{53}, bax, bcl_2 and caspase3. Images of representative immunoblots (A) show decreased expression level of p53, bax and caspase3 and increased expression of bcl2, in both the resident (GOT-R) and transplanted (GOT-T) ovaries of the ovary-transplanted (GOT) group, as compared to the expression of the corresponding factors by the follicle-deficient ovary of the fat-transplanted (GFT) group. The histograms (B) represent the densitometric analyzes with relative intensity of the bands normalized to loading control, β-actin.

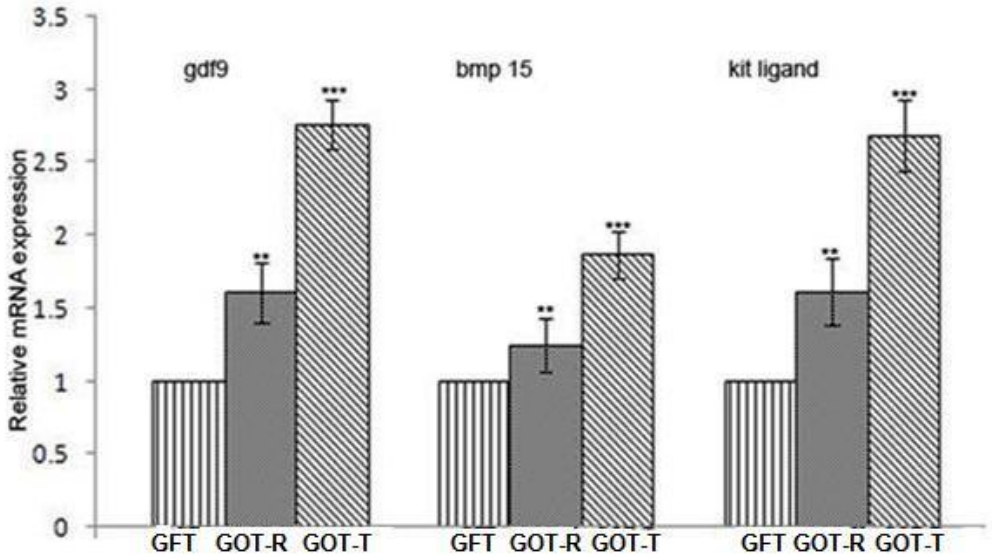

Figure 2. Relative mRNA expression of gdf9, bmp15 and kit ligand as assessed by real-time RT-PCR. The expression levels of gdf9, bmp15 and kit ligand are normalized with internal control, β-actin, and expressed as fold-change with respect to fat-transplanted group (GFT). The expression level of gdf9 is 1.61-fold higher in the resident (GOT-R) and 2.76-fold higher in the transplanted (GOT-T) ovaries, while the expression of bmp15 increased by 1.24-fold in GOT-R and 1.87-fold in GOT-T ovaries. The expression of kit ligand in the GOT-R and GOT-T is 1.62-fold and 2.69-fold higher, respectively. Data are presented as mean ±SEM of five independent determinations, each from individual rats of the corresponding gtoup (***P*<0.05 vs. GFT; ****P*<0.001 vs. GFT).

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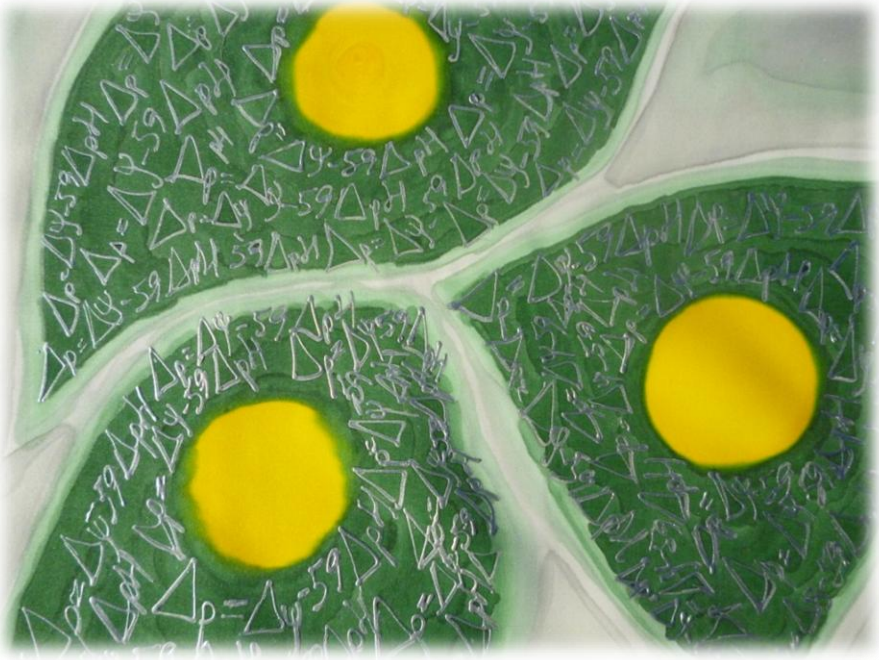

C4 Diagnosis and prevention of mitochondrial dysfunction

C4-01 Cool or heat? Bioenergetic and ROS homeostatic approach to therapeutic hypothermia.

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Acute ischemia-reperfusion injury of the brain affects millions of people. Currently there is no really efficient neuroprotective therapy, however, a simple physical procedure, therapeutic hypothermia, can have beneficial effects. Although there is agreement that in this group of diseases oxidative stress is an important factor, the effects of temperature changes on the reactive oxygen species (ROS) formation and on the ROS elimination have not been clarified yet. A few publications in high profile journals claimed that mitochondrial ROS formation was inversely related to increasing temperature. In the present work, the effects of temperature changes on H_2O_2 formation and elimination were investigated in isolated guinea pig brain mitochondria in association with oxygen consumption.

Mitochondrial ROS production was measured using Amplex UltraRed fluorescence, the rate of H_2O_2 elimination was measured using a hydrogen peroxide-sensitive electrode (WPI). Oxygen consumption of mitochondria was measured using an OROBOROS Oxygraph-2k. In order to energize mitochondria glutamate plus malate, succinate and alpha-glycerophosphate substrates were used. The bioenergetic and ROS parameters of mitochondria were investigated at 33, 37 and 41 °C.

The rate of substrate oxidation showed a strong increase with temperature, whereas the efficiency of oxidation was decreased. Considering the ROS homeostasis both the formation of H_2O_2 and the elimination of H_2O_2 became faster with increasing temperature. With Complex I substrates at resting respiration, H_2O_2 production was increased by 31%, as a consequence of elevating the temperature from 33 °C to 41 °C. Using succinate or alpha-glycerophosphate, results were similar. The biggest difference (59% between 33 °C and 41 °C) was detected when H_2O_2 production was measured in the presence of the Complex I inhibitor rotenone. The rate of H_2O_2 elimination was also elevated by 24% with increased temperature (from 33 °C to 41 °C), in mitochondria supported by glutamate and malate.

Rising the temperature from hypothermic to hyperthermic conditions resulted in an increase in mitochondrial oxygen consumption, H_2O_2 production and H_2O_2 elimination. The increase of ROS production was higher than that of H_2O_2 elimination; thus, according to our results, the elevation of temperature created oxidative stress conditions. We conclude that the neuroprotective effects of therapeutic hypothermia are also based on the decreased rate of mitochondrial H_2O_2 production.

Supported by OTKA (NK 81983), and Hungarian Academy of Sciences MTA TKI 02001, and Hungarian Brain Research Program - Grant No. KTIA_13_NAP-A-III/6.

MiP2013: Laszlo Tretter

C4-02 The hot heart: cardiac mitochondrial energetics during hyperthermia.

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Heart failure is a common cause of death with hyperthermia [1], while the exact cause of hyperthermic heart failure appears elusive. It has been shown that there is an increase in inner-mitochondrial membrane permeability with hyperthermia starting at 42 °C [3]. We hypothesized that this would result in an impaired ATP supply by oxidative phosphorylation (OXPHOS) and hence compromise normal working heart function.

Sprague Dawley rat *ex vivo* working heart function was assessed with a graded increase in perfusion temperature up to 43.5 °C. Mitochondrial high-resolution respirometry coupled to fluorometry was employed to determine the effects of moderate (40 °C) and severe (43 °C) hyperthermia on both ATP production (using magnesium green) and mitochondrial membrane potential ($\Delta\Psi_{mt}$; using safranine) *in vitro,* using a comprehensive metabolic substrate complement with isolated mitochondria [2].

Ex vivo working rat hearts showed breakpoints in all functional parameters (heart rate, cardiac output and ventricular contractility) at ~40.5 °C. Relative to 37 °C and 40 °C, 43 °C elevated LEAK O_2 flux and depressed OXPHOS O_2 flux and $\Delta\Psi_{mt}$. Measurement of steady-state ATP flow from mitochondria revealed decreased ATP synthesis capacity and a negative steady-state ~P/O ratio at 43 °C. This approach offers a more powerful analysis of the effects of temperature on OXPHOS that cannot be measured using simple measures, such as the traditional RCR or ~P/O ratio, which respectively can only approach 1 or 0 with inner-membrane failure.

At 40 °C there was only a slight enhancement of the LEAK O_2 flux and this did not significantly affect ATP production rate. Therefore, during mild hyperthermia (40 °C) there is no enhancement of ATP supply by mitochondria, to accompany increasing cardiac energy demands, while between this and critical hyperthermia (43 °C), mitochondria become net consumers of ATP. This consumption would contribute to cardiac failure or permanent tissue damage during severe hyperthermia.

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C4-03 Function of mitochondrial energy provision apparatus is compromised in patients with chronic heart failure.

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Epidemic increase in the prevalence of heart failure is a common feature in all developed countries. Regardless of the underlying aetiology, defects in myocardial mitochondrial energetics play a central role in its pathogenesis, since the heart function places enormous demands on the range and dynamics of energy provision. Diminished functional capacity of mitochondria can, therefore, lead to heart failure. Our aim was to elucidate prevalence and significance of abnormalities of the mitochondrial energetic apparatus in the myocardium of patients with advanced heart failure, and to describe putative mechanisms leading to such abnormalities.

We analyzed left ventricular myocardial tissue of patients undergoing heart transplantation harvested at the time of the heart explanation (*N*=62) and samples of control myocardial tissue harvested from hearts of organ donors not used for cardiac transplantation (*N*=20). In the heart failure samples, we found profound markers of mitochondrial dysfunction. Content of mitochondria was decreased both when detected as mtDNA content (76% of controls) and citrate synthase activity (74% of controls). Similarly, we observed a decrease in activities of OXPHOS enzymes: NCCR (122±5.7 vs. 161±13, *P*<0.01), SCCR (66±4.7 vs. 111±7.9, *P*<0.001) and cytochrome *c* oxidase (472±18 vs. 767±56, *P*<0.001). Using high-resolution respirometry, we detected a decrease in succinate supported respiration (451±23 vs. 598±47, *P*<0.01). SDS PAGE showed a decrease in content of Complexes CI, CII and CIII and a significant decrease in the endonuclease G, a mitochondrial protein recently associated with heart failure. Concerning aetiology, no differences were observed between patients with ischemic (coronary arterial disease) and non-ischemic heart failure.

Taken together, our data implicate the important role of mitochondrial respiratory capacity in the development of human heart failure.

Supported by the Ministry of Health of the Czech Republic project no. NT14050.

C4-04 Understanding the utility and limitations of autopsy samples for mitochondrial studies - evaluation of oxidative stress in the central nervous system of patients with diagnosed or highly probable mitochondrial diseases.

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Genetic mitochondrial diseases occur as a result of mutations in the nuclear or mitochondrial genome. Such impairment is often associated with dysfunctions in proteins which are part of the respiratory system. First, our aim was to study whether the time elapsed from death until dissection has a significant impact on the detected protein damage and thus affects or distorts the results.

To address this topic, brain samples were obtained from mice. After sacrificing the animals, they were stored under the same conditions as the remains of patients, and dissected in a similar time regime as when obtaining autopsy samples from deceased patients.

Our results demonstrated no significant alterations in protein levels and damage analyzed at successive time points. Our results suggest that autopsy samples can be used for the study of oxidative damage, which can extend and direct further research toward the diagnosis of mitochondrial diseases, which are accompanied by elevated levels of reactive oxygen species.

Supported by the Internal Projects of CMHI 125/2012, Statutory Founding from Nencki Institute of Experimental Biology, Polish Ministry of Science, Higher Education grant W100/HFSC/2011 and a grant from the Polish National Science Centre (UMO-2011/01/M/NZ3/02128).

C4-05 Proximal ubiquinone binding site in mitochondrial respiratory Complex II is a target for vitamin E analog MitoVES, an experimental anti-cancer agent.

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Previously, we showed that mitochondrialy targeted vitamin E analog MitoVES kills cancer cells by inducing apoptosis, especifically in transformed cells. The binding of MitoVES to the proximal ubiquinon binding (UbQp) site of Complex II (CII), accompanied by the

inhibition of electron transfer within CII, and ensuing reactive oxygen species production were suggested as the molecular mechanism underlying apoptosis induction by MitoVES [1], but the direct experimental evidence for MitoVES interaction with the UbQp site was missing.

In the present study we focused on the interaction of MitoVES with this site in more detail. By site-directed mutagenesis, we prepared cells with UbQp site–directed point mutations in the SDHC subunit of CII. Next, we characterized their respiratory status, focusing on CII activity and assembly. Mutations S68L and R72C resulted in nonfunctional CII; although, in R72C, mutant CII was partially assembled. In cells with S68A and I56F mutations, CII-linked respiration was similar to wild type cells under coupled conditions; although, in case of S68A, mutant uncoupling with FCCP did not lead to significant increase in oxygen consumption. This suggests that this mutation compromised CII activity to some degree, but under physiological conditions this might be barely recognizable. In confirmation, mass spectrometry measurements did not reveal any increase in succinate levels for the S68A mutant, in contrast to S68L and R72C mutations, associated with nonfunctional CII.

In cells with functional CII (wild type, I56F and S68A mutants), we determined the inhibitory effect of MitoVES on succinate-driven respiration and found IC_{50} values to be significantly higher for the mutants. To better characterize the mutated UbQp site we also carried out these experiments with two control inhibitors of CII: malonate, which binds to the succinate binding site in SDHA subunit, and TTFA, which binds to the UbQp site. Inhibitory curves and IC_{50} values for malonate showed very little, if any, differences between mutant and wild type CII, but the TTFA effect on CII-linked respiration in S68A mutant was almost one magnitude stronger than in cells with wild type SDHC. This suggests that the effect of mutations is local and UbQp site-specific. Further, all the mutants were assessed for their susceptibility to various apoptosis inducing agents, including MitoVES, TTFA, hydrogen peroxide and staurosporine. MitoVES-induced apoptosis was reduced in cell lines with mutations in SDHC, particularly in those carrying S68A substitution. While there were some differences between the variant cell lines in apoptotic response to control compounds, they did not copy the MitoVES treatment response.

The findings, that for the I56F and S68A mutants the inhibition of CII-linked respiration and the pro-apoptotic activity of MitoVES were found reduced, suggest that the UbQp site of CII is the target of MitoVES, and that the observed diminished pro-apoptotic effect of the drug is not secondary due to the lack of electron flow through CII (as might be the case in cells with nonfunctional CII). Instead, it is directly linked to the reduced interaction of MitoVES with the UbQp site.

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C4-06 Very long chain ceramides interfere with C_{16} ceramide-induced channel formation: rheostatic control of apoptosis.

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Mitochondria mediate both cell survival and cell death. The intrinsic apoptotic pathway is initiated by the permeabilization of the mitochondrial outer membrane to pro-apoptotic intermembrane space proteins, such as cytochrome *c*. A number of pathways are known to cause the egress of mitochondrial intermembrane space (mtIMS) proteins. Of particular interest is the ability of the lipid, ceramide, to self-assemble into dynamic water-filled channels. The formation of ceramide channels is regulated extensively by Bcl-2 family proteins as well as dihydroceramide, the immediate precursor in the *de novo* ceramide biosynthetic pathway. Here, we present evidence that the chain length of

biological ceramides serves as an important regulatory factor. Ceramides are synthesized by a family of six mammalian ceramide synthases (CerS), each of which produces a subset of ceramides that differ in their fatty acyl chain length. Indeed, we find that various ceramides function to permeabilize mitochondria differentially. Interestingly, the presence of very long chain ceramides reduces the potency of C_{16}-mediated permeabilization of mitochondria, indicating that the intercalation of the lipids in the dynamic channel is destabilizing, reminiscent of dihydroceramide inhibition of ceramide channel formation [1]. Moreover, cells overexpressing the ceramide synthase responsible for the production of C_{16}-ceramide (CerS5) are more vulnerable to etoposide, compared to cells overexpressing CerS2 (very long chain fatty acyl ceramides). We also find that co-overexpression of CerS2 and CerS5 reduces the fraction of dead cells upon etoposide treatment, indicating that the product of CerS2 inhibits C_{16}-channel formation *in vivo*. This interplay between different ceramide metabolic enzymes and their products adds a new dimension to the complexity of mitochondrial-mediated apoptosis and emphasizes its importance as a key regulatory step that commits cells to life or death.

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C4–07 Bioenergetics: arts meets gentle science in sickness and in health.

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Recent scientific reports have highlighted the increasing proportion of childhood obesity and mitochondrial diseases, leading to energy failures [1-4]. Equally important is the fact that mitochondrial dysfunction is at the core of numerous diseases that affect energy production. An urgent cell-to-society integrative approach is needed to address this problem, to increase awareness for improving nutrition and bioenergetics of young children and their families, and ultimately improve the quality of life. This wake-up call presents an urgent need to educate the public about the importance of "bioenergetics" in their daily lives. Our goal is to create novel educational modules to teach and engage a wide audience for understanding a variety of disorders related to energy failure.

Faculty and students from Schools of Engineering and Arts along with the Science Museum of Virginia and patient advocates, affiliated with the United Mitochondrial Disease Foundation- Virginia Chapter, are developing creative STEAM-H (Science, Technology, Engineering, Arts, Mathematics, Health) approaches to communicate this important public health concern.

In spring 2015, the outcome of this project will be displayed at an open exhibition at the Science Museum of Virginia. It is high time that such cell-to-society interactive modules are being created for explaining bioenergetics to children and adults.

Supported by the Virginia Commonwealth University Quest for Innovation Fund.

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www.mitophysiology.org/?MiPcalendar

MiP*schools* 2015

7th MiP*school* on Mitochondrial Physiology

20 to 24 Apr 2015

University College
London, UK

Organizer: Michael Duchen (*University College London, UK*), The MiP*society*.
Email: *m.duchen@ucl.ac.uk*

www.bioblast.at/index.php/MiPschool London 2015

8th MiP*summer* School on Mitochondrial Physiology

10 to 14 Aug 2015

East Carolina Diabetes and Obesity Institute
East Carolina University
Greenville, NC, USA

Organizer: P Darrel Neufer (*East Carolina Diabetes Obesity Inst, East Carolina University, Greenville, NC, USA*), The MiP*society*.
Email: *neuferp@ecu.edu*

www.bioblast.at/index.php/MiPschool Greenville 2015

MiP2015

The next world-summit on Mitochondrial Physiology

11th Conference on Mitochondrial Physiology

07 to 11 Sep 2015, Luční Bouda, Czech Republic

Organizers: Petr Pecina, Tomas Mráček, Josef Houštěk (*Inst Physiol, Czech Academy of Sciences, CZ*), The MiP*society*.
Email: *petr.pecina@biomed.cas.cz*

www.bioblast.at/index.php/MiP2015

Location: Luční Bouda (Meadows Chalet; 1410 m - http://www.lucnibouda.cz/en) is located in the Giant Mountains National Park. Luční Bouda is reached from Prague in 2.5 hours. Participants **arriving in Prague on Sunday Sep 6** will enjoy a social programme with a visit of the city centre and a boat trip. Transfer by bus from Prague to the conference venue will be arranged with several departures throughout Monday, adjusted to participants' travel arrangements with arrival before the conference opening in the evening, to enjoy a taste of PAROHÁČ (The STAG) – the locally brewed beer. Bus return transfer to Prague, early morning departure from Luční Bouda.

Number of participants: 120.

Becoming a MiP*member*

Operating as in informal group since MiP2003 in Schroecken, the MiP*society* was formally established at MiP2011 in Bordeaux as an international non-government organization with its legal base in Innsbruck, Austria. The MiP*society* organizes annual summer schools and biannual conferences, bringing together international leading scientists and young researchers in the rapidly expanding field of Mitochondrial Physiology.

MiP2013: Verena Laner, Erich Gnaiger, Vilma Borutaite, Steven Hand, Petr Pecina

MiP*members* benefit from

1. Information on MiP*events*
2. MiP*circulars*
3. Reduction of registration fees at MiP*events*

MiP in the **Mitochondrial Global Network: MitoGlobal** aims at providing a WorldWide information platform for scientific mitochondrial organizations and mitochondrial research consortia.

Two options for MiP*membership*

- **MiP*member*** bi-annual fee of EUR 70.- (student fee EUR 30.-)
- **MiP*lifetime-member*** EUR 350.-

Application

The application form is available for download at www.mitophysiology.org. Submit your application to join as a MiP*member*. Your application will be confirmed by the Executive MiP*committee*.

Honorary Gentle Science Member of the MiP*society*

Nobel laureate Professor Sir John Walker (Cambridge MBU, UK; Nobel prize 1997 in chemistry) has joined the MiP*society* as the first '**Honorary Gentle Science Member**' of the Mitochondrial Physiology Society, following his presentation of 'The ATP Synthase' at the MiP*summer* School 2012 on July 10 in Trinity Hall, Cambridge, UK.

Contact

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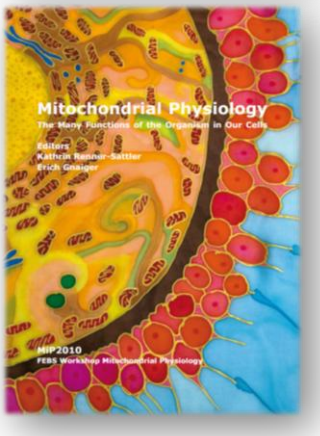

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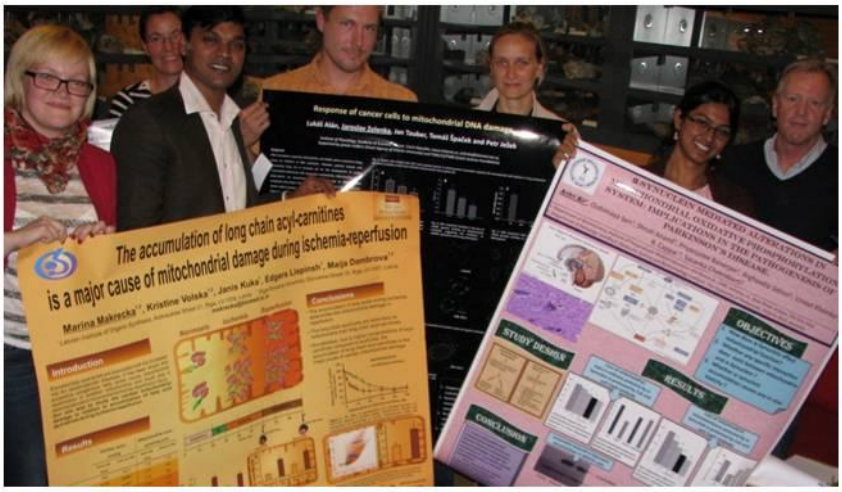

MiP2013 Poster prizes: Marina Makrecka-Kuka, Katrin Lindenberg, Vijay Sonkar, Jaroslav Zelenka, Lydie Plecita-Hlavata, Aritri Bir, R Boushel