GIBB
Gruppo Italiano di Biomembrane e Bioenergetica

Annual Meeting of the Italian Group of Biomembranes and Bioenergetics (GIBB)

Udine, June 18-20, 2015
Scuola Superiore dell'Università di Udine
Palazzo Garzolini - di Toppo Wassermann
Via Gemona, 92

http://www.gibb-be.org

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PROGRAMME

Thursday 18th June

14:00-15:00	**Registration**
15:00-15:15	**Welcoming address**
15:15-15:30	**Opening** Giancarlo Solaini (GIBB President), Giovanna Lippe
15:30-16:30	**Opening Lecture** MITOCHONDRIA AND CALCIUM SIGNALING IN ENERGY HOMEOSTASIS AND CELL DEATH Michael Duchen (University College London, U.K.)
16:30-18:45	***CALCIUM SIGNALING & MITOCHONDRIA STRUCTURE*** ***Chairpersons: Giancarlo Solaini – Alessandro Giuffrè***
16:30-16:45	Ca^{2+} BINDING TO THE CATALYTIC Me^{2+} BINDING SITE OF F-ATP SYNTHASE PROMOTES FORMATION OF THE PERMEABILITY TRANSITION PORE Valentina Giorgio, Victoria Burchell, Marco Schiavone, Michael Forte, Valeria Petronilli, Giovanna Lippe and Paolo Bernardi
16:45-17:00	MODULATION OF F-ATP SYNTHASE/PTP BY pH Manuela Antoniel, Barbara Spolaore,Valentina Giorgio, Federico Fogolari,Valeria Petronilli, Paolo Bernardi and Giovanna Lippe
17:00-17:15	MODULATION OF THE F_1F_O-ATPASE AND MITOCHONDRIAL PERMEABILITY TRANSITION PORE ACTIVITY BY THE MITOCHONDRIAL PROTEASE HTRA2 Victoria S. Burchell, Hélène Plun-Favreau and Paolo Bernardi
17:15-17:30	DISCOVERY AND OPTIMIZATION OF NOVEL INHIBITORS OF THE MITOCHONDRIAL PERMEABILITY TRANSITION PORE Justina Šileikytė, Paolo Bernardi and Michael Forte
17:30-17:45	HUMAN ND1 SUBUNIT IS ESSENTIAL FOR COMPLEX III AND SUPERCOMPLEXES STABILITY Luisa Iommarini, Maria Antonietta Calvaruso, Concetta Valentina Tropeano, Anna Ghelli, Ivana Kurelac, Giulia Leone, Renaud Vatrinet, Sara Vidoni, Massimo Zeviani, Anne Lombes, Michela Rugolo, Giuseppe Gasparre and Anna Maria Porcelli
17:45-18:00	OPA1 ISOFORMS AND PROTEIN DOMAINS IN THE MITOCHONDRIAL FUNCTIONS Valentina Del Dotto, Prashant Mishra, Sara Vidoni, Mario Fogazza, Guy Lenaers, Valerio Carelli, David Chan, Michela Rugolo and Claudia Zanna
18:00-18:15	MITOCHONDRIAL TRANSLOCATION OF APE1 RELIES ON THE MIA PATHWAY Arianna Barchiesi, Michal Wasilewski, Agnieszka Chacinska, Gianluca Tell, and Carlo Vascotto
18:15-18:45	*CAREER DEVELOPMENT BY BRIDGING RESEARCH* WITH *SOCIETY: THE EXPERIENCE* AND THE RESULTS OF *TRANS2CARE* PROJECT. Sabina Passamonti
19:00	***Get together party***

Friday 19th June

8:30- 9:30	**Plenary lecture** MITOCHONDRIA AND HYDROGEN SULFIDE Frederich Bouillaud (University of Paris Descartes, France)
9:30-10:45	***MITOCHONDRIA DYSFUNCTION I*** **Chairpersons: Luigi Palmieri - Michela Rugolo**

9:30-9:45 THE MITOCHONDRIAL CARNITINE/ACYLCARNITINE CARRIER IS REGULATED BY HYDROGEN SULFIDE VIA INTERACTION WITH C136 AND C155
Lara Console, Nicola Giangregorio, Annamaria Tonazzi, Imma Lorusso, Annalisa De Palma, Cesare Indiveri

9:45-10:00 PEROXYNITRITE DECOMPOSITION BY *E. coli* CYTOCHROME BD OXIDASE
Elena Forte, Vitaliy B. Borisov, Micol Falabella, Sergey A. Siletsky, Marzia Arese, Albert Davletshin, Daniela Mastronicola, Paolo Sarti and Alessandro Giuffrè

10:00-10:15 NITRIC OXIDE: A ROLE IN LEBER'S HEREDITARY OPTIC NEUROPATHY
Micol Falabella, Elena Forte, Kristina Radić, Maria Chiara Magnifico, Marzia Arese, Alessandro Giuffrè, Marco Salvetti and Paolo Sarti

10:15-10:30 ACTIVATION OF COMPLEX II BY HYDROGEN PEROXIDE IN COMPLEX III DEFICIENT CELLS
Concetta Valentina Tropeano, Leonardo Caporali, Valerio Carelli, Michela Rugolo and Anna Maria Ghelli

10:30-10:45 DO REACTIVE OXYGEN SPECIES INCREASE OR DECREASE IN HUMAN FIBROBLASTS ADAPTED TO HYPOXIA?
Giulia Gorini, Gianluca Sgarbi, Simona Barbato, Alessandra Baracca and Giancarlo Solaini

10:45-11:15 ***Coffee break***

11:15-13:00 ***MITOCHONDRIA DYSFUNCTION II***
Chairpersons: Marcello Pinti – Anna Maria Tonazzi

11:15-11:30 IMPAIRMENT OF MUSCLE OXIDATIVE FUNCTION IN VIVO FOLLOWING MUSCLE DISUSE: WHAT IS THE ROLE OF MITOCHONDRIA?
Desy Salvadego, Michail E. Keramidas, Jörn Rittweger, Irene Mavelli, Ola Eiken, Igor B. Mekjavic and Bruno Grassi

11:30-11:45 SUCCESSFUL TREATMENT WITH INHIBITORS OF THE MITOCHONDRIAL PERMEABILITY TRANSITION PORE IN A ZEBRAFISH MODEL OF COLLAGEN VI MUSCULAR DYSTROPHY
Marco Schiavone, Justina Šileikytė, Alessandra Zulian, Erika Rizzo, Francesca Tagliavini, Luciano Merlini, Patrizia Sabatelli, Sudeshna Roy, Frank Schoenen, Michael Forte, Francesco Argenton and Paolo Bernardi

11:45-12:00 TARGETING MITOCHONDRIAL METABOLISM TO MODULATE THE MACROPHAGE IMMUNE RESPONSE: A PRELIMINARY STUDY WITH PARA-HYDROXY-PHENYLPYRUVATE
Rosella Scrima, Marta Menga, Simona Siliberti, Alessandra De Gregorio, Giuliana Villani, Antonella Cotoia, Julia V. Gefter, Gilda Cinnella and Nazzareno Capitanio

12:00-12:15 ATPase-INHIBITORY FACTOR 1 AND OXPHOS COMPLEXES IN HUMAN GLIOMAS
Alessia Buso, Marina Comelli, Veronica Candotti, Daniela Cesselli and Irene Mavelli

12:15-12:30 INHIBITION OF LON PROTEASE BY TRITERPENOIDS CDDO AND CDDO-ME ALTERS MITOCHONDRIA AND CAUSES CELL DEATH IN RKO HUMAN COLON CANCER CELLS
Regina Bartolomeo, Lara Gibellini, Simone Pecorini, Elena Bianchini, Sara De Biasi, Antonella Cormio, Andrea Cossarizza and Marcello Pinti

12:30-12:45 TARGETING MITOCHONDRIAL COMPLEX I TO INDUCE PSEUDONORMOXIA: AN ADJUVANT ANTI-CANCER STRATEGY
Ivana Kurelac, L. Iommarini, R. Vatrinet, L. Amato, C. Calabrese, L. Giulia, G. Girolimetti, M. Vidone, M. De Luise, C. Bergamini, F. Marzano,MF. Caratozzolo, M. Columbaro, R. Fato, M. Rugolo, A. Tullo, G. Gasparre and A.M. Porcelli

12:45-13:00 CALPAINS, CASPASES, HEAT SHOCK PROTEINS AND TENDERNESS OF BEEF
Elena Saccà, Mirco Corazzin, Nicoletta Pizzutti, Giovanna Lippe and Edi Piasentier

13:00-14:30 ***Lunch***

14:30-15:30 ***Plenary lecture***
GLUTAMINE MEMBRANE TRANSPORT: FROM ENERGY SUPPLY TO NUTRIENT SENSING
Cesare Indiveri (University of Calabria)

15:30-16:30 ***Membrane transporters: structure & function I***
Chairpersons: Mario Zoratti – Francesco Francia

15:30-15:45 STRATEGIES FOR BACTERIAL EXPRESSION AND LARGE SCALE PURIFICATION OF HUMAN AMINO ACID TRANSPORTERS
Michele Galluccio, Lorena Pochini, Mariafrancesca Scalise, Manuele Rebsamen, Giulio Superti Furga and Cesare Indiveri

15:45-16:00 SLC7A5 (LAT1) PLAYS THE ROLE OF CATALYZING AMINO ACID TRANSPORT IN THE LAT1/CD98 HETERODIMER.
Lara Napolitano, Mariafrancesca Scalise, Michele Galluccio, Lorena Pochini, Leticia Albanese and Cesare Indiveri

16:00-16:15 IDENTIFICATION OF NEW HIGHLY SELECTIVE INHIBITORS OF THE HUMAN ADP/ATP CARRIERS.
Giandomenico Redavid, Angelo Onofrio, Simona Todisco, Maria Antonietta DiNoia, Giovanni Parisi, Giuseppe Punzi, Anna De Grassin and Ciro Leonardo Pierri

16:15-16:30 ALS-LINKED MUTANT SOD1 G93A INTERACTING WITH VDAC1 CHANNEL AFFECTS ITS VOLTAGE-GATING ACTIVITY
Ramona Belfiore, Andrea Magrì, Simona Reina, Vito De Pinto and Angela Messina

16:30-17:00 ***Coffee break***

17:00-18:00 ***Membrane transporters: structure & function II***
Chairpersons: Nazzareno Capitanio – Giovanna Lippe

17:00-17:15 TRANSFORMATION WITH hSOD1 OF *S. cerevisiae* DEVOID OF ENDOGENOUS VDAC1 IMPROVES THE DEFECTIVE PHENOTIPE AND RE-ESTABLISH THE MITOCHONDRIAL METABOLISM
Maria Carmela Di Rosa, Francesca Guarino, Andrea Magrì, Simona Reina, Flora Tomasello, Angela Messina and Vito De Pinto

17:15-17:30 ALTERNATIVE 5'-UTR SEQUENCES OF VDAC GENE IN *Drosophila melanogaster* REGULATE THE TRANSLATION OF DOWNWARD CODING SEQUENCE
Loredana Leggio, Massimo Tommasino, Vito De Pinto and Angela Messina

17:30-17:45 IN VIVO ASSAY TO MONITOR FLAVONOID UPTAKE ACROSS PLANT CELL MEMBRANES
Antonio Filippi, Elisa Petrussa, Carlo Peresson, Alberto Bertolini, Angelo Vianello and Enrico Braidot

17:45-18:00 MITOCHONDRIAL TRANSPORT OF ADMA BY SLC25A2
Vito Porcelli, Antonella Longo, Luigi Palmieri, Ellen Closs and Ferdinando Palmieri

18:00-19:00 ***GIBB member assembly***

20:30 ***Social dinner (Ristorante "Concordia", p.zza I maggio)***

Saturday 20th June

9:00- 10:00 **Plenary lecture**
DISSECTING MITOCHONDRIAL ENERGY PHYSIOLOGY AND REGULATION BY IN VIVO IMAGING IN PLANTS
Markus Schwarzländer (University of Bonn, Germany)

10:00-10:30 ***BIOENERGETIC IN PHOTOSYNTHETIC ORGANISMS I***
Chairpersons: Vito De Pinto – Marco Zancani

10:00-10:15 ATP SYNTHASE DIMERS FROM PEA STEM MITOCHONDRIA FORM THE PTP
Valentina De Col, Carlo Peresson, Elisa Petrussa, Valentino Casolo, Sonia Patui, Alberto Bertolini, Valentina Giorgio, Vanessa Checchetto, Enrico Braidot, Giovanna Lippe, Ildiko Szabò, Angelo Vanello, Paolo Bernardi and Marco Zancani

10:15-10:30 ALTERNATIVE SPLICING-MEDIATED TARGETING OF THE ARABIDOPSIS GLUTAMATE RECEPTOR 3.5 TO MITOCHONDRIA AFFECTS ORGANELLE MORPHOLOGY
Enrico Teardo, Luca Carraretto, Sara De Bortoli, Alex Costa, Smrutisanjita Behera, Richard Wagner, Fiorella Lo Schiavo, Elide Formentin and Ildiko Szabò

10:30-11:00 ***Coffee break***

11:00-12:00 ***BIOENERGETIC IN PHOTOSYNTHETIC ORGANISMS II***

11:00-11:15	MUTATION OF CYTOCHROME B H291 IMPAIRS UBIQUINOL OXIDATION AT THE QO SITE OF THE BACTERIAL CYTOCHOME BC1 COMPLEX Marco Malferrari, Fevzi Daldal, Giovanni Venturoli and Francesco Francia
11:15-11:30	UNCOVERING THE REDOX REGULATION AND THE STRUCTURAL FEATURES OF TRANSKETOLASE FROM C*hlamydomonas reinhardtii* Miriam Pasquini, Chiara Sciabolini, Simona Fermani, Laure Michelet, Stéphane D. Lemaire, Francesco Francia and Mirko Zaffagnini
11:30-11:45	ZmDUR3, CHARACTERIZATION OF A HIGH AFFINITY UREA TRANSPORTER IN MAIZE ROOTS Laura Zanin, Nicola Tomasi, Corina Wirdnam, Stefan Meier, Doris Rentsch and Roberto Pinton
11:45-12:00	CHARACTERIZATION OF A GENISTEIN TRANSPORTER IN CLUSTER ROOTS OF WHITE LUPIN Silvia Venuti, Laura Zanin, Enrico Martinoia, Roberto Pinton and Nicola Tomasi

12:15-13:00 ***Awards and closing remarks***

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ABSTRACT BOOK

MITOCHONDRIA AND CALCIUM SIGNALING IN ENERGY HOMEOSTASIS AND CELL DEATH

Michael R Duchen

Department of Cell and Developmental Biology and Mitochondrial Biology Group, University College London, Gower St., London WC1E 6BT.

Calcium signaling is fundamental to cell physiology, usually driving an increase in work, as it initiates core physiological processes including fertilization, contraction, migration, secretion etc. A rise in intracellular Ca^{2+} concentration acts through multiple convergent pathways that couple the associated increased energy demand to increased ATP synthesis, matching energy supply to demand. Thus, increased cytosolic Ca^{2+} increases metabolite transfer across the inner mitochondrial membrane through activation of Ca^{2+}-regulated mitochondrial carriers, while increased matrix Ca^{2+} concentration stimulates the citric acid cycle and the ATP synthase. Calcium signals increase mitochondrial biogenesis, at least in muscle, while calcium signals may also control mitochondrial trafficking positioning mitochondria at points of high energy demand.

The field has been transformed by the recent identification of the molecular substrates for mitochondrial calcium handling. Although the specific physiological importance of the mitochondrial calcium uptake (MCU) complex in work-induced stimulation of respiration is not yet clarified, abnormal mitochondrial calcium signaling causes pathology. Loss of function mutations in MICU1, a regulator of the MCU, are associated with neuromuscular disease in children, causing learning difficulties, a progressive extrapyramidal motor disorder and muscle weakness. In MICU1 deficient fibroblasts from the patients, resting mitochondrial matrix [Ca^{2+}] is increased and mitochondria fragmented. Thus, the fine tuning of mitochondrial Ca^{2+} signals is critical in shaping energy homeostasis. Pathological, excessive calcium signals coincident with oxidative or nitrosative stress cause cell death through opening of the permeability transition pore (PTP), best characterised as a cause of reperfusion injury following cardiac ischaemia. Recent studies point to reverse electron flow from complex II, driven by the specific accumulation of succinate during ischaemia, as the source of oxidative stress at reperfusion. The coincidence of the resultant oxidative stress and mitochondrial calcium uptake at reperfusion opens the ptp. Understanding these pathways is may pave the way to development of novel therapeutic strategies to protect tissues from reperfusion injury.

MITOCHONDRIA AND HYDROGEN SULFIDE

F. Bouillaud[1,2,3]

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Hydrogen sulfide (H_2S) is highly toxic to mitochondria because it is a complex IV (cytochrome oxidase) inhibitor as potent as cyanide, so are CO and NO. These three gases (NO, CO and H_2S) are known as gasotransmitters in mammals. Two different sources of hydrogen sulfide exist in the mammalian organism: enzymatic activities inside mammalian cells and the microbiota present in the gut. This second source generates levels of hydrogen sulfide able to poison the surrounding eukaryotic cells. Hydrogen sulfide rich environments challenge their eukaryotic inhabitants, mostly invertebrates, which use a sulfide quinone reductase to oxidize sulfide into the less toxic thiosulfate. In 2007 it became clear that human colonic wall uses similar mechanisms to protect itself and the rest of the organism against the microbiota born hydrogen sulfide. Later on it was observed that H_2S is considered by most mitochondria as a substrate. SQR takes priority over others electron donors to quinone with the aim to prevent self-poisoning by the endogenous rates of H_2S release. Maximal activity of SQR may be high enough to saturate mitochondrial respiratory chain with electrons originating from sulfide. Moreover in colonocytes reversion of mitochondrial complex I may further increase the sulfide detoxication rate. A notable exception is the central nervous system that appears to be devoid of SQR. The presence of the SQR is therefore likely to interfere with the signaling role of sulfide, which is considered with an increasing interest because of its possible relevance to cardiovascular diseases and inflammation.

DISSECTING MITOCHONDRIAL ENERGY PHYSIOLOGY AND REGULATION BY IN VIVO IMAGING IN PLANTS

Markus Schwarzländer

Plant Energy Biology Lab, INRES – Chemical Signalling, University of Bonn, Friedrich-Ebert-Allee 144, 53173 Bonn, Germany

Mitochondria are the major cellular source of ATP in most complex life. Respiratory ATP provision needs to match cellular needs requiring tight control. In plants, which often experience dramatic changes in their environment and where photosynthesis strictly depends on mitochondrial function, respiration requires particularly sophisticated regulation. However, the key mechanisms by which this control is achieved are poorly understood. We use genetically encoded fluorescent protein sensors, such as redox sensitive GFPs for glutathione redox potential and cameleon-type probes for free calcium levels, in combination with confocal micro-imaging and high-throughput fluorimetry to monitor the dynamics of energy physiology in mitochondria *in organello* and *in planta*. By developing and applying dedicated sensing strategies for a growing set of parameters, also including membrane potential, pH, H_2O_2 and ATP levels, over the recent years, we have made several fundamental insights into how mitochondrial physiology works in the context of the living plant cell. The dynamics of mitochondrial physiology over time has offered several surprises in particular. This lecture will highlight key insights from our studies on energy dynamics in single mitochondria, the control of the thiol redox machinery and mitochondrial calcium regulation, in which we combine imaging with genetic, biochemical and cell biological approaches. The significance of mitochondrial physiology in orchestrating respiration with plant growth performance and stress responses will be discussed.

GLUTAMINE MEMBRANE TRANSPORT: FROM ENERGY SUPPLY TO NUTRIENT SENSING

Cesare Indiveri

Department DiBEST (Biologia, Ecologia, Scienze della Terra) Unit of Biochemistry and Molecular Biotechnology, via Bucci 4C, University of Calabria, 87036 Arcavacata di Rende, Italy

Glutamine is essential for body's homeostasis. It is involved in many biosynthetic, regulatory and energy production processes as well as in nutrient sensing. Several membrane transporters are needed to manage glutamine traffic in human body by coordinating absorption, reabsorption, delivery to tissues and sub-cellular compartments. Transporters involved in glutamine homeostasis belong to different protein families (SLCs), are redundant and ubiquitous. Function of glutamine transporters is studied in cells and, more recently, in proteoliposomes harboring the proteins extracted from animal tissues or over-expressed in heterologous systems. Most glutamine transporters share specificity also for other amino acids. Na^+-dependent co-transporters efficiently accumulate glutamine; while antiporters regulate the intra-cellular pools of glutamine and other amino acids. The most acknowledged glutamine transporters belong to the SLC1, 6, 7 and 38 families. The members involved in the homeostasis are the co-transporters SLC6A19 (formerly called B0AT1) and the SLC38A- (SNAT-) 1, 2, 3, 5 and 7; the antiporters are SLC1A5 (ASCT2), SLC7A5 and 6 (LAT1 and 2). We described B0AT1 as a very efficient Na^+ dependent transporter, which provides cells with glutamine and neutral amino acid for energy purposes. More recently we elucidated function, regulation and trafficking properties of ASCT2 which is over-expressed in cancers where it plays a crucial role in energy metabolism (1). Glutamine is also involved in nutrient sensing that is a fundamental process for controlling cell growth and proliferation. Activation of mTORC1 at the surface of lysosomes needs to sense amino acid availability. However, the molecular entity of sensor was still elusive. We have identified, by proteomic analysis, the orphan member 9 of the SNAT family (SLC38A9) as the amino acid-sensing component of the machinery controlling mTOR activation. The protein, over-expressed in *E. coli* has been inserted in proteoliposomes with the same orientation of the lysosomal membrane. SLC38A9 is a low capacity transporter mediating slow glutamine and arginine efflux (2). The dealt with transporters are very interesting druggable targets, thus the pointed out proteoliposome models constitute suitable tools for drug screening and discovery.

1. Console L, Scalise M, Tarmakova Z, Coe IR, Indiveri C. Biochim Biophys Acta. 2015 Jul;1853(7):1636-45.
2. Rebsamen M, Pochini L, Stasyk T, de Araújo ME, Galluccio M, Kandasamy RK, Snijder B, Fauster A, Rudashevskaya EL, Bruckner M, Scorzoni S, Filipek PA, Huber KV, Bigenzahn JW, Heinz LX, Kraft C, Bennett KL, Indiveri C, Huber LA, Superti-Furga G. Nature. 2015 Mar 26;519(7544):477-81.

Ca^{2+} BINDING TO THE CATALYTIC Me^{2+} BINDING SITE OF F-ATP SYNTHASE PROMOTES FORMATION OF THE PERMEABILITY TRANSITION PORE

Valentina Giorgio[a]*, Victoria Burchell[a]*, Marco Schiavone[a], Michael Forte[b], Valeria Petronilli[a], Giovanna Lippe[c] and Paolo Bernardi[a]

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A crucial mitochondrial effector of cell death is the permeability transition pore (PTP), an inner membrane channel whose molecular composition has long remained a mystery (1). Our group has recently demonstrated that the PTP forms from dimers of the F-ATP synthase (2). Under stress conditions F-ATP synthase forms Ca^{2+}-dependent channels in a transition that turns the energy-conserving enzyme of life into the death-inducing PTP. The mechanism through which Ca^{2+} promotes PTP formation from the F-ATP synthase remains unknown. A Thr159Ser mutation of the β subunit of *R. rubrum* abolishes Ca-ATP but not Mg-ATP hydrolysis (3). We tested the hypothesis that Ca^{2+} binding to the β subunit is involved in PTP formation. We found that in HeLa cells the Thr213Ser mutation (which corresponds to Thr159 of *R. rubrum*) (i) abolishes Ca-ATP but not Mg-ATP hydrolysis; (ii) does not affect oligomycin-sensitive respiration in intact cells; (iii) decreases PTP sensitivity to Ca^{2+}; and (iv) protects cells from death induced by arachidonic acid or ionomycin. Expression of the human Thr213Ser β subunit in 48 hour-post-fertilization zebrafish embryos decreased apoptosis *in vivo*. Taken together these results suggest that the transition of F-ATP synthase dimer to the PTP occurs after interaction of the Ca^{2+} with the F-ATP synthase metal binding site of the catalytic β subunit.

1. Bernardi P. The mitochondrial permeability transition pore: A mystery solved? Front Physiol 2013; 4:95.
2. Giorgio V, von Stockum S, Antoniel M, Fabbro A, Fogolari F, Forte M, Glick GD, Petronilli V, Zoratti M, Szabó I, Lippe G and Bernardi P. Dimers of mitochondrial ATP synthase form the permeability transition pore, Proc Natl Acad Sci USA 2013; 110:5887-92.
3. Nathanson L, Gromet-Elhanan Z. Mutations in the β-Subunit Thr_{159} and Glu_{184} of the *Rhodospirillum rubrum* F_0F_1 ATP Synthase Reveal Differences in Ligands for the Coupled Mg^{2+}- and Decoupled Ca^{2+}-dependent F_0F_1 Activities, J Biol Chem 2000; 275(2):901 –905.

MODULATION OF F-ATP SYNTHASE/PTP BY pH

Manuela Antoniel[1,3], Barbara Spolaore[4],Valentina Giorgio[1,2], Federico Fogolari[4],Valeria Petronilli[1,2], Paolo Bernardi[1,2], Giovanna Lippe[3]

[1] Department of Biomedical Sciences and [2]Consiglio Nazionale delle Ricerche Institute of Neuroscience, University [r] Padova, Padova, Italy; [3]Department of Food Science, University of Udine, Udine, Italy;[4]CRIBI Biotechnology entre, University of Padova, Padova, Italy; [5]Department of Biomedical Sciences, University of Udine, Udine, Italy

Recent findings of our group demonstrated that: (i) ATP synthase dimers from mammals, yeast and *Drosophila melanogaster* generate Ca^{2+}-dependent currents indistinguishable from those of the permeability transition pore (PTP), an inner membrane channel whose opening can precipitate cell death; (ii) in mammalian mitochondria the PTP inducer cyclophilin D (CyPD) binds to the F_O OSCP subunit, while the CyPD inhibitor CsA displaces CyPD from OSCP. While these findings leave little doubt that the PTP forms from the F-ATP synthase dimers under conditions of high Ca^{2+} and oxidative stress, it remains unclear how many modulators of the PTP may act on the ATP synthase (1).

The most potent physiological PTP inhibitors are protons, indeed, the pore is blocked at acidic matrix pH that also promotes CyPD release from the inner membrane.Both PTP inhibition and CyPD release occur through reversible protonation of still unknown His residues that can be blocked by diethylpyrocarbonate (DPC)(2). Aim of this study is to identify His residues responsible for H+ modulation of the PTP function.

The low PTP open probability at acidic pH may suggest the involvement of the inhibitor protein IF_1, whose binding to ATP synthase is strongly favored by low pH, resulting in full inhibition of the enzyme. Furthermore, bovine IF_1 contains His residues responsible for the pH dependence of its binding. However, the conditions of high Ca^{2+} favouring the PTP opening seem to prevent the inhibitory binding of IF_1 to ATP synthase, making unlikely a role of IF_1 His residues in PTP inhibition by low pH.

On the other hand, the unique conserved His of OSCP (His112, bovine numbering) appears a candidate modulator of the CyPD-ATP synthase interaction by its pronation at low pH. By immune-precipitation of ATP synthase from bovine heart mitochondria we found that acidic pH induces release of CyPD from ATP synthase and that pre-treatment of mitochondria with DPC prevents such release. Consistently, when OSCP subunit was separated from mitochondria treated with DPC and analysed by ESI-MS/MS, a mass shift of +72 Da of the OSCP 95-113 peptide was determined, which is consistent with DPC-dependent carbethoxylation of the His112. Experiments are in progress to address the role of OSCPHis112 in the CyPD-ATP synthase interaction by site-directed mutagenesis.

1. Bernardi P, Di Lisa F, Fogolari F, Lippe G. From ATP to PTP and Back: A Dual Function for the Mitochondrial ATP Synthase, Circ Res. 2015; 116(11):1850-1862.
2. Nicolli A, Basso E, Petronilli V, Wenger RM, Bernardi P. Interactions of cyclophilin with the mitochondrial inner membrane and regulation of the permeability transition pore, and cyclosporin A-sensitive channel, J Biol Chem. 1996;271(4):2185-92.

MODULATION OF THE F_1F_O-ATPASE AND MITOCHONDRIAL PERMEABILITY TRANSITION PORE ACTIVITY BY THE MITOCHONDRIAL PROTEASE HTRA2

Victoria S Burchell[1], Hélène Plun-Favreau[2], Paolo Bernardi[1]

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HtrA2 is a mitochondrial protease with a dual function: upon its release from the intermembrane space HtrA2 is pro-apoptotic, but under basal conditions it carries out a crucial neuroprotective function revealed by mutation or targeted deletion of the gene in mice. To better understand this neuroprotective function, we have previously investigated the mitochondrial phenotype of HtrA2 knockout (KO) mouse neurons (1), revealing an unexpected effect of HtrA2 on the ATP synthase. We are now further investigating the effect of HtrA2 on ATP synthase function and, following the recent finding that dimers of the ATP synthase form the mitochondrial permeability transition pore (PTP; 2), its effect on the PTP. In mitochondria isolated from HtrA2 KO mouse livers, we observe a shift in the dose-dependence of PTP inhibition by ADP suggestive of an increase in affinity of the PTP for adenine nucleotides. Strikingly, this difference is normalised by inhibition of the ATP synthase with oligomycin, indicating that the catalytic activity of the enzyme is critical for this difference. We further observe a dramatic reduction in the stimulation of respiration by ADP in HtrA2 KO mitochondria compared to wild-type (WT) and a reduction in Pi/O ratio, indicating that the catalytic activity of the ATP synthase is compromised in HtrA2 KO mitochondria. These data indicate that the ATP synthase is functionally altered in HtrA2 KO mice and this alteration corresponds to an effect on the PTP. Further work will now focus on understanding how the difference in the catalytic activity of the ATP synthase relates to the effect on the PTP, and how the deletion of HtrA2 affects both.

1. Plun-Favreau H *et al*. HtrA2 deficiency causes mitochondrial uncoupling through the F1F0-ATP synthase and consequent ATP depletion. *Cell Death and Disease* 2012;3:e335
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DISCOVERY AND OPTIMIZATION OF NOVEL INHIBITORS OF THE MITOCHONDRIAL PERMEABILITY TRANSITION PORE

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The mitochondrial permeability transition pore (PTP) is a Ca^{2+}-requiring mega-channel that under pathological conditions leads to deregulated release of Ca^{2+} and mitochondrial dysfunction, ultimately resulting in cell death. Although the PTP is a potential therapeutic target in diseases such as multiple sclerosis, amyotrophic lateral sclerosis, Alzheimer's disease, muscular dystrophies, myocardial infarction, diabetes, its potential as a drug target is currently unrealized. Thus using resources within the NIH Molecular Libraries Probe Production Centers Network (MLPCN) we screened a ~360,000-compound chemical library in order to identify small molecules that serve as inhibitors of the PTP. Here we report the screening strategy, identification of the isoxazole screening hit **1** (which inhibits mitochondrial swelling with $EC_{50} < 0.39$ μM) and chemical optimization of the molecule which yielded a number of potent compounds. The isoxazole probes inhibit mitochondrial swelling at nanomolar concentration, increase calcium retention capacity up to 16-fold and neither affect the inherent function of F-ATP synthase nor impact cell viability at effective concentrations. Compared to the prior art, the new compounds are the best-in-class inhibitors of the PTP and is a promising basis for the development of the therapeutic agents for some of the most wide-spread human diseases.

HUMAN ND1 SUBUNIT IS ESSENTIAL FOR COMPLEX III AND SUPERCOMPLEXES STABILITY

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Mammalian CI is composed of 44 subunits, 7 of which are encoded by mitochondrial DNA (mtDNA) and constitute the hydrophobic membrane arm (ND subunits). The remaining 37 subunits are encoded by the nuclear genome (nDNA) and assembled within both the hydrophilic and hydrophobic arms, to form an L-shape structure [1]. A large fraction of mammalian respiratory complex I (CI) is organized together with complex III (CIII) and complex IV (CIV) in the respirosome ($CI+CIII_2+CIV_n$), whereas isolated CIII and CIV may dynamically interact to form the $CIII_2$+CIV supercomplex [2]. Among the CI subunits encoded by mitochondrial DNA, ND1 is an evolutionary conserved core subunit, whose role in mammalian CI biogenesis and dynamic organization of supercomplexes has been only inferred so far.

Taking advantage from cell models with unique mitochondrial genetic features developed in our laboratory, we demonstrate that the lack of human ND1 induces a stall in the multi-step process of CI assembly. Hence, human ND1 is essential for CI stability, and therefore permitted respirosome formation. Moreover, we show for the first time that CI is required for CIII maintenance and function, highlighting a reciprocal dependence between the two complexes.

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OPA1 ISOFORMS AND PROTEIN DOMAINS IN THE MITOCHONDRIAL FUNCTIONS

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OPA1 is a dynamin-related GTPase inserted in the inner mitochondrial membrane, displaying a mitochondrial targeting sequence, a predicted transmembrane domain (TM), a first coiled-coil domain, followed by three highly conserved regions: a GTPase domain, a middle domain and a second coiled-coil domain at the C-terminus, named GTPase effector domain (GED). Human OPA1 is present in eight isoforms, resulting from alternative splicing of exons 4, 4b and 5b, which exhibit a tissues-specific pattern. These isoforms undergo proteolytic processing at the cleavage sites S1 and S2 (in exons 5 and 5b, respectively), to generate long and short isoforms, these latter lacking the TM domain. OPA1 is involved in several functions, such as mitochondrial dynamics and *cristae* organization, oxidative phosphorylation efficiency, mitochondrial DNA (mtDNA) maintenance and control of apoptosis (Belenguer et al., 2013).

Accordingly, the lack of OPA1 causes the complete fragmentation of the mitochondrial network, a drastic disorganization of the *cristae* ultrastructure and a significant reduction of the mtDNA content (Song et al., 2009; Patten et al., 2014). Whether a specific protein variant is implicated in one or more of these functions is at present poorly understood. To address this issue we expressed individual OPA1 isoform in OPA1 null MEFs, generating stable cell lines.

Surprisingly, all the isoforms were able to completely rescue the energetic efficiency and the mtDNA content of OPA1 null MEFs, whereas the mitochondrial morphology and the *cristae* integrity were only partially recovered. To identify the OPA1 protein domains crucial for its functions, we modified the isoform 1 to generate OPA1 variants with a different N-terminus portion or unable to generate short forms or with a defective GTPase domain. Our results indicate that an intact GTPase domain is a prerequisite for all functions. Furthermore, both N-terminal and C-terminal OPA1 domains, also present in separate polypeptides, are essential for the mtDNA stability and a cleavable isoform generating short forms ensures complete energetic efficiency.

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MITOCHONDRIAL TRANSLOCATION OF APE1 RELIES ON THE MIA PATHWAY

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APE1 is a multifunctional protein with a fundamental role in repairing nuclear and mitochondrial DNA lesions caused by oxidative and alkylating agents. Unfortunately, comprehensions of the mechanisms regulating APE1 intracellular trafficking are still fragmentary and contrasting. Recent data demonstrate that APE1 interacts with the mitochondrial import and assembly protein Mia40 suggesting the involvement of a redox-assisted mechanism, dependent on the disulfide transfer system, to be responsible of APE1 trafficking into the mitochondria. The MIA pathway is an import machinery that uses a redox system for cysteine enriched proteins to drive them in this compartment. It is composed by two main proteins: Mia40 is the oxidoreductase that catalyzes the formation of the disulfide bonds in the substrate, while ALR reoxidizes Mia40 after the import.

In this study, we demonstrated that: i) APE1 and Mia40 interact through disulfide bond formation; and ii) Mia40 expression levels directly affect APE1's mitochondrial translocation and, consequently, play a role in the maintenance of mitochondrial DNA integrity. In summary, our data strongly support the hypothesis of a redox-assisted mechanism, dependent on Mia40, in controlling APE1 translocation into the mitochondrial inner membrane space (IMS) and thus highlight the role of this protein transport pathway in the maintenance of mitochondrial DNA stability and cell survival.

CAREER DEVELOPMENT BY BRIDGING RESEARCH WITH SOCIETY: THE EXPERIENCE AND THE RESULTS OF TRANS2CARE PROJECT

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Trans2Care - Transregional Network for Innovation and Technology Transfer to Improve Health Care is a strategic project funded by the Cross-border Cooperation Programme Italy-Slovenia 2007-2013.
It has involved 13 Partners, of which 6 were in Slovenia and 6 were in Italy, with the University of Trieste as Lead Partner. It has received € 2.61 million to achieve two objectives of the Operational Programme Italy-Slovenia 2007-2013: i) Promote R & D and the knowledge-based economy; ii) Improve and qualify the potential employment in the sector through coordinated education.
A group of 14 researchers have been recruited for 3 years. The researchers have perfected their technological training, providing impetus to research and cross-border collaboration and have carried out an entrepreneurial training programme. They now have skills suitable for working with industry, healthcare professionals and associations.
The main R & D results are: i) 2 markers for early disease detection (mad cow disease and metabolic syndrome); ii) 2 new approaches to disease prevention (colorectal cancer and metabolic syndrome); iii) 2 methods for diagnosis (syncope and coeliac disease); iv) an improved biomaterial for orthopaedic implants; v) new computational and analytical methods (laser and electrochemical technologies, bioseparations) and diagnostic bio-reagents.
Besides that, we have created an organic innovation model, the first we are aware of in the cross-border area.

THE MITOCHONDRIAL CARNITINE/ACYLCARNITINE CARRIER IS REGULATED BY HYDROGEN SULFIDE VIA INTERACTION WITH C136 AND C155

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Hydrogen sulfide plays important roles in human physiology [1]. The main actions of this gas consist of relaxation of the cardiovascular systems, control of inflammation and neuroprotection [2]. The mechanism of action is mainly mediated by interaction with protein Cys residues. The mitochondrial carnitine/acylcarnitine carrier (CAC), that is essential for completion of the β-oxidation pathway, contains six Cys residues whose structure/function relationships have been well defined by site-directed mutagenesis and chemical modification. It has been demonstrated that two of these residues, C136 and C155, play a role in GSH mediated redox regulation of the protein function [3].

Incubation of CAC reconstituted in proteoliposomes with NaHS, an H_2S releasing compound, led to significant impairment of [3H]carnitine/carnitine antiport reaction catalyzed by the transporter. From dose-response analysis an IC50 of 0.70 μM was derived for NaHS. After longer times of incubation, the inhibition was reversed. Indeed, the inhibition or activation effect of H2S depended on the initial redox state of the protein, i.e. reduced or oxidized, respectively. The Cys residues involved in the H_2S regulation were identified by site-directed mutagenesis. The protein containing the residues C136 and C155 responded to the reagent as the WT. Absence of one or both these Cys residues led to significant impairment or loss of H_2S sensitivity. Among mitochondrial carriers, only the CAC possesses two Cys residues in the position sensitive to H_2S, i.e., on the 3rd transmembrane segment and on the first hydrophilic loop. This indicated that CAC is a mitochondrial inner membrane target for H_2S sensing.

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PEROXYNITRITE DECOMPOSITION BY E. COLI CYTOCHROME BD OXIDASE

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Cytochrome bd [1] is prokaryotic respiratory quinol:O_2 oxidoreductase phylogenetically unrelated to heme–copper oxidases (HCOs), that was shown to promote virulence in some bacterial pathogens. Beyond contributing to cell bioenergetics, this terminal oxidase has been shown to enable bacterial survival to a number of stress conditions. Interestingly, cytochrome bd-I from Escherichia coli was previously suggested to enhance bacterial tolerance to oxidative and nitrosative stress conditions, owing to an unusually fast dissociation of nitric oxide (NO) from the active site and a notable H_2O_2-degrading activity (reviewed in [2]). As a novel finding, we report here that the enzyme is highly resistant to peroxynitrite ($ONOO^-$) [3], a harmful species produced by the host immune system through the reaction of NO with superoxide anion. Moreover, by directly monitoring the kinetics of ONOO- decomposition using time-resolved spectroscopy, it was found that the enzyme is able to catalyze the reductive decomposition of ONOO- with turnover rates as high as ~10 mol ONOO- (mol enzyme)-1 s-1 (at 25°C). All together these results suggest that the preferential (over HCOs) expression of cytochrome bd likely represents a defence strategy against the nitroxidative stress conditions created by the host immune system, which makes bd-type oxidases of interest also as potential drug targets.

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NITRIC OXIDE: A ROLE IN LEBER'S HEREDITARY OPTIC NEUROPATHY

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Leber's hereditary optic neuropathy (LHON) is the most common primary mitochondrial genetic disorder. The disease is caused by specific mitochondrial DNA (mtDNA) point mutations affecting complex I subunit genes. The most common pathogenic mutations are at position 3460/ND1, 11778/ND4 and 14484/ND6. Usually LHON affects young males leading to a rapid painless bilateral vision loss. The disease results from a striking death of retinal ganglion cells (RGCs) and optic nerve atrophy accompanied by a severe demyelination. Biochemical data reveal that LHON leads to respiratory defects with increased reactive oxygen species (ROS) production and apoptotic death. Several studies carried out on cellular systems and animal models unravelled some of the main LHON features, but many unsolved questions still persist. The observed bioenergetic failure and enhanced ROS production do not exhaustively explain the late disease onset, the preferential involvement of RGCs and optic nerve, and the incomplete penetrance of the pathology. It is reported that in most LHON families all the individuals on the maternal line usually harbour homoplasmic mtDNA mutations, but not all of them develop the disease. Thus the mtDNA mutation seems to be a necessary but not sufficient condition to cause the disorder, pointing to the existence of other triggering factors. Working on lymphoblasts derived from a LHON patient, we investigated the effect of nitric oxide (NO) on cell respiration. NO, a fairly stable and highly diffusible gasotransmitter, is a well-established regulator of many physiological processes, including mitochondrial activity. Here we propose a higher susceptibility of LHON patients to those events (e.g. inflammation) that lead to an increase of NO production.

ACTIVATION OF COMPLEX II BY HYDROGEN PEROXIDE IN COMPLEX III DEFICIENT CELLS

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Mitochondria are the principal source of cellular reactive oxygen species (ROS) in both physiological conditions and as a result of inefficiencies in the electron transport chain due to dysfunctions in the respiratory complexes. In this regard, it has been recently reported that loss of complex I activity, induced by both a null *MTND6* gene mutation or by rotenone treatment, markedly increased hydrogen peroxide production, leading to elevation of the complex II redox activity, through phosphorylation mediated by the Src type Fgr tyrosine kinase. Complex II activation was reported to be specific for complex I-deficient cells, since it was not detected in cells lacking complex III or IV. Accordingly this was proposed to be a compensatory mechanism for impaired activity of CI, thus allowing the respiratory chain to reset its capacity for processing NADH- versus FADH2-derived electrons [1].

We have recently characterized in details two mutations in the MTCYB gene: (i) a missense mutation at m.15579A>G nucleotide position, that replaces the Tyr 278 with a Cys (p.278Y>C) [2] and (ii) a micro-deletion 18-base pair long encompassing nucleotide positions 15649-15666, causing the loss of six amino acids (I300-P305) in the sixth trans-membrane helix of the protein, leaving the remaining of the MT-CYB sequence in frame [3]. Here we report that in homoplasmic cybrids for these mutations, complex III activity was almost completely abolished. Conversely, the complex II redox activity was markedly increased in cells harboring the micro-deletion, but not in those with the missense mutation. Hydrogen peroxide production was measured in both cell lines, revealing a marked increase in those with the micro-deletion only. In these cells, prolonged treatment with the antioxidant N-acetylcysteine dampened complex II activity stimulation. Furthermore, analysis by western blotting revealed that the band corresponding to FpSDH subunit only was positive to anti P-tyr antibody, suggesting the involvement of a phosphotyrosine-dependent mechanism.

In conclusion our results indicate that enhanced CII activity triggered by mitochondrial hydrogen peroxide production is a more general phenomenon in cell adaptation to respiratory chain dysfunction, being detected also in complex III-deficient cells.

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DO REACTIVE OXYGEN SPECIES INCREASE OR DECREASE IN HUMAN FIBROBLASTS ADAPTED TO HYPOXIA?

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Mitochondria are considered oxygen sensors and play a role stabilizing and activating the main factor, HIF-1, involved in cellular adaptation to hypoxia [1]. Mitochondria are the major source of reactive oxygen species (ROS) in the cell [2], and ROS of mitochondrial origin are considered essential to the HIF-1 action according to many researchers [3], but not influent by others [4]. Therefore, this issue needs to be clarified. Although the role of mitochondria has been widely investigated, particularly in tumor cells, bioenergetics and oxidative stress in normal cells exposed to prolonged hypoxic conditions have been scarcely addressed or resulted in conflicting data, respectively. To clarify these issues, we set up experiments exposing human fibroblasts to 1% oxygen tension (moderate hypoxia) and supplying either glucose or glucose-deficient media to induce different OXPHOS and ROS producing conditions. Fibroblasts grown in glucose underwent mitochondrial fission and lost some 50 % of their mass by autophagy, whereas fibroblasts grown in glucose-free medium adapted to hypoxia maintaining their mitochondrial mass and network, as previously reported [5]. However, under both conditions (i.e. glucose or glucose-free culture medium) cells exposed to hypoxia showed a time-dependent decrease of ROS level. Independently of the oxygen tension, ROS were always lower in cells grown in glucose-free than in glucose containing medium. We then assayed the mitochondrial membrane potential ($\Delta\Psi$mit) and found that it was strongly dependent on glucose presence. Indeed, whatever the oxygen tension, fibroblasts mitochondrial membrane potential was higher when grown in the presence of glucose. In addition, fibroblasts exposed to hypoxia showed a significant $\Delta\Psi$mit increase compared to normoxic conditions when grown in glucose-free medium only. All the results will be discussed and related to hypoxia induced changes of both reduced/oxidized glutathione level and antioxidant enzymes expression in human fibroblasts.

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IMPAIRMENT OF MUSCLE OXIDATIVE FUNCTION IN VIVO FOLLOWING MUSCLE DISUSE: WHAT IS THE ROLE OF MITOCHONDRIA?

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Skeletal muscle dysfunction is nowadays considered a hallmark sign of many disease states and a key predictor of exercise intolerance and effort perception during daily-life activities. Alterations of mitochondrial respiratory function and muscle oxidative capacity can contribute to determine muscle (dys)fuction and can have important implications on health status. However, whether (and to what extent) a mitochondria functional remodeling can modulate muscle oxidative function in vivo under "stressful" conditions is poorly clear.

We studied, in healthy young men, the effects of prolonged muscle disuse (by 10 and 21-d bed rest campaigns), alone and combined with a hypoxic stimulus (simulated altitude 4,000m above sea level), on mitochondrial respiratory function ex vivo and in vivo skeletal muscle oxidative function.

Mitochondrial respiration (by high-resolution respirometry) was assessed in permeabilized v. lateralis muscle fibers. v. lateralis muscle fractional O_2 extraction (by near-infrared spectroscopy) and pulmonary O_2 uptake ($V'O_2$) were assessed during incremental one-leg knee extension (KE) exercise up to the limit of tolerance.

Mitochondrial oxidative phosphorylation capacity ex vivo was not affected by bed rest, nor by hypoxia, nor by the combination of both stimuli in a 10-d time period, whereas it decreased significantly (by 15-20%) after all the interventions in a 21-d period.

Peak muscle fractional O_2 extraction and peak $V'O_2$ in vivo during KE were significantly reduced by bed rest (by ~12% and 8%, respectively) after both 10 and 21 days; both variables were not affected by hypoxia, either after 10 or 21 days. Superimposed to bed rest, hypoxia did not alter the impairment in vivo induced by bed rest, either after 10 or 21 days.

Muscle oxidative function in vivo is significantly impaired by muscle disuse, irrespective of mitochondria functional responses and muscle oxidative capacity ex vivo.

Alterations within the intramuscular matching of O_2 delivery/O_2 utilization would represent the trigger factor of muscle dysfunction in vivo, at least in healthy populations.

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SUCCESSFUL TREATMENT WITH INHIBITORS OF THE MITOCHONDRIAL PERMEABILITY TRANSITION PORE IN A ZEBRAFISH MODEL OF COLLAGEN VI MUSCULAR DYSTROPHY

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Ullrich Congenital Muscular Dystrophy (UCMD) and Bethlem Myopathy (BM) are inherited muscle diseases due to mutations in the genes encoding the extracellular matrix protein collagen VI. Opening of mitochondrial permeability transition pore(s) (mPTP) is the causative event in disease pathogenesis, and a potential pharmacological target for therapy. In a previous work, we investigated cyclophilin inhibitors able to desensitize the mPTP in vitro, i.e. cyclosporin (Cs) A and two non immunosuppressive cyclosporine derivatives, D-MeAla3-EtVal4-undecapeptide (Alisporivir) and N-methyl-4-isoleucine-undecapeptide (NIM811); then, we selected novel mPTP inhibitors after high-throughput screening of a library of 363,827 compounds from the NIH Molecular Libraries Small Molecule Repository. To study the effects of these compounds on muscular dystrophy, we used a zebrafish model of collagen VI myopathy obtained by deletion of the N-terminal region of the col6a1 triple helical domain, a common dominant mutation of UCMD. Treatment with antisense morpholino sequences targeting col6a1 exon 9 at the 1-4 cell stage (within 1 hour post fertilization, hpf) caused severe ultrastructural and motor abnormalities as assessed by electron and fluorescence microscopy, birefringence, spontaneous coiling events and touch-evoked responses measured at 24-48 hpf. These structural and functional abnormalities were largely corrected via cyclophilin D inhibition with NIM811 or Alisporivir (which both proved significantly more effective than CsA) administered at 21 hpf. In contrast, FK506 (tacrolimus), a calcineurin inhibitor that does not bind to cyclophilins, was ineffective. Motor abnormalities and birefringence were significantly rescued by compound 147, which acts independently of cyclophilin inhibition. Since the mPTP of zebrafish shares the key regulatory features of the mammalian mPTP, these results suggest that early treatment with mPTP inhibitors could be beneficial as a potential therapy for UCMD and BM. A possible synergy between cyclophilin inhibitors and new compounds could be useful for a combined therapy of muscular dystrophies.

TARGETING MITOCHONDRIAL METABOLISM TO MODULATE THE MACROPHAGE IMMUNE RESPONSE: A PRELIMINARY STUDY WITH PARA-HYDROXYPHENYLPYRUVATE

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Recent studies on the metabolism of immune cells have highlighted the tight link between metabolic state and phenotype of these cells. An emerging concept is that metabolism not only sustains diverse immune cell phenotypes as a consequence of alterations in cellular signalling, but metabolism also feeds back and alters signalling to drive immune-cell phenotype. In this contest mitochondria are being increasingly recognized as signalling organelles for both the maintenance and establishment of immune cell phenotypes. On this basis, targeting mitochondrial metabolism is becoming an attractive therapeutic strategy for modulation of the immune response in human diseases.

Sepsis is a systemic infection-induced syndrome characterized by a generalized inflammatory state and it represents a leading cause of death in the intensive care unit attributed to a multiple organ dysfunction syndrome (MODS). Although the precise mechanisms through which organ dysfunction develops remain unknown a growing body of evidence indicates that sepsis-induced organ failures are associated with fundamental alterations of cellular metabolism. Severe sepsis caused by Gram-negative invasive infection is associated to endotoxemia because of the presence of circulating lipopolysaccharide (LPS), which potently activates macrophages toward a highly inflammatory phenotype with high phagocytic and bactericidal potential.

In the presented *in vitro* study we used the murine macrophage RAW 264.7 cell line as a model to investigate the consequence of lipopolysaccharide (LPS)-mediated activation on mitochondrial metabolism and tested on this the effect of p-hydroxyphenylpyruvate (pHPP). pHPP is an intermediate of the phe/tyr catabolic pathway that in addition to provide intermediates of the TCA cycle (i.e. fumarate and acetylCoA) proved to exhibit antioxidant and anti-inflammatory activities.

Treatment of RAW 264.7 cell line with a concentration of LPS comparable with that detected in sera of septic patients resulted in up-regulation of the inducible nitric oxide synthase (iNOS) expression and consequently of NO production and in increased production and release of the pro-inflammatory cytokine IL-6. Oxymetric assay of LPS-treated RAW 264.7 cells resulted in a significant ≈50 % decrease of the endogenous and of the ATP synthase-linked mitochondrial respiration. Notably, the LPS-mediated inhibition of respiration was fully prevented by the PDH activator dichloroacetate (DCA). The observed respiratory failure was accompanied with inhibition of the respiratory chain Complex I and enhanced production of reactive oxygen species. Co-incubation of RAW 264.7 with mM concentration of pHPP prevented all the observed pro-inflammatory and metabolic alteration caused by LPS. Interestingly, pHPP caused also marked morphological changes in RAW 264.7 along with enhanced adhesiveness.

Of note, inhibition of the mitochondrial respiratory activity was also observed following incubation of neonatal fibroblasts (NHDF) with sera from septic patients and pHPP was able to prevent the respiratory impairment in this case as well.

On the basis of the results presented a working model linking pathogen-associated molecular patterns (PAMPs)-mediated immune response to mitochondrial metabolism will be put forward along with suggestions for its therapeutic control.

ATPase-INHIBITORY FACTOR 1 AND OXPHOS COMPLEXES IN HUMAN GLIOMAS

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In the last decade the importance of the bioenergetic phenotype of cancer's cells has gained new interest due to new molecular, proteomic and bioinformatic studies, and a marked heterogeneity is emerging in the mitochondrial competence of different tumors independently of up-regulation of glycolysis. In this framework, the attention was focused in many types of carcinomas (lung, breast and colon) on down-regulation of β-F1-ATPase and, more recently, on up-regulation of ATPase-Inhibitory Factor 1 (IF1) as important factors in defining the glycolytic phenotype and as biomarkers of prognosis [1-3]. So far nothing was reported about gliomas.

The situation about gliomas is very complex and not all agree on the exact phenotype these cancer cells use. Some authors reported a mixed phenotype both glycolytic and oxidative, others reported a complete oxidative phenotype especially in the most aggressive case of glioblastoma [4]. Moreover it seems now to be accepted the idea of OXPHOS heterogeneity respect other metabolic pathways in different glioma types, but also inside the same tumor mass, in relation to tissue conditions such as vascularization and necrosis [5].

The purpose of the present study was to investigate the expression levels of IF1 in human gliomas in relation to their aggressiveness (WHO grading). The expression levels of OXPHOS complex I–V proteins and in particular of β-F1-ATPase, Citrate Synthase and HSP60 were also determined.

45 chirurgic specimens were analysed by immunohistochemistry and grouped as follows: II grade gliomas, n=17; gliomas with anaplastic transformation, n=8; and IV grade gliomas, n=20; and 21 by quantitative immunoblotting: n=6; n=7; n=8 respectively. No peritumoral or healthy tissues were possible to collect and the histology of low grade gliomas was very heterogenic.

Immunohistochemistry analyses have shown a significant marked increase of IF1 in IV grade gliomas and a small increase of β-F1-ATPase.

Immunoblotting analyses have shown that IF1 and β-F1-ATPase expression levels do not present any significant change through the different groups. These results, apparently in contrast, are due to the fact that only vital tumor cell somata were evaluated by immunohistochemistry, whereas immunoblotting results reflect the overall protein expression of the whole tissue sample, containing large areas of necrosis besides tumor tissue in the case of IV grade gliomas. Nevertheless, IF1expression levels have an interesting pattern with an increase concomitant with the anaplastic transformation. This pattern appears to be more evident when only astrocitomas were analysed. Further analyses to investigate this aspect will clarify if IF1 might be proposed as a biomarker in this histologic subtype.

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INHIBITION OF LON PROTEASE BY TRITERPENOIDS CDDO AND CDDO-ME ALTERS MITOCHONDRIA AND CAUSES CELL DEATH IN RKO HUMAN COLON CANCER CELLS

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Background: Lon is a nuclear-encoded, mitochondrial ATP-dependent protease that assists protein folding, degrades oxidized or damaged proteins and participates in maintaining mitochondrial DNA (mtDNA) levels. Lon is up-regulated in several cancer cells, and its down-regulation causes profound alterations of mitochondrial proteome and function, and cell death. Lon enzymatic activity is activated by ATP, and inhibited by the triterpenoid 2-cyano-3,12-dioxooleana-1,9-dien-28-oic acid (CDDO) or by its C-28 methyl ester derivative (CDDO-Me).

Objectives: The aim of the work was to characterize the effects of triterpenoids on mitochondria of cancer cells, paying a particular attention to parameters such as membrane potential, mass, morphology, dynamics and ROS content, and Lon proteolytic activity.

Methods: We used RKO colorectal carcinoma cell line as cellular model. Lon was pharmacologically inhibited by the treatment with CDDO, or its methylated derivative CDDO-Me. The expression level of Lon protease and of its two representative substrates, namely human transcription factor A (TFAM) and aconitase (Aco2), were analysed by western blot. Lon was up-regulated by using a retroviral vector, harbouring the cDNA encoding for Lon protease (pMSCV-Lon), to stably transduce RKO cells. Apoptosis, content of mitochondrial hydrogen peroxide and mitochondrial superoxide, mitochondrial membrane potential and mitochondrial mass were measured by flow cytometry. Mitochondrial morphology was analysed by confocal microscopy.

Results: CDDO and CDDO-Me resulted to be potent stressors for mitochondria. In particular, they inhibited cell growth in a dose-dependent manner and induced apoptosis, increased mitochondrial ROS, depolarized mitochondrial membrane, altered mitochondrial morphology and mitochondrial mass, affected the levels of Lon and those of Aco2 and TFAM, two targets of Lon proteolytic activity. Lon overexpression reduced apoptotic cell death induced by CDDO and CDDO-Me.

Conclusions: Lon is a non-oncogenic molecule, which does not initiate tumourigenesis, but is essential for maintaining mitochondrial functionality and morphology; its pharmacological inhibition could represent a possible new anticancer strategy.

TARGETING MITOCHONDRIAL COMPLEX I TO INDUCE PSEUDONORMOXIA: AN ADJUVANT ANTI-CANCER STRATEGY

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Mitochondrial DNA mutations are known modifiers of tumorigenesis (1). According to Warburg hypothesis mitochondrial damage underlies the metabolic changes cancer cells undergo to persist proliferation. Moreover, mtDNA mutations were shown to contribute to the metastatic potential of cancer cells by inducing reactive oxygen species and hypoxia-inducible factor-1a (HIF1a) stabilization (2). Conversely, our group described the loss of respiratory complex I (CI) in lowproliferative oncocytic tumors, where the metabolic rearrangements that follow CI disassembly were shown to induce HIF1a degradation, inhibiting the adaptation to hypoxia (3-4). Such metabolic alterations may originate from the imbalance of NAD^+/NADH and αketoglutarate/succinate (αKG/SA) ratio, which in turn may be responsible for constitutive activation of prolyl-hydroxylases (PHDs) and chronic HIF1a destabilization (pseudonormoxia). We hypothesize that, whereas mild mitochondrial impairment may sustain tumorigenesis, a severe CI dysfunction would induce pseudonormoxia, preventing HIF1a stabilization and progression to malignancy. Thus, pseudonormoxia induction may represent a potential adjuvant anti-cancer strategy and CI may be envisioned as a lethality target. To this aim, CI deficient cell models were generated by using zinc finger endonucleases to induce the knock-out of *NDUFS3* (*NDUFS3* KO), which encodes an essential CI assembly subunit. The biochemical characterization revealed a strong decrease of CI activity leading to a severe energetic impairment in *NDUFS3* KO clones. Metabolomic analysis showed, among other, a marked difference in Krebs cycle metabolite levels between *NDUFS3* KO and wild-type clones, in particular an increase of αKG/SA ratio in *NDUFS3* KOs. This was followed by a decreased amount of stabilized HIF1a protein and a lower expression of HIF1a responsive genes in *NDUFS3* KO clones, without affecting *HIF1A* gene expression. *In vitro* evaluation of tumorigenic potential showed that lack of CI reduces two-dimensional migration in NDUFS3 KO cells, rather than inducing significant changes in their clonogenic ability, suggesting that lack of CI and subsequent HIF1a destabilization most likely affect later stages of tumor progression, such as invasion and metastases formation. This hypothesis is supported by the transcriptome analysis, which identified "focal adhesion" and "cytoskeletal protein binding" as the most significantly modified gene ontology categories in *NDUFS3* KO cells. Experiments in immunodeficient mice are currently ongoing to understand whether lack of CI may prevent metastases formation *in vivo*. Final results will be discussed.

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CALPAINS, CASPASES, HEAT SHOCK PROTEINS AND TENDERNESS OF BEEF

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Tenderness is one of most appreciated characteristics of meat by consumer, and, during beef ageing, it mainly depends by the proteolysis of myofibrillar proteins. From this point of view, it is widely known that calpain-1 has a key role, however, recently, it has been hypothesized that the onset of cell death follows an apoptotic process and activates the caspase-9 – caspase-3 cascade. The objective of this trial was to assess the *post mortem* activity and expression of enzymes belonging to calpain and caspase systems and the expression of genes of the Heat shock proteins (Hsp), in two muscle types of Italian Simmental young bulls characterized by different tenderization levels. Samples of *Longissimus lumborum* (LL; white/type IIB muscle) and *Infraspinatus* (IS; red/type I muscle) muscles of 10 Italian Simmental young bulls were collected at slaughter and during beef ageing. The mRNA abundances of calpain-1, calpastatin, calpain-2, caspase-3, caspase-9, Hsp27, Hsp40, and Hsp70 were detected by q-PCR. At different beef ageing times, by SDS-PAGE and Western Blot, the degradation products of αII spectrin, 145 kDa (SBDP145) and 120 kDa (SBDP120) were analyzed to assess calpains and caspase-3 activities respectively.

The development of a tender beef in LL was related to a greater calpain-1/calpastatin mRNA ratio abundance ($P=0.05$) and a lower Hsp27 ($P<0.05$) than IS. No differences were found in the gene expression of calpain-2, caspase-3, caspase-9, Hsp40, and Hsp70 ($P>0.05$). Consistently, the full-length αII spectrin level was lower in LL than IS ($P<0.01$), whereas SBDP145 level was higher in LL than in IS ($P<0.01$), suggesting that calpains was more active in LL than in IS. Conversely, SBDP120 fragment was detected, but its level was not different between muscles ($P>0.05$), indicating that caspase activity was similar in LL and IS.

In conclusion, despite caspase-3 was active in early *post mortem* confirming the onset of an apoptotic process, it seems to play a minor role respect to calpain-1 in the tenderization of beef. Conversely, the small Hsp27 could have a role in the protection of myofibrillar proteins from proteolysis during beef ageing.

STRATEGIES FOR BACTERIAL EXPRESSION AND LARGE SCALE PURIFICATION OF HUMAN AMINO ACID TRANSPORTERS

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Amino acid transporters are crucial in nitrogen metabolism and some of them are involved in cancer development. *E. coli* is the most used experimental tool for high level expression of a target protein for both structural and functional studies. However, bacterial expression of human amino acid transporters is hampered by their toxicity for bacterial metabolism [1]. Here we report the successful over-expression of SLC7A5 (LAT1) with its heterodimer counterpart SLC3A2 (CD98) and of SLC38A9, which is involved in amino acid sensing and control of mTORC1. The cDNA coding for LAT1 protein has been cloned in the pH6EX3 vector under the control of taq promoter with a N-terminal 6-His tag. *E. coli* Rosetta(DE3) strain has been used to supply tRNA specific for eukaryotic codons. After induction with 0.4 mM IPTG, cells have been cultured at 28 °C for 4 hours. Purification of hLAT1 homogeneous protein has been achieved using the AKTA start FPLC system on His Trap HP column. SLC3A2 has been over-expressed as GST-tagged protein after cloning in pGEX-4T1 plasmid. The same expression conditions adopted for LAT1 have been used. The protein present in the soluble fraction of induced lysate has been purified using glutathione Sepharose 4B. Protein was eluted and cleaved from GST tag by using thrombin. Differently from the other SLCs, the expression of SLC38A9 protein, has been obtained only after codon optimization. Nevertheless, the protein was highly toxic causing bacterial death immediately following addition of the inducer. To overcome this problem *E. coli* Lemo21(DE3) strain, carrying an inhibitor of RNA polymerase, has been cultured at 39 °C for only two hours after IPTG induction. The expressed protein purified by Ni-affinity chromatography was suitable for functional assays [2].

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SLC7A5 (LAT1) PLAYS THE ROLE OF CATALYZING AMINO ACID TRANSPORT IN THE LAT1/CD98 HETERODIMER

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LAT1 (SLC7A5) is an insoluble transmembrane protein that is assembled via a disulfide with CD98 (SLC3A2), a soluble glycoprotein with a single transmembrane segment, forming an heterodimeric membrane complex. The heterodimer has the function of catalyzing transport of neutral amino acids [1]. However, it is still no clear which of the two protein subunits plays a major role in amino acid transport function. The interest in solving this issue relies on the documented over-expression of LAT1 in human cancers [2]. Indeed, cancer cells have an increased demand for amino acids. Different experimental models have been employed for establishing which of the two subunits is competent for transport function. Firstly, BCH sensitive [3H]His transport has been measured in SiHa intact cells, in absence or presence of the disulfide reducing agent DTE in order to measure activity of heterodimer or LAT1 alone; similar activity was found in the two different conditions. Then, the LAT1/CD98 heterodimer has been extracted from the same cells and reconstituted in proteoliposomes. BCH sensitive [3H]His uptake has been measured in proteoliposomes preloaded with His or other amino acids showing that Cys, Tyr and Gln are counter substrates for the antiport reaction; DTE stimulated transport indicating that covalent interaction of LAT1 with CD98 is not required. Finally, recombinant over-expressed human LAT1 and CD98 have been reconstituted in proteoliposomes. Using the sole hLAT1, results overlapping those obtained with intact cells or reconstituted native protein were obtained. No transport could be detected in hCD98 proteoliposomes. The described results demonstrate that LAT1 is the transport competent unit of the heterodimer.

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IDENTIFICATION OF NEW HIGHLY SELECTIVE INHIBITORS OF THE HUMAN ADP/ATP CARRIERS

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ADP/ATP carriers (AAC) play a key role in the cell viability because it translocates the synthesized ATP from the mitochondrial matrix to the cytosol [1] and it appears to be deeply involved in mitochondrial apoptosis [2, 3]. Carboxyatractyloside (CATR) and Bongkrekic acid (BKA) are powerful and highly specific AAC inhibitors and are known to be able to induce mitochondrial dysfunction at molecular level and poisoning at physiological level. For identifying other specific AAC inhibitors to be used as chemotherapeutic agents we performed docking-based virtual screening of an in-house developed chemical library. In our analyses we used the crystallized AAC-CATR complex and the Autodock program suite for screening a chemical library consisting of 10,000 bioactive compounds (that include also CATR and BKA as reference inhibitors) to identify a set of molecules with a high binding affinity for mitochondrial AAC, according to our validated protocols [4]. Autodock analyses are based on a Free Energy scoring function which evaluated the fitness between the AAC and the screened ligands. From the virtual screening we identified 15 ligands predicted to have similar physical-chemical properties (in terms of binding energy) to those shown by CATR and BKA. The 15 candidates were tested in in vitro transport assays on the recombinant AAC2 protein and it was found that 4 of them were good AAC inhibitors. The inhibition constants (Ki) of the known AAC inhibitors, i.e. CATR and BKA, were found to be 7 nM and 1,8 µM respectively, whereas the inhibition constants of the 4 newly identified inhibitors range between 0.2 and 2 µM. The same inhibitors were also tested on other mitochondrial carriers like ORC1, AGC1 and APC1 and it was found that at the same concentration they were not able to inhibit those carriers. Thus, we can propose that our proposed small molecules are highly selective AAC2 inhibitors.

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ALS-LINKED MUTANT SOD1 G93A INTERACTING WITH VDAC1 CHANNEL AFFECTS ITS VOLTAGE-GATING ACTIVITY

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Amyotrophic Lateral Sclerosis (ALS) is a neurodegenerative disease that selectively affects motor neurons in specific regions of central nervous system, leading to the death of patients in 2-5 years from the onset of the disease (1). Majority of the familiar ALS forms are associated to over 150 mutated forms of Cu/Zn Superoxide dismutase (SOD1), a ubiquitous enzyme that plays a key role in cell defense mechanisms against ROS. Aggregates of mutant SOD1 associate with the cytoplasmic face of the outer mitochondrial membrane in spinal cord mitochondria. The mutant SOD1 aggregations are believed responsible of the mitochondrial morphological degeneration and malfunctioning. The dismutase-active mutant G93A is the most frequent among mutations occurring in fALS and it has been proposed to be capable to bind the Voltage Dependent Anion Channel isoform 1 (VDAC1), the most abundant pore-forming protein in the mitochondrial outer membrane (MOM) (2). VDAC1 is considered the master regulator of mitochondrial-cytosol cross-talk, thanks to its possible action in gating many ions and metabolites, as ATP/ADP, NAD+ or glutamate (3-4). Thus, the interaction between VDAC1 and SOD1 G93A has been proposed to affect the functionality of mitochondrial respiratory chain, resulting in decreased ATP synthesis and mitochondrial membrane potential, in addition to alterations of calcium homeostasis (3). In this regard, our study was aimed to investigate the molecular mechanisms of the interaction between SOD1 G93A and VDAC1, in *in vitro* systems and also in NSC-34 cells, by using different experimental approaches. Moreover, we analyzed the effect of this interaction on the electrophysiological properties of VDAC1 channel. Our results confirmed the affinity of the mutated SOD1 G93A to VDAC1 , affinity not present for the physiological form. Understanding the domains involved in the interaction between VDAC1 and SOD1 mutants could be exploited to design of a new class of biological drugs, able to restore the VDAC1 activity.

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TRANSFORMATION WITH hSOD1 OF *S. cerevisiae* DEVOID OF ENDOGENOUS VDAC1 IMPROVES THE DEFECTIVE PHENOTIPE AND RE-ESTABLISH THE MITOCHONDRIAL METABOLISM

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The Cu/Zn Superoxide Dismutase (SOD1) protects the cells from oxidative stress catalyzing the disproportionation of superoxide anion. Recently, it was shown that SOD1 is involved in the regulation of glucose metabolism in yeast *Saccaromyces cerevisiae*. In the presence of glucose, yeast uses mainly glycolysis for energy production and inhibits the mitochondrial respiration; when glucose level drops, mitochondrial respiration is resumed. In yeast cells lacking SOD1 (*ΔSod1*) the mitochondrial respiration is not totally repressed even though the glucose is abundant (1-2). The physiological activity of mitochondria requires an ongoing exchange of metabolites between cytosol and mitochondria, provided by the pore-forming protein Voltage Dependent Anion Channel isoform 1 (VDAC1) located on the mitochondrial outer membrane (MOM). VDAC1 is essential for yeast viability: its absence (*Δpor1* strain) strongly reduces its growth on not fermentable carbon source, such as glycerol at 37°C. Many evidences suggest a relationship between SOD1 and VDAC1. In *Δpor1* cells, the activity and expression of SOD1 fraction located in mitochondrial inter membrane space is significantly reduced. *ΔSod1* cells show down-regulated VDAC1 and TOM40 levels, and VDAC shows a less pronounced voltage dependence and conductance. To investigate the SOD1 metabolic role in relation to VDAC1 -mediated metabolism, we expressed human SOD1 in yeast devoid of endogenous VDAC (*Δpor1*). Our results indicates that the over expression of hSOD1 in *Δpor1* strain restore the growth defect, suggesting that SOD1 is involved in the mitochondrial metabolism mediated by VDAC1. Our data supports the report by Tsang et al (3) showing a specific translocation of SOD1 into the nucleus to act as gene inducer.

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ALTERNATIVE 5'-UTR SEQUENCES OF VDAC GENE IN *DROSOPHILA MELANOGASTER* REGULATE THE TRANSLATION OF DOWNWARD CODING SEQUENCE

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Voltage Dependent Anion Channel (VDAC), or eukaryotic porin, is a pore-forming protein found in the outer mitochondrial membrane and highly conserved from yeast to human [1]. In *Drosophila melanogaster* the porin gene produces two splice variant transcripts termed 1A-VDAC and 1B-VDAC [2]. Although these transcripts comprise the same coding sequence, only 1A-trascript is translated in a functional protein. 1B-trascript is not translated in any tissue or in any development stage of the fly. Transformation of *Δporin1* yeast cells with a construct carrying the 1A-VDAC results in the expression of the corresponding protein and in the complementation of the defective cell phenotype. In contrast, no protein was detected in *Δporin1* yeast cells transformed with 1B-VDAC. Identical results were obtained in *D. melanogaster* embryonal SL2 cells or in HeLa cells by transfection with hybrid constructs containing the two 5'-untranslated regions fused to luciferase or GFP reporter genes. Moreover, either 1A-VDAC and 1B-VDAC transcripts bind to ribosomes in VDAC-deleted yeast strain.

In this work, by scanning mutagenesis of the 1B-5'UTR sequence, we identified one stretch of 16 nucleotides responsible of the translation inhibition of any tested ORF linked to it. A RNA electrophoretic mobility shift assay shows that this sequence could act by linking RNA Binding Proteins (RBPs) and, by proteomic analysis, we found some translation initiation factors as interactors. In addition, a computational analysis on 1B-5'UTR sequence shows the ability of different stretches of this sequence to interacts with exposed helices of the 18S rRNA. We hypothesize that a direct base-pairing of a specific sequence of the 1B-5'UTR to exposed-regions of 18S rRNA prevents the pre-initiation complex (PIC) from moving along the mRNA for recognition of the authentic start codon.

Since the coding potential for the two alternative 1A-VDAC and 1B-VDAC mRNAs is identical, the interesting question arises as to the functional significance of this 5′-UTR feature, which is evolutionarily conserved only in the melanogaster subgroup of the *Drosophila* genus.

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IN VIVO ASSAY TO MONITOR FLAVONOID UPTAKE ACROSS PLANT CELL MEMBRANES

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Flavonoids represent one of the most important molecules of plant secondary metabolism, playing a plenty of different biochemical and physiological roles. Although their essential role in plant life and human health has been elucidated by many studies, their subcellular transport and accumulation in plant tissues still remain unclear, because of the absence of a convenient and simple method to monitor their transport. In the present work, we suggest an assay able to follow *in vivo* the transport of quercetin, the most abundant flavonoid in plant tissues. This uptake was monitored by using 2-aminoethoxydiphenyl borate (DPBA), as fluorescent probe, in non-pigmented *Vitis vinifera* cell cultures.

MITOCHONDRIAL TRANSPORT OF ADMA BY SLC25A2

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Asymmetric dimethylarginine (ADMA) is generated within cells and mitochondria when proteins with dimethylated arginine residues are degraded. The aim of this study was to identify the carrier protein(s) that transport ADMA across the inner mitochondrial membrane. It was found that the recombinant, purified mitochondrial solute carrier SLC25A2 when reconstituted into liposomes efficiently transports ADMA in addition to its known substrates arginine, lysine and ornithine and in contrast to the other known amino acid mitochondrial transporters SLC25A12, SLC25A13, SLC25A15, SLC25A18, SLC25A22 and SLC25A29. The widely expressed SLC25A2 transports ADMA across the liposomal membrane in both directions by unidirectional transport or by an exchange mechanism against arginine or lysine. The SLC25A2-mediated ADMA transport follows first-order kinetics, is nearly as fast as the transport of the best SLC25A2 substrates and is highly specific as SDMA is not transported at all. Furthermore, ADMA inhibits SLC25A2 activity with an inhibition constant of 0.38 ± 0.04 mM, whereas SDMA inhibited it poorly. The identification of SLC25A2 as mitochondrial ADMA transporter suggests that a major function of SLC25A2 is to export ADMA from the mitochondria of the cells missing the mitochondrial ADMA-metabolizing enzyme AGXT2. In cells expressing AGXT2 there are indications that ADMA can be imported into mitochondria, a possibility that can also be fulfilled by SLC25A2.

ATP SYNTHASE DIMERS FROM PEA STEM MITOCHONDRIA FORM THE PTP

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The Permeability Transition Pore (PTP) is a mitochondrial mega-channel well characterized in mammals, yeast and Drosophila. In these organisms, it has been recently demonstrated that the PTP is a channel formed by dimers of F-ATP synthase. Even if plant mitochondria possess all the components needed for PTP formation, the evidence for permeability transition (PT) in plants is still circumstantial. Therefore, this work was undertaken to characterize the plant mitochondria PTP and to verify if plant F-ATP synthase possesses features similar to those already seen in other species. This would help to understand the evolution of the F-ATP synthase channel functions.

Pea stem mitochondria showed a substrate-dependent electrical potential difference formation. In the presence of the ionophore ETH129, trains of Ca^{2+} additions induced transient spikes, indicating a calcium uptake into the mitochondrial matrix. When Ca^{2+} concentration reached approx. 0.3 mM, an abrupt collapse of the membrane potential was observed, significantly delayed by Cyclosporin A (CsA) and Pi. Similarly to membrane potential, Ca^{2+} Retention Capacity (CRC) showed a trend suggesting that plant mitochondria, when permeabilized to Ca^{2+}, can undergo a PT. Purified mitochondria were also subjected to Blue-native (BN)-PAGE and F-ATP synthase activity was detected in situ. The active bands, corresponding to the dimers, were eluted from the gel, and analyzed for the presence of F-ATP synthase components and of Cyclophylin D (CyPD). The active bands of the F-ATP synthase dimers were also inserted into an artificial bilayer, to evaluate the channel activity by electrophysiology experiments.

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ALTERNATIVE SPLICING-MEDIATED TARGETING OF THE ARABIDOPSIS GLUTAMATE RECEPTOR 3.5 TO MITOCHONDRIA AFFECTS ORGANELLE MORPHOLOGY

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Since the discovery of 20 genes encoding for putative ionotropic glutamate receptors in the Arabidopsis (Arabidopsis thaliana) genome, there has been considerable interest in uncovering their physiological functions. For many of these receptors, neither their channel formation and/or physiological roles nor their localization within the plant cells is known. Here, we provide, to our knowledge, new information about in vivo protein localization and give insight into the biological roles of the so-far uncharacterized Arabidopsis GLUTAMATE RECEPTOR 3.5 (AtGLR3.5), a member of subfamily 3 of plant glutamate receptors. Using the pGREAT vector designed for the expression of fusion proteins in plants, we show that a splicing variant of AtGLR3.5 targets the inner mitochondrial membrane, while the other variant localizes to chloroplasts. Mitochondria of knockout or silenced plants showed a strikingly altered ultrastructure, lack of cristae, and swelling. Furthermore, using a genetically encoded mitochondria-targeted calcium probe, we measured a slightly reduced mitochondrial calcium uptake capacity in the knockout mutant. These observations indicate a functional expression of AtGLR3.5 in this organelle. Furthermore, AtGLR3.5-less mutant plants undergo anticipated senescence. Our data thus represent, to our knowledge, the first evidence of splicing-regulated organellar targeting of a plant ion channel and identify the first cation channel in plant mitochondria from a molecular point of view.

MUTATION OF CYTOCHROME B H291 IMPAIRS UBIQUINOL OXIDATION AT THE QO SITE OF THE BACTERIAL CYTOCHOME BC1 COMPLEX

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Although the basic catalytic mechanism of the cytochrome (cyt) bc_1 complex has been elucidated, electron-proton coupling and proton efflux pathways remain unclear. Divalent metal ions (e.g. Zn^{2+}) inhibit proton-coupled electron transfer in the cyt bc_1 complex [1] and in other energy transducing redox complexes [2-5]. The determination of metal ion binding sites provides a valuable approach to localize proton pathways (see e.g. [5]). In view of this, the coordination of Zn^{2+} has been investigated by EXAFS spectroscopy in avian, bovine and bacterial cyt bc_1 [9]. The binding site in the complex from *Rhodobacter capsulatus* was found structurally homologous to those of the avian and bovine cyt bc_1, but exhibited a different pseudo-octahedral coordination. EXAFS analysis indicated as ligand residues H276, D278, N279, and E295 of the *b* subunit plus two water molecules [9], or, alternatively, a cluster formed, besides H276 and D278, by a second histidine, H291, plus three water molecules [9]. These residues are located in an hydrophilic region connecting the Q_o site to the water exposed periplasmic surface of the complex, suggested to contain the exit pathway of protons released by the oxidation of QH_2 [10]. To test this proposal we mutated residues H276, D278, N279, E295 and H291. Interestingly, among these mutants, only H291L was totally unable of photosynthetic growth. Here we report on the effect of this mutation on the thermodynamic and kinetic properties of the cyt bc_1 studied in chromaphores. The H291L mutation does not affect the midpoint potential of the hemes b_L and b_H, but distorts the bandshape of heme b_L, suggesting a perturbation of its environment. Kinetics of flash-induced cyt c and cyt b redox changes indicate that H291L inhibits electron transfer to the high potential FeS-cyt c chain, as well as reduction of cyt b_H in the presence of antimycin, demonstrating an important role of H291 in the catalytic mechanisms of quinol oxidation. Since in the mutant cyt c redox changes following a single flash retain some sensitivity to stigmatellin, we suggest that mutation does not prevent the movement of the FeS domain, but rather retards the delivery of the first electron form QH_2 to FeS, possibly impairing the first proton release.

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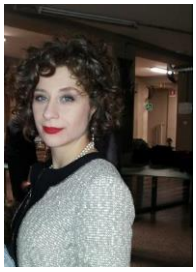

UNCOVERING THE REDOX REGULATION AND THE STRUCTURAL FEATURES OF TRANSKETOLASE FROM *CHLAMYDOMONAS REINHARDTII*

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The Calvin-Benson Cycle (CBC) is a metabolic pathway, occurring in the chloroplasts' stroma of the photosynthetic organisms, responsible for carbon dioxide fixation. Many studies identified the CBC as a redox-regulated pathway. New biochemical and proteomic surveys suggest that all the CBC enzymes may withstand redox regulation through post-translational modifications (PTMs), including disulfide bond formation, oxidation, glutathionylation and nitrosylation. All these redox changes are involved in the short term adaptation to light/dark transitions and also play a role in response to hostile environmental conditions, like oxidative stress.
Our purpose is to get insight into the redox regulation and the biochemical and structural peculiarities of the chloroplastic transketolase (CrTK) from the single-cell green alga *Chlamydomonas reinhardtii*. CrTK is a thiamine pyrophosphate-dependent enzyme, which catalyzes the transfer of a glycolaldehydic fragment from a ketose donor to an aldose acceptor in double substrate reactions. It was previously proposed as a putative thioredoxin (TRX) target (*i.e.* disulfide bond formation) and it was recently found to contain a glutathionylated cysteine residue (Cys84). The recombinant form of CrTK was expressed in *E. coli* and purified to homogeneity through metal affinity chromatography. *In vitro* activity assays showed that the CrTK activity is inhibited by alkylating agents, suggesting the presence of cysteine residues directly or indirectly involved in the enzymatic catalysis. The redox sensitivity of CrTK was further investigated by evaluating the effects of copper chloride, which is known to induce disulfide bond formation in proteins: this analysis revealed that CrTK activity is reversibly regulated by copper chloride, strongly suggesting the presence of one or more regulatory disulfide bond(s). In addition, we confirmed that CrTK is target of glutathionylation, as shown from activity assays (enzymatic inhibition in the presence of GSSG) and from anti-biotin Western Blot performed on CrTK exposed to gluthathion-conjugated biotin. Dynamic Light Scattering and Size-Exclusion Chromatography suggested an homo-dimeric nature for CrTK and, solving its three-dimensional structure at 1.7 Å resolution by X-ray chrystallography, this result was upheld. From the structure, we can now identify cysteine residues potentially involved in the redox induced PTMs by mass spectrometry analysis and site-directed mutagenesis, whereas, in the following step, we will investigate the role of chloroplastic TRX(s) in modulating the redox state of the protein.

ZmDUR3, CHARACTERIZATION OF A HIGH AFFINITY UREA TRANSPORTER IN MAIZE ROOTS

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In the last decades urea has become an extensively used nitrogen (N) fertilizer. However, despite its great agricultural importance the role of urea as a direct N source for plants is still barely understood. Among higher plants, the molecular and physiological basis of its transport have been investigated only in Arabidopsis and recently in rice for which a high affinity urea transporter, named DUR3, has been isolated and characterized. To date the only information regarding the urea acquisition in maize concerned the identification of acquaporins, permeable to urea, In order to characterize a high affinity urea transporter from maize, we isolated the ORF of *ZmDUR3* from maize roots and characterized its activity.

The isolated *ZmDUR3* ORF shows high homology (84% identity on nucleotide level) to the rice urea transporter *OsDUR3*. In particular, *ZmDUR3* encodes an integral membrane protein with 731 amino acid residues. The urea transport activity of ZmDUR3 was verified by complementing the *dur3* yeast mutant strain (YNVWI), which is unable to grow on a medium containing less than 5 mM urea, as sole N source. The expression of *ZmDUR3* in YNVWI cells complemented the urea-uptake deficiency and restored the ability of the yeast strain to grow on a medium containing ≤ 3 mM urea. Moreover, to describe the kinetic features of transport activity of ZmDUR3, ^{14}C-urea uptake experiments were conducted in *X. laevis* oocytes expressing ZmDUR3. Data indicated a saturable kinetic of urea accumulation in *ZmDUR3*-injected oocytes with a K*m* value around 20 μM, comparable kinetic parameters to its orthologs of Arabidopsis (AtDUR3, K*m* of 3 μM) and rice (OsDUR3, K*m* of 10 μM).

To provide convincing evidence on the ZmDUR3 functionality in urea acquisition from the soil and its use within the plant, complementation experiments were performed overexpressing *ZmDUR3* ORF in a *dur3* Arabidopsis knockout mutant line (*atdur3-3*). The overexpression of ZmDUR3 ORF restored the wild type phenotype, allowing plants to grow on a medium containing N in the form of urea (<5mM).

In conclusion, the identification and characterization of ZmDUR3 by heterologous expression in *dur3* yeast mutant and *X. laevis* oocytes strongly support the presence of a high affinity urea transporter in maize. Moreover, the transformation of an Arabidopsis mutant provide a clear evidence in planta for a role of ZmDUR3 in urea acquisition from the extra-radical solution. This work helps to clarify the relative contribution of urea uptake from the soil to the overall N acquisition in maize roots and may provide a key to understanding the molecular basis of the use efficiency of urea fertilization in cultivated plants. *(FIRB grant RBFR127WJ9).*

CHARACTERIZATION OF A GENISTEIN TRANSPORTER IN CLUSTER ROOTS OF WHITE LUPIN

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White lupin (*Lupinus* albus L.) has developed a highly efficient strategy to mobilize nutrients from soil, in particular when P is scarcely available. This strategy is based on the modification of the root architecture with the formation of cluster roots. From these structures, a huge quantity of root exudates (organic acids and flavonoids) are released. These exudates mobilize sparingly available nutrients in soils via complexation, ligand exchange and, in the case of flavonoids, reduction . These secondary metabolites can also influence the biological characteristics of the rhizosphere, affecting the presence and activity of the microorganisms. Hence acquiring a better comprehension of physiological, biochemical and molecular mechanisms that regulates roots exudation processes is fundamental for the improvement of the mineral nutrition of plants.

It is well known that in P-deficient conditions, in the mix of flavonoids released by white lupin, genistein is the one mainly rappresented and it is exuded mainly from the early stages of the proteoid roots; nevertheless up to now there are no data on proteins involved in this processes even if in some plant species transmembrane flavonoid transport via MATE or ABC transporters has been already characterized.

The aim of this work is the physiological, biochemical and molecular characterization of a protein putatively involved in the release of genistein from white lupin plants.

Via cDNA-AFLP a gene sequence sharing a high similarity with known MATE proteins involved in flavonoid transport had been isolated. This gene was called *LaMATE2* and analyses of its expression shown that it is upregulated in P-deficient white lupin roots. To investigate the role of LaMATE2 in genistein exudation, a RNA interference approach was used with the aim to knock down the expression of this gene and to analyses its effect on the exudate release activity of the transformed roots. qPCR results confirmed that the *LaMATE2* expression was reduced and HPLC-analyses of the composition of root exudates revealed that these roots released less genistein.

To biochemically characterize the activity of LaMATE2, the full-length cDNA of the gene was clone and expressed in *Saccharomyces cerevisiae*. The membrane vesicles isolated from transformed yeast were then used to perform transport assay of ^{3}H-genistein.

Microsomes isolated from yeast cells that express *LaMATE2*, compared to the control, accumulated a higher amount of the flavonoid in short time (30 s) and long time of the reaction (10 min). These data demonstrate that LaMATE2 protein is involved in the transport of genistein. Finally, it has been demostrated to be localized on the plasma membrane of *Arabidopsis* transformed protoplast.