SECOND INTERNATIONAL GIBB MEETING

GRUPPO ITALIANO DI BIOMEMBRANE E BIOENERGETICA

HOTEL "I MELOGRANI"

VIESTE (FG), JUNE 13-15 2019

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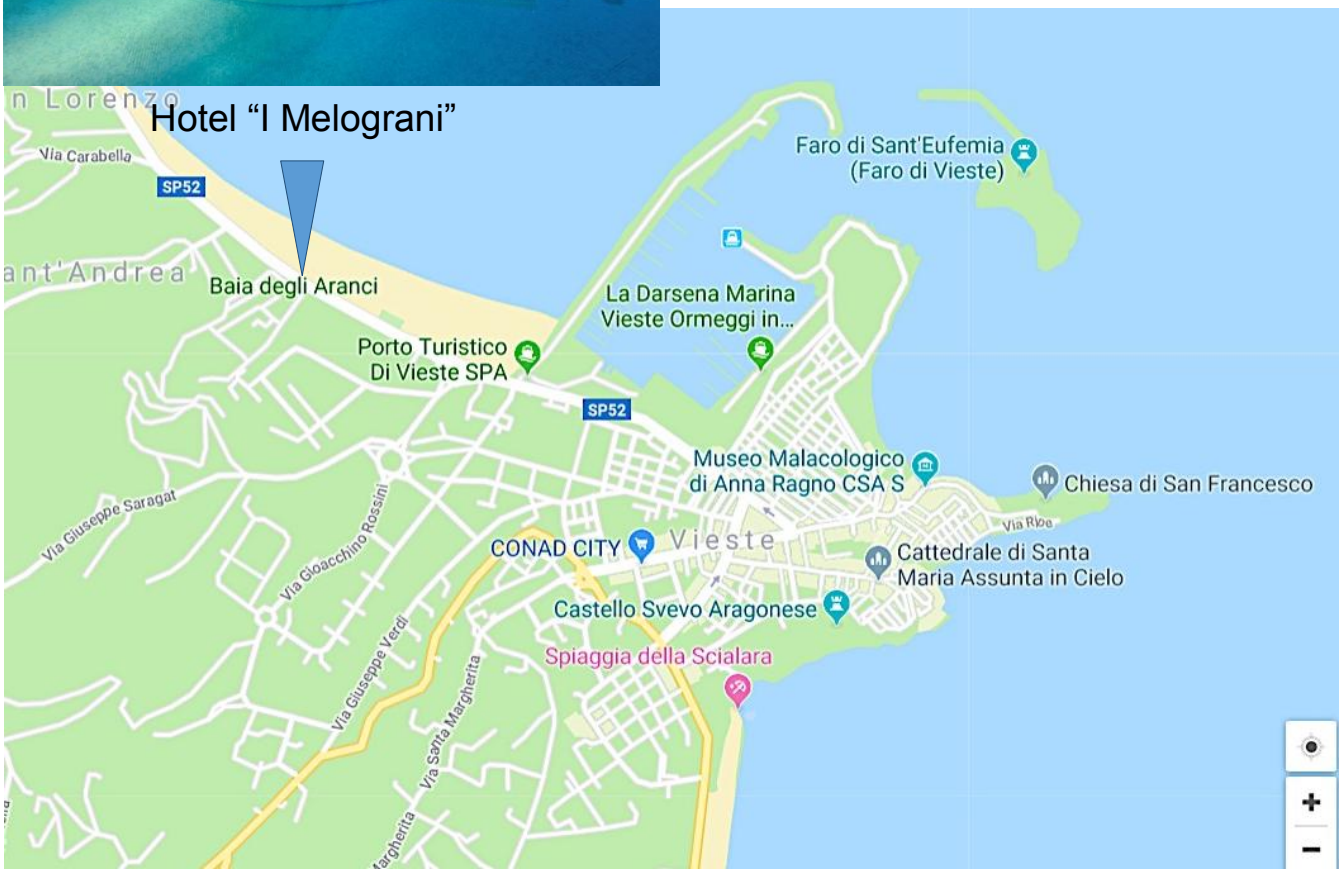


PROGRAM GIBB 2019 – Vieste, June 13-15

Thursday 13th

13.00-15.00 **Registration**

15.00-15.30 **Welcome Address** (Authorities, GIBB President and Organizing Committee)

Opening Plenary Lecture
Chair: **Alessandro Giuffrè** (CNR, Rome)

15.30-16.15 **Barbara Schoepp-Cothenet** (BIP Laboratory, UM7281 AMU/CNRS, Marseille, France)
Using phylogenetics in studying bioenergetic metalloenzymes: the case of enzymes working in arsenic metabolism

Session I. Respiratory Chain Complexes, ATP Synthase, Redox Biology

Chairs: **Alessandro Giuffrè** (CNR, Rome), **Francesco Francia** (University of Bologna)

16.15-16.30 **Chiara Galber** (University of Padova)
Role of F-ATP synthase ƒ subunit in dimer formation and PTP modulation

16.30-16.45 **Carolina Hierro-Yap** (University of South Bohemia)
The mitochondrion of Trypanosoma brucei's infectious stage: how minimalism can broaden your limits

16.45-17.00 **Leonid A. Ivontsin** (Institute of Cytochemistry and Molecular Pharmacology, Moscow)
Proton transport through the inlet half-channel of F0F1-ATP synthase: new insights and perspective

17.00-17.15 **Luigi Leonardo Palese** (University of Bari)
What about the structures of the cytochrome c oxidase tell us about the mechanism

17.15-17.30 **Piero Leone** (University of Bari)
FAD forming and destroying via human FAD synthase: a puzzle of modulated and modulating activities

17.30-17.45 **Francesco Bellanti** (University of Foggia)
Defective transdifferentiation of senescent hepatic progenitor-like cells is associated with impairment of mitochondrial bioenergetics

18.00-19.00 **Welcome cocktail**

20.00-22.00 **Guided tour to Vieste**

Friday 14th

Opening Plenary Lecture
Chair: **Nazzareno Capitanio** (University of Foggia)

08.45-09.30 **Yaarit Adamovich** (Weizmann Institute of Science, Rehovot, University of Tel Aviv, IL)
The Interplay Between Oxygen and Circadian Clocks

Session II. Bioenergetics Metabolism and Mitochondrial (Dys)Function I

Chairs: **Paolo Bernardi** (University of Padova), **Annamaria Tonazzi** (CNR, Bari)

09.30-09.45 **Giuseppe Cannino** (University of Padova)
TRAP1 regulation in cancer metabolism: identification of new interactors

09.45-10.00 **Consiglia Pacelli** (University of Foggia)
Parkin mutation affects the clock genes-dependent energy metabolism

10.00-10.15 **Francesca Liuzzi** (University of Bologna)
IF_1 promotes cancer cells survival under anoxic conditions

10.15-10.30 **Claudio Laquatra** (University of Padova)

Zebrafish (Danio rerio) as a model to study the pathophysiological role of the mitochondrial chaperone TRAP1

10.30-10.45 **Pauline Dao** (Medical University of Vienna)
Dissecting mitochondrial functions in brain, behavior and timing with the human disease gene LETM1

11.00-13.45 Tour on Boat to the Sea-Caves and Swim in the Sea

14.00-15.00 Lunch

Session II. Bioenergetics Metabolism and Mitochondrial (Dys)Function I (continued)

15.00-15.15 **Karim Zuhra** (CNR Institute of Molecular Biology and Pathology, Rome)
N-acetylcysteine serves as substrate of 3-mercaptopyruvate sulfurtransferase and boosts sulfide metabolism in colon cancer cells

15.15-15.30 **Francesca Agriesti** (IRCSS – CROB, Rionero in Vulture, (PZ))
Nandrolone induces a stem cell-like phenotype inhibiting mitochondrial respiratory activity

15.30-15.45 **Francesco Tucci** (University of Foggia)
Cell metabolism profile of different embryonic stem cells

Session III. Transporters and Pores: Structure and Physio-Pathology I

Chairs: **Giovanna Lippe** (University of Udine), **Vito de Pinto** (University of Catania)

15.45-16.00 **Lara Console** (University of Calabria)
Role of Tryptophan 224 in the transport mechanism of the mitochondrial carnitine/acylcarnitine carrier

16.00-16.15 **Rosaria Saletti** (University of Catania)
Oxidation state of methionine and cysteine residues in human mitochondrial Voltage-Dependent Anion Selective Channel isoforms (hVDAC) investigated by high resolution mass spectrometry

16.15-16.45 Coffee break

16.45-17.00 **Veronica Fiorito** (University of Torino)
Metabolic adaptation in colorectal cancer: heme export is required for the down-modulation the tricarboxylic acid cycle

17.00-17.15 **Emma Aloe** (University of Calabria)
Effect of cholesterol on the human ASCT2 amino acid transporter

17.15-17.30 **Marco Schiavone** (University of Padova)
A mitochondrial therapy for Duchenne muscular dystrophy

17.30-17.45 **Anna Stocco** (University of Padova)
Cyclophillins in Duchenne muscular dystrophy

17.45-18.00 **Andrea Urbani** (University of Padova)
Ca^{2+}-dependent high-conductance channel activity of F-ATP synthase matches the permeability transition pore

18.00 **GIBB member annual meeting**

20.30 **Social dinner**

Saturday 15th

09.00-09.15 Technical presentation
Enrico Tatti (Biolog, Hayward Inc. California, USA)
Biolog Mitoplates™: a novel tool to assess mitochondrial functions in eukaryotic cells

Session III. Bioenergetics Metabolism and Mitochondrial (Dys)Function II

Chairs: **Luigi Palmieri** (University of Bari), **Elena Forte** (University of Rome, La Sapienza)

09.15-09.30 **Silvia Russo** (University of Bari)
Mitochondrial signature in human ovarian cancer tissue: involvement of cAMP pathway, SIRT3, OPA1 and PHB2 proteins

09.30-09.45 **Vidyasagar Naik Bukke** (University of Foggia)
Effect of obethicholic acid on liver mitochondria in a model of secondary biliary cirrhosis

09.45-10.00 **Federica Taurino** (University of Bari)
Proteomic-driven analysis reveals a dysregulation of a key mitochondrial protein in Short-Term Type-1 Diabetes Mellitus

10.00-10.15 **Arcangela Iuso** (Institute of Human Genetics, Helmholtz Zentrum Munich, Germany)
Development of treatment strategy for MPAN, a sub-type of Neurodegeneration with Brain Iron Accumulation (NBIA)

10.15-10.30 **Anna Ferretta** (University of Bari)
Increased levels of cAMP by the calcium-dependent activation of soluble adenylyl cyclase in parkin-mutant fibroblasts

10.30-10.45 **Elisabetta Wyart** (University of Torino)
Iron availability regulates cancer related skeletal muscle wasting

10.45-11.15 Coffee Break

Session IV. Transporters and Pores: Structure and Physio-Pathology II

Chairs: **Cesare Indiveri** (University of Calabria), **Marco Zancani** (University of Udine)

11.15-11.30 **Mariafrancesca Scalise** (University of Calabria)
The human SLC38A9 transceptor: insights into the transport side and structure/function relationship

11.30-11.45 **Andrea Carrer** (University of Padova)
Electrophysiological characterization of channel formed by F-ATP synthases

11.45-12.00 **Ruggero Gorgoglione** (University of Bari)
Molecular identification and functional characterization of a novel glutamate transporter in yeast and plant mitochondria

12.00-12.15 **Maria Tolomeo** (University of Bari)
Function and dysfunction of human riboflavin transporters

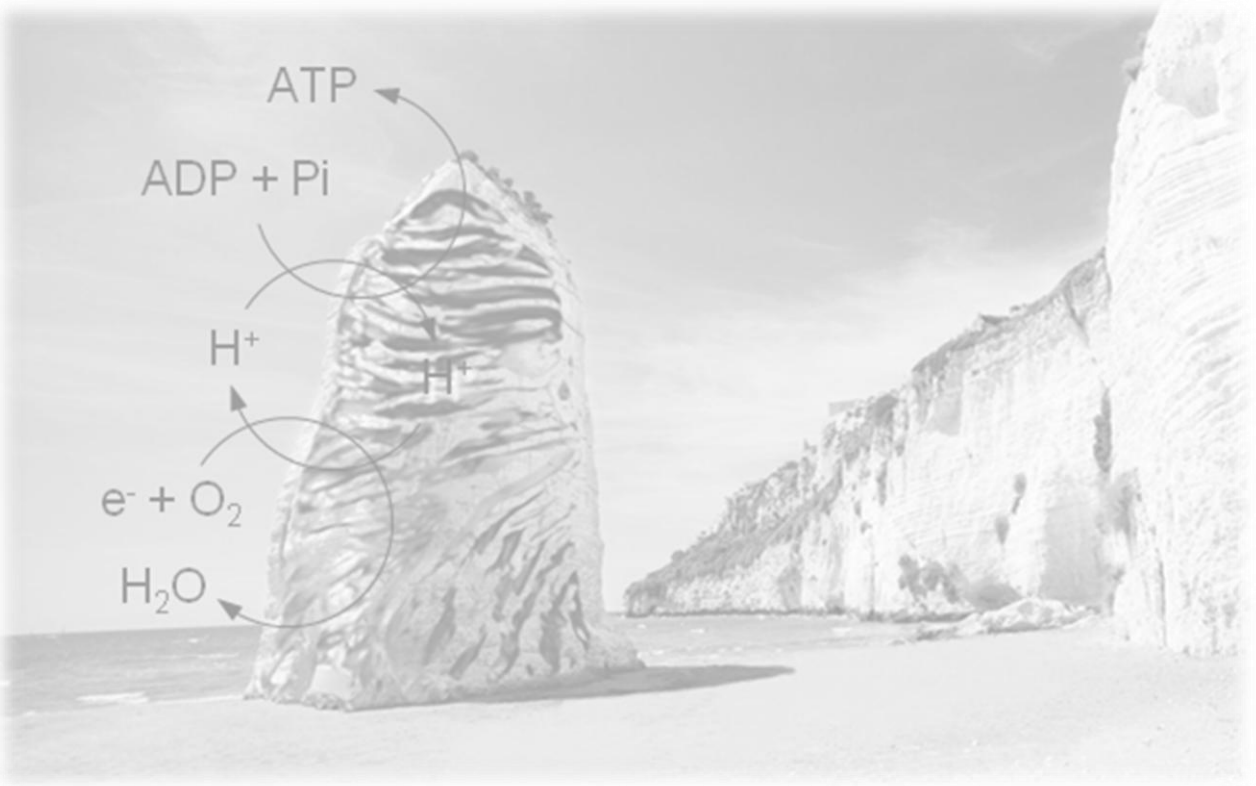
12.15-12.30 **Angela Messina** (University of Catania)
Post-translational analysis of VDAC1 revealed a marginal role of kinase Nek1 in the phosphorylation of the pore

12.30-13.00 **Concluding remarks and awards**

13.00 Lunch / lunch box

14.00 End of the meeting

Abstracts Book



Using phylogenetics in studying bioenergetic metalloenzymes: the case of enzymes working in arsenic metabolism

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One of the most exiting question addressed by fundamental research is to elucidate the evolutionary history of Bioenergetics. Phylogenetics is one of the most powerful approach to answer this issue. This approach being based on the reconstruction of phylogenetic trees, the primary step to be overcome is this reconstruction itself. The sequence and structural data collected during the past decades, reveal that Bioenergetics is a “construction kit”, with a very limited number of basic building blocks [1], among them, proteins carrying a Molybdenum-*bis*PGD [2]. We will illustrate, with our studies of enzymes involved in the arsenic metabolism, how the sparsity of structural modules used by Bioenergetics provides the solution for tree reconstruction and its interpretation [3-4]. Another challenge for fundamental research is to elucidate the rules underlying the bioenergetic chemical reactions, for example the catalytic redox mechanisms. Again, we will illustrate the contribution of phylogenetics to this question, with some of our results [5].

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Role of F-ATP synthase f subunit in dimer formation and PTP modulation

Chiara Galber[1], Giovanni Minervini[1], Andrea Carrer[1], Giuseppe Cannino[1], Valeria Petronillli[1], Silvio Tosatto[1], Ildikò Szabò[2], Giovanna Lippe[3], Valentina Giorgio[1] and Paolo Bernardi[1]

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The Permeability transition pore (PTP) is a mitochondrial channel the opening of which induces cell death; its molecular nature has long remained a mystery [1]. We proposed that dimers of F-ATP synthase form the PTP [2], but how this Ca^{2+}-dependent transition may occur and which subunits are involved in its formation and regulation remain open issues. Structural data suggest an important role of f subunit in dimer stabilization in *Yarrowia lipolytica* and *Saccharomyces cerevisiae*. To investigate the role of the f subunit in PTP formation we generated human cells lackingthe f subunit or with decreased expression levels of the protein. We found that decreased expression of f subunit does not affect mitochondrial respiration or F-ATP synthase assembly but makes it more prone to destabilization by detergent while affecting cristae morphology and desensitizing PTP opening. This last feature was confirmed with electrophysiological studies on f-null cells, which display a PTP of lower conductance. These data suggest that the PTP forms at the interface between F-ATP synthase monomers and involves subunit f.

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The mitochondrion of *Trypanosoma brucei*'s infectious stage: how minimalism can broaden your limits

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Trypanosoma brucei, the causative agent of African sleeping sickness, alternates between a mammalian host (human or cattle) and an insect vector (the tsetse fly). The infectious stage of the parasite (bloodstream form, BF) thrives in the glucose-rich bloodstream of the mammalian host, where glycolysis provides the majority of ATP required for cellular processes. Since the canonical electron transport chain is missing in this stage, the function of the F_oF_1-ATP synthase complex (cV) is to maintain mitochondrial membrane potential (mΔψ) at the expense of ATP hydrolysis [1]. We observed that BF cells can withstand the loss of nearly 90% of the membrane-bound complexes, and that the drop in mΔψ is compensated by the electrogenic exchange of ATP^{4-} for ADP^{3-} by the ATP/ADP carrier (AAC). We hypothesise that this ability may have facilitated the emergence of mt DNA-lacking trypanosomes (dyskinetoplastic, Dk), which were able to extend their distribution beyond Africa, as they are no longer transmitted by the tsetse fly [2]. Dk cells lack subunit a, and consequently, the proton-pumping function of cV. Hence, they generate mΔψ by the above mentioned electrogenic mechanism mediated by the AAC, which relies solely on the F_1-ATPase activity of cV. Our results imply that the membrane attachment of cV may not be essential in Dk cells, as OSCP null mutants showed the same infectivity as wild-type cells. In conclusion, Dk trypanosomes are an example of reductionism that provided an evolutionary advantage for these parasites.

References:

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Proton transport through the inlet half-channel of F_oF_1-ATP synthase: new insights and perspectives

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ATP is one of the most frequently updated molecules in the organism. In the living cell, ATP is mostly synthesized by the protein complex named F_oF_1-ATP synthase, which is a universal molecular motor that uses the transmembrane gradient. In spite of significant advance and diversity in the investigations of the enzyme membrane part, many issues still remain unresolved.

In the work the preferable locations of water molecules in two existing structures of inlet half-channel (normally and laterally oriented) were determined. A combined approach was used for a proton motion modeling. Probability of a proton transfer was determined by a quantum-mechanical approach of one-dimensional motion. The problem of its transport through a sequence of charged centers in the half-channel was considered in the framework of the stochastic approach.

The proton transport rate in the laterally oriented structure of a-subunit appeared to be in two orders of magnitude greater than it was in normally oriented one. However, significant differences in the number of water molecules inside were not discovered. H^+ transport along the operative direction of the transmembrane gradient depended more on the presence and level of potential difference. Nevertheless, this effect was not crucial. Thus, the half-channel orientation in the membrane can have an important influence on F_oF_1-ATP synthase functioning.

What the structures of the cytochrome *c* oxidase tell us about its mechanism

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Cytochrome c oxidase (CcO) reduces molecular oxygen in a process coupled with proton pumping [1,2]. Models for proton pumping activity of CcO can be divided into two groups: one in which haem *a* is the key player, and another where this role is covered by the oxygen reduction site [1-3]. However, all currently accepted models require, more or less explicitly, an ordered sequence of events. We show that available structures of CcO can be clustered in four groups. These structural observations, and the experimental data on which there is a general consensus, suggest a four-state, stochastic pump model [4]. This model implies the observed convex dependence of the stoichiometry of the pump on the electron transfer rate [5], while to explain this phenomenon strictly deterministic models require a series of ad hoc assumptions (e.g. slipping mechanisms) [1,3,5]. Therefore, these results lead us to conclude that a stochastic conformational coupling could be in action in the energy transduction operated by this protein machine [4].

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FAD forming and destroying *via* human FAD synthase: a puzzle of modulated and modulating activities

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FAD synthase (FADS or FMN:ATP adenylyl transferase) coded by human *FLAD1* gene, is the last enzyme in the pathway converting riboflavin into the redox cofactor FAD.Mutations in FLAD1 gene are responsible for Riboflavin-Responsive and Non-Responsive MADD and Combined Respiratory-Chain Deficiency[1].

Alternative splicing of the *FLAD1* gene generates different hFADS isoforms: the mitochondrial isoform 1 and the cytosolic isoform 2. These are bi-functional enzymes containing two domains: a PAPS domain at the C-terminus able to catalyze FAD synthesis (EC 2.7.7.2), a molybdopterin-binding domain at the N-terminus able to perform FAD hydrolysis (EC 3.6.1.18).We show here that theCo^{2+}-dependent hydrolytic activity of hFADS2 is strongly stimulated in the presence of K^+, reaching a Vmax even higher than that of FAD synthesis. hFADS2acts as a non-NuDiX hydrolaseand it could interconnect FAD and NAD homeostasis.

Recently,in patients suffering for frameshift mutations in the FLAD1 gene, we revealed ashort transcript variant corresponding to isoform 6,containing the sole PAPS domain[1]. We overproduced and characterized this emergency protein, which is relevant for patient survival. It is able to synthesize, but not to hydrolyze FAD [2].In the aim to find a target for therapy intervention in patients harboring FADS defects, a variant of hFADS6 carrying the site-directed mutation D238A, expected to exhibit a higher K_{cat}, was also over-produced, purified and characterized.

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Defective transdifferentiation of senescent hepatic progenitor-like cells is associated with impairment of mitochondrial bioenergetics

Francesco Bellanti, Giorgia di Bello, Rosanna Tamborra, Bukke Vidyasagar Naik, Archana Moola, Gaetano Serviddio, Gianluigi Vendemiale

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INTRODUCTION: Aging is characterized by a decline in stem cell function, with loss of tissue homeostasis and regenerative capacity. The liver is the only visceral organ with high regenerative capacity due to replication of mature hepatocytes. During aging, hepatocytes are increasingly senescent, and liver regeneration might be dependent on the activation of hepatic progenitor cells (HPCs). However, the impact of senescence on HPCs is not yet known. Thus, this study aimed at characterizing the effects of aging on the differentiation capacity and mitochondrial function of the human hepatic HepaRG® cells, bi-potent progenitors with the ability to differentiate into both hepatocyte-like and biliary-like epithelial phenotypes *in vitro*.

METHODS: To induce aging in HepaRG cells, we applied a replication-induced senescence protocol. We then compared the differentiation capacity of cells over 10 passages (P10) versus 20 passages (P20). To this, both P10 and P20 cells underwent a 2-step procedure to obtain differentiation. Aging was evaluated by senescence-associated (SA) beta-galactosidase activity and the comet assay. Differentiation into hepatocyte-like cells was analyzed by confocal microscopy (expression of CD34, CD49a, CD49f, CD184, and CK19), and by real-time RT-PCR analysis (expression of albumin, CYP3A4, CEA, GGT and CK19). Mitochondrial respiration, the ATP and the NAD/NADH content were also measured.

RESULTS: SA beta-galactosidase staining was higher in P20 than P10 HepaRG cells; in parallel, the comet assay showed a consistent DNA damage in P20 HepaRG cells. With respect to P10, P20 HepaRG cells exhibited a reduction of CD34, CD49a, CD49f, CD184, and CK19 after the induction of differentiation. Furthermore, a lower gene expression of albumin, CYP3A4, and GGT was reported in differentiated P20 HepaRG cells as compared to P10; on the contrary, gene expression level of CEA and CK19 was similar in both groups. Of note, both cellular and mitochondrial oxygen consumption were lower in P20 rather than P10 differentiated HepaRG cells. Finally, both ATP and NAD/NADH were depleted in P20 cells with respect to P10 cells.

CONCLUSION: HepaRG cells display a bipotent proliferating progenitor phenotype and escape from aging process because of hTERT expression and p27/p21 kinase inhibition. Nevertheless, our results show that late-passage cells present markers of cellular senescence and lose the capacity of trans-differentiate into hepatocyte-like cells, showing an impairment in mitochondrial bioenergetics. To conclude, the present study suggests that senescence-associated mitochondrial dysfunction limits the hepatic differentiation potential of HepaRG cells. Future studies are needed to investigate the impact of mitochondrial restoration (e.g. with NAD precursors) on the trans-differentiation process of senescent HepaRG cells.

The Interplay Between Oxygen and Circadian Clocks

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The mammalian timekeeping system is composed of clocks that reside in nearly every cell in the body. The central clock located in the brain keeps all clocks in synchrony with respect to each other, and with the external environment. Together, they produce circadian rhythms that enable the organism to fit its physiology and behavior to changes associated with light dark cycles. Keeping this system in synchronization is important to the wellbeing of the organism. How all these clocks tick in harmony is therefore a fundamental question. Food and body temperature are well known for being powerful time signals. Moreover, nutrient processing and heat production are tightly linked to oxygen levels. This led us to hypothesize that oxygen can convey time to circadian clocks. We monitored oxygen levels in vivo at multiple levels and identified principles that control its rhythmicity. We showed that when these rhythms are recapitulated in cell culture they can synchronize the molecular clock through the oxygen-sensing transcription factor, HIF-1. Taken together, our findings attribute a central role for oxygen in the mammalian circadian clock work.

TRAP1 regulation in cancer metabolism: identification of new interactors

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The metabolic rewiring is a central component of cell progression to malignancy and how mitochondria contribute to it remains partially defined. We have demonstrated that the mitochondrial chaperone TRAP1 is essential for neoplastic growth in several models through its activity of metabolic regulator. In particular TRAP1 binds and inhibits succinate dehydrogenase with the consequent succinate accumulation. TRAP1 causes HIF1α stabilization even under normal oxygen tension, setting a pseudohypoxic phenotype instrumental in prompting tumor growth. In addition, ERK-dependent phosphorylation enhances this oncogenic effect of TRAP1.It is conceivable that, in analogy with its cognate chaperone Hsp90, TRAP1 has multiple interacting partners, and these could have relevant functions in the oncogenic process. In order to identify novel TRAP1 interactors, we used a mass spectrometry analysis on TRAP1 immunoprecipitated by a human glioblastoma cell model.
Among the potential TRAP1 partners fished out by MS, we found proteins involved in OXPHOS, tricarboxylic acid cycle and glutaminolysis. Interestingly, we found some subunits of ATP synthase, the central enzymatic complex in the process of energy conservation. We used different methods to confirm the interaction between TRAP1 and ATP synthase and we were able to observe such interaction in living cells with a new approach to detect protein-protein interactions based on tripartite split-GFP association. In addition, our data show that the absence of TRAP1 markedly inhibits the activity of ATP synthase dimers. Taken together, our data indicate that TRAP1 modulates ATP synthase activity.

Parkin mutation affects the clock genes-dependent energy metabolism

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Growing evidence highlights a tight connection between circadian rhythms, molecular clockworks and mitochondrial function. In particular, mitochondrial quality control and bioenergetics proved to undergo circadian oscillations driven by core clock genes. Parkinson's disease (PD) is a chronic neurodegenerative disease characterized by a selective loss of dopaminergic neurons. Almost half of the autosomal recessive forms of juvenile parkinsonism has been associated with mutations in the *PARK2* gene coding for parkin, shown to be involved in mitophagy-mediated mitochondrial quality control. The aim of this study was to investigate in fibroblasts from genetic PD patients carrying parkin mutations the interplay between mitochondrial bioenergetics and the cell-autonomous circadian clock. Using two different *in vitro* synchronization protocols, we demonstrated that normal fibroblasts displayed rhythmic oscillations of both mitochondrial respiration and glycolytic activity. Conversely, in fibroblasts obtained from PD patients a severe damping of the bioenergetics oscillatory patterns was observed. Analysis of the core clock genes showed deregulation of their expression patterns in PD fibroblasts, which was confirmed in induced pluripotent stem cells (iPSC) and induced neural stem cells (iNSC) derived thereof. The results from this study support a reciprocal interplay between the clockwork machinery and mitochondrial energy metabolism, point to a parkin-dependent mechanism of regulation and unveil a hitherto unappreciated level of complexity in the pathophysiology of PD and eventually other neurodegenerative diseases.

IF_1 promotes cancer cells survival under anoxic conditions

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The ATPase inhibitor, IF_1, is a mitochondrial protein that has raised interest due to its overexpression in many human cancers. However, what role IF_1 plays in the control of the mitochondrial F_1F_0-ATP synthase/ATPase activity in cancer cells is still a matter of debate [1-3]. The only IF_1 function stated, in ischemic cardiomyocytes, is the capability to inhibit the ATP hydrolytic activity of the ATP synthase to avoid cellular ATP-wasting [4].

Since the paucity of vasculature in solid tumors generates hypoxic/anoxic areas, we investigated the bioenergetics of both IF_1-expressing and IF_1-silenced tumor cells under hypoxic and anoxic conditions. In different tumor cell lines, we showed that IF1 preserves the ATP cellular levels by inhibiting the ATP-synthase hydrolytic activity in anoxia [5]. Under this condition, both mitophagy and mitochondrial biogenesis markers were found up-regulated in IF_1-expressing cells only. Indeed, the presence of IF_1 promoted a continuous renewal and preservation of the mitochondrial mass. Taken together, our results support the idea that IF_1 overexpression promotes cell survival under prolonged anoxia and favors proliferation as cells are re-oxygenated by preserving ATP level and providing functioning mitochondria.

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Zebrafish (*Danio rerio*) as a model to study the pathophysiological role of the mitochondrial chaperone TRAP1

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Expression of the Hsp90-family chaperone TRAP1 is restricted to mitochondria and increased in most tumor types. We have previously shown that TRAP1 elicits the stabilization of the transcriptional factors HIF1α, thus generating a pseudohypoxic state that supports neoplastic growth. Following oncogenic activation of the Ras/ERK pathway, TRAP1 is phosphorylated by ERK1/2, leading to inhibition of succinate dehydrogenase (SDH) and to the ensuing accumulation of the oncometabolite succinate that eventually stabilizes HIF1α. However, little is known about the timing and importance of TRAP1 induction during the process of neoplastic onset and growth, as well as on the role of TRAP1 in the physiology of non-transformed cells.

Here we exploit the Zebrafish (*Danio rerio*) model, to investigate how TRAP1 contributes to cell bioenergetics during embryonic development and in a model of neoplastic progression. We found that TRAP1 is highly expressed during the early stages of Zebrafish embryo development, but its levels are dramatically decreased at 96 (hpf). Knocking-out TRAP1 expression delays early phases of larvae development. Notably, we detected an increase in TRAP1 mRNA and protein levels after HIF1α stabilization that caused a down-regulation of SDH activity and mitochondrial respiration. An *in silico* analysis of Zebrafish and human TRAP1 promotor unveiled the presence of hypoxia-responsive elements motifs that are the target of HIF1 transcriptional activity. Moreover, we found that TRAP1 is absent in Zebrafish pancreas, but it is highly expressed in a model of pancreatic adenocarcinoma induced by $KRas^{G12D}$ expression, where it causes a strong decrease of SDH activity. Altogether, these data suggest the existence of a positive feedback loop between HIF1 α and TRAP1, in which HIF1α stabilization induces TRAP1 expression acting at the transcriptional level, and in turn TRAP1, through SDH inhibition, leads to HIF1 α stabilization. This regulatory mechanism could play an important role for the adaptation of cells to fluctuating level of oxygen both in embryogenesis and during the process of neoplastic transformation.

Dissecting mitochondria functions in brain, behavior and timing with the human disease gene LETM1

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The inner mitochondrial membrane protein LETM1 is a key player in mitochondrial cation and volume homeostasis. Above all, genotype-phenotype studies correlated hemizygous loss of LETM1 with epilepsy in the Wolf Hirschhorn Syndrome (WHS), a complex disorder caused by multiple mono allelic gene deletions on the chromosome 4 [1]. However, the identity of LETM1 as a potassium or calcium exchanger is still controversial and its substantial implication in many of the features observed in the WHS, in terms of brain function, metabolism and timing is not clear [2]. In our ongoing study, we investigate mitochondrial ion homeostasis, metabolism, brain function and circadian clock in order to understand the impact of LETM1, in the context of a human disease. We are using the zebrafish *Danio rerio* as a highly suitable model organism for disease and circadian clock studies, since like humans- but unlike mice or rats- it is a diurnal animal. We present novel data on the expression level of the core circadian clock genes in function of *letm1*, highlighting a nexus between LETM1 and rhythmicity. Whether LETM1 is a calcium or potassium exchanger is a critical information and will be assessed using our mutant *letm1* fish. Further investigation will clarify whether the clock deregulations are linked to the imbalance of cation homeostasis or metabolic defects.

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N-acetylcysteine serves as a substrate of 3-mercaptopyruvate sulfurtransferase and boosts sulfide metabolism in colon cancer cells

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Hydrogen sulfide (H_2S) is known to play a regulatory role in cell bioenergetics [1]. Endogenously synthesized by the cytosolic enzymes cystathionine β-synthase (CBS) and cystathionine γ-lyase (CSE) along with 3-mercaptopyruvate sulfurtransferase (MST), partly localized in mitochondria, H_2S is oxidatively catabolized in the mitochondrion, where H_2S-derived electrons are transferred to coenzyme Q by sulfide:quinone oxidoreductase (SQR), thus stimulating ATP production. Both MST and SQR contribute to formation of persulfides and polysulfides, collectively known as “sulfane sulfur”. Dysregulation of H_2S metabolism was reported in several cancer types, including colon cancer, where H_2S supports cellular proliferation and energy metabolism [2]. Oxidative stress is one of the cancer hallmarks. Nevertheless, recent clinical trials showed that antioxidants may not be beneficial or even adversely affect anticancer therapy [3]. Recently, N-acetylcysteine (NAC), a widely used pharmacological antioxidant, was suggested to act by increasing intracellular levels of sulfane sulfur [4]. Here, working on SW480 colon cancer cells, we evaluated the effect of NAC on H_2S metabolism. After exposing cells to 10 mM NAC for 24 hours, increased expression of MST and SQR (but not of CSE and CBS) was observed. Accordingly, based on colorimetric and fluorimetric assays, higher MST and SQR activities were detected in lysates of NAC-incubated cells. In addition, NAC was shown to persist for at least 24 hours inside colon cancer cells and be able to act as a substrate for human MST, as shown working on the isolated enzyme recombinantly produced. In conclusion, this work shows that chronic exposure of colon cancer cells to NAC stimulates H_2S metabolism, thus shedding new light on the mechanism of action of this drug and providing a possible explanation for its failure in anticancer therapy.

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A Stem Cell-Like Phenotype is Induced by Nandrolone through Inhibition of the Mitochondrial Respiratory Activity

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Nandrolone (ND) is a testosterone analogue with anabolic properties commonly abused worldwide, recently utilized also as therapeutic agent in chronic diseases, cancer included.

Here, we firstly investigated the impact of ND on the mitochondrial bioenergetics in an *in vitro* model of hepatocellular carcinoma. The results attained show that pharmacological dosage of ND significantly inhibited HepG2 cell growth hampering cell cycle and repressed severely the mitochondrial respiratory activity with no effect on glycolysis. The latter effect was traceable back to specific inhibition of the respiratory chain complexes I and III and accompanied with enhanced production of mitochondrial reactive oxygen (ROS) species. Intriguingly, ND caused a significant increase of the $CD133^+$ cells as well the expression of other stemness markers. Insightfully, antimycin A, a specific inhibitor of CxIII, mimicked the nandrolone-mediated up-regulation of $CD133^+$ cells, which was prevented by antioxidant co-treatment, thus resulting CxIII-ROS dependent.

Moreover, ND negatively affected differentiation of additional cell models such as the hematopoietic umbilical cord blood (UCB) $CD34^+$ stem/progenitor cells and the dental pulp mesenchymal stem (DPMS) cells, both primary normal stem cells. Notably, ND inhibited colony formation in $CD34^+$ cells and osteogenic differentiation of DPMS cells.

Finally and importantly, up-regulation of the stemness markers was also found *in vivo*, in healthy mice, particularly in kidney, liver and spleen tissues.

Our observations show, for the first time, that chronic administration of nandrolone, favouring persistence of stem cells in different tissues could represent a precondition that, in addition to multiple hits, could enhance risk of carcinogenesis raising warnings about its abuse and therapeutic utilization.

Cell metabolism profile of different embryonic stem cells

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Embryonic stem cells (ESC) are emerging as a composite of subpopulations with different self-renewal capacity and differentiative proficiency. *Zscan4* is a pluripotency key factor which marks ESC undergoing a transient metastate which is thought to confer to ESC higher genomic stability and reduced replicative senescence. In this study we combined proteomic, metabolomics and functional analyses to define the metabolic phenotype of highly purified zscan4+ ESC. The results attained unveils that arginase II, a mitochondria localized isoenzyme, is the major over-expressed protein in Zscan4+ cells (validated by WB, qRT-PCR and reduced level of intracellular arginine), likely linked to the metabolism of polyamines. Conversely the cellular level of methionine and aspartate were significantly higher. Metabolic flux analysis showed a more oxidative metabolic phenotype in Zscan4+ as compared with zscan4- ESC indicated by higher OCR/ECAR and NAD+/NADH ratios and mitochondrial production of reactive oxygen species. Interestingly, the relative contribution of pyruvate, long chain fatty acids and glutamine to the mitochondrial respiration was lower in Zscan4+ ESC suggesting reliance on alternative catabolic fuels thereof. Accordingly, a higher content of short chain acyl-carnitines was detected in Zscan+ ESC. All together the biochemical characterization emerged from this study suggests in Zscan+ transient ESC the occurrence of a distinctive metabolic signature which in addition to bioenergetic support provides epigenetic. substrates/modulators.

Role of Tryptophan 224 in the transport mechanism of the Mitochondrial Carnitine/Acylcarnitine Carrier.

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The mitochondrial carnitine/acylcarnitine carrier (CAC) belongs to the SLC25 family. Members of this family share common structural features. They are constituted by six transmembrane α-helices, which line a central cavity closed to the bottom by a salt bridges network of six amino acid residues. The substrate-binding site is located in the central cavity at about mid of the membrane.

CAC catalyzes carnitine/acylcarnitine antiport and, in the absence of counter-substrate, a slower carnitine uniport. The antiport results from two uniport reactions in opposite directions. Extensive conformational changes allow the transition of the carrier from an outward open conformation to an inward open one and vice versa. These transitions are much faster for the carrier-substrate complex than for the unbound carrier. Thus, we have investigated the molecular determinants that couple the binding of the substrate with the conformational transitions underlying the antiport mechanism. We have studied the role of Trp residues which are known to be crucial for gating and substrate recognition in some bacterial antiporters. Alteration of the transport function was observed in the mutant of CAC in which W224 was substituted with Alanine. This mutation led to loss of the antiport but not of the uniport function. In this mutant, the substrate binding on the external side of the transporter was impaired, while the binding on the internal site was not altered. The data highlights that W224 is crucial for coupling of the substrate binding with the matrix gate opening and hence for the antiport mechanism.

Oxidation state of methionine and cysteine residues in human mitochondrial Voltage-Dependent Anion Selective Channel Isoforms (hVDACs) investigated by high resolution mass spectrometry

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Recently, we have reported the characterization of cysteines over-oxidation of VDACs in rat liver mitochondria (rVDACs) by means of an “in solution” proteolysis and UHPLC/High Resolution nanoESI-MS/MS procedure originally developed by us [1].

Using the same approach, this structural investigation has now been extended to the hVDACs, obtained by mitochondria from HAP1 cells. Our results demonstrate that, in physiological state, Cys^{127} in hVDAC1 is detected exclusively as cysteic acid, while Cys^{232} is observed totally reduced. In hVDAC2, cysteine residues 8, 13, 133, and 227 are exclusively reduced, Cys^{138} is trioxidized, and the cysteines 47, 76,103, and 210 are partially in the form of cysteic acid. In hVDAC3 cysteines 2, 8, 122, and 229 are reduced and Cys36 and Cys65 partially oxidized. This peculiar behavior of hVDACs may be related with the regulation of the activity of this trans-membrane pore proteins.

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Metabolic adaptation in colorectal cancer: heme export is required for the down-modulation of the tricarboxylic acid cycle

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Heme synthesis is a constitutive process, crucial for oxidative metabolism as heme is a key component of the electron transport chain complexes and heme biosynthesis, by consuming succynil-CoA, is a tricarboxylic acid (TCA) cycle cataplerotic pathway. Moreover, heme production is one of the major cellular iron-consuming processes, competing with the biogenesis of iron-sulfur (Fe-S) clusters, key cofactors for both TCA cycle and electron transport chain enzymes.

Here we show that the heme exporter Feline Leukemia Virus subgroup C Receptor 1 (FLVCR1) is overexpressed in colorectal cancer. Our data demonstrate that increased heme export is required in colorectal cancer cells to sustain heme biosynthesis, in order to down-modulate the TCA cycle and respiratory chain activity. Indeed, *FLVCR1a* silencing results in reduced heme synthesis, increased TCA cycle flux, enhanced TCA cycle anaplerosis and high oxidative phosphorylation. Inhibited heme synthesis in *FLVCR1a* down-modulated cells is associated to increased activity of Fe-S cluster containing enzymes that contribute to potentiate cell respiration. The metabolic alterations caused by the inhibition of heme biosynthesis lead to mitochondrial dysfunction and impaired cancer cell survival/proliferation both *in vitro* and *in vivo*.

Our data support a model in which the heme synthesis/heme export axis modulates TCA cycle flux and oxidative metabolism by regulating the availability of succynil-CoA and heme and, indirectly, that of Fe-S clusters. This work identifies the heme synthesis/heme export axis as a targetable site to interfere with metabolic adaptation of cancer cells, a key process for tumor cell growth, dissemination, immune escape and establishment of drug resistance.

Effects of cholesterol on the human ASCT2 amino acid transporter

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The human ASCT2 (SLC1A5) transporter mediates Na^+-dependent antiport of neutral amino acids across plasma membrane that balances intracellular amino acid pools. Despite the acronym (Ala, Ser, Cys Transporter 2), Cys is not a substrate but acts as a modulator and Gln is the preferred substrate. Gln taken up by ASCT2 is also used for energy purposes under proliferative conditions in both physiological and pathological states. Indeed, the transporter is over-expressed in stem cells and in most human cancers. To characterize the human transporter, the protein was produced in *P. pastoris*, purified and assayed in proteoliposomes for transport function. A functional and kinetic asymmetry has been demonstrated. The kinetic mechanism of the transport is random simultaneous in agreement with the recently solved trimeric structure of the protein [1]. Using the proteoliposome experimental model the effect of cholesterol on the transport activity of the human ASCT2 was studied. Cholesterol (7% of total lipids) stimulated Gln transport. Kinetic analysis showed increased Vmax with respect to the control while the Km for Gln did not change. hASCT2 harbors in its structure cholesterol binding motifs, such as the CARC-CRAC at the level of transmembrane segment 6 and the R-W-L between the scaffold and the lift domains of the protein. Cys residues present in the CARC motif could be critical for the binding/effect of cholesterol. This was confirmed by the different IC50 values for $HgCl_2$ and MTSES in the presence or absence of cholesterol. Trp residue(s), including the one present in the R-W-L motif, may be also involved in the interaction with cholesterol. Indeed, targeting of Trp residues with the Koshland's reagent, lowered the transport stimulation by cholesterol.

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A mitochondrial therapy for Duchenne muscular dystrophy

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Duchenne muscular dystrophy (DMD) is a life-threatening X-linked muscle disease caused by mutations in the DMD gene encoding for dystrophin. It is characterized by progressive muscle degeneration and an effective, or generally applicable therapy is lacking. The presence of mitochondrial dysfunction during the disease pathogenesis was widely studied. Opening of the mitochondrial Permeability Transition Pore (PTP), due to an increase of [Ca^{2+}] in both sarcoplasm and mitochondria, is one of the main mechanisms leading to muscle fiber death. Since the *mdx*$^{-/-}$ mouse (the best-characterized mouse model of the disease) displays a rather mild dystrophic phenotype, we took advantage of the severe *sapje* zebrafish mutant, which lacks dystrophin and shows muscle defects close to those of DMD patients. Our aim is to explore *in vivo* a possible mitochondrial therapy targeting the PTP with non-immunosuppressive derivatives of cyclosporine A and new PTP inhibitors developed by our research group. As previously observed [1] in muscle biopsies from DMD patients, we showed that *sapje* zebrafish (i) display a dramatic disruption of muscle structure, (ii) a strong decrease of respiratory reserve capacity and (iii) are prone to mitochondrial dysfunction due to opening of the PTP, whose features in zebrafish are the same as those of mammals. Treatment with the FDA-approved cyclophilin inhibitor Alisporivir - a cyclosporin A derivative that desensitizes the PTP but does not inhibit calcineurin - and the new PTP inhibitor MF1 extended *sapje* life and led to a striking recovery of muscle structure, motor abilities and respiratory function. As Alisporivir has an excellent safety profile, it could be used in combination with MF1 for treatment of DMD.

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Cyclophilins in Duchenne muscular dystrophy

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Duchenne muscular dystrophy (DMD) is a severe X-linked muscle disease caused by mutations in the *DMD* gene encoding for dystrophin. Muscle weakness, low resistance to stress and impairment of degeneration/regeneration balance appear during disease progression. Our hypothesis is that a progressive increase of Ca^{2+} levels in the sarcoplasm of the affected fibers leads to mitochondrial dysfunction that eventually triggers fiber death. One of the potential mechanisms involved in DMD pathogenesis is opening of the mitochondrial permeability transition pore (PTP), the activity of which is positively regulated by Cyclophilin (CyP) D and high Ca^{2+} levels. PTP is desensitized by Cyclosporin A and by its non-immunosuppressive analogs NIM811 and Alisporivir, which inhibit CyPD. Alisporivir treatment restored normal mitochondrial function, respiration, muscle ultrastructure and improved survival of *sapje* zebrafish, a severe model of DMD [1]. However, Alisporivir became less effective at concentrations higher than 5 µM. Our experiments reveal that Alisporivir decreases the levels of CyPB, the isoform present in the endoplasmic reticulum, both in cells and in zebrafish. We also found that CyPB levels are decreased in the *sapje* zebrafish. Knockdown of CyPB in zebrafish affected embryo development and resulted in increased expression of proteins involved in Store Operated Ca^{2+} Entry (SOCE), which could contribute to an overall rise in Ca^{2+} levels. These data suggest that decreased CyPB levels in *sapje* zebrafish may contribute to disease pathogenesis, and that further decrease induced by Alisporivir may be at the basis of its decreased efficacy at higher concentrations.

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Ca^{2+}-dependent high-conductance channel activity of F-ATP synthase matches the permeability transition pore

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Permeability transition (PT) in mitochondria leads to increased permeability to ions and solutes of the inner mitochondrial membrane (IMM) and finally to cell death. PT is mediated by a Ca^{2+}-regulated pore, named the Permeability Transition Pore (PTP) and identified, by means of patch clamp on mitoplasts, as a high-conductance channel, named Mitochondrial Mega Channel (MMC). Despite many years of study the molecular nature of the pore remains controversial. In 2013 Giorgio et al. provided evidence that dimers of mammalian F_OF_1 (F)-ATP synthase purified from native gels can form the PTP/MMC [1] but the exact molecular mechanism is still unclear. F-ATP synthase was purified from bovine hearts using the very mild, lipid-like detergent LMNG [2] and analyzed by clear native PAGE, SDS-PAGE, mass spectrometry, and negative stain electron microscopy. The protein was shown: (i) to be intact and inclusive of all subunits; and (ii) to be active and highly sensitive to oligomycin both in its soluble form and after reconstitution in proteoliposomes. After incorporation into planar lipid bilayers we tested whether mammalian F-ATP synthase generates high conductance channels by electrophysiology. Channel activity was elicited by Ca^{2+} and potentiated by Bz-423, a specific activator of the MMC/PTP, and was indistinguishable from activity of the *bona fide* MMC/PTP measured in the native inner membrane by patch-clamp experiments. The channel maintained sensitivity to MMC/PTP inhibitors but not to bongkrekate and König's polyanion.

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Biolog Mitoplates™: a novel tool to assess mitochondrial functions in eukaryotic cells

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We have developed a new mitochondrial function assay technology that measures the rates of metabolism of mitochondrial substrates and the sensitivity of metabolism of these substrates to mitochondrial inhibitors. The technology employs saponin permeabilized cells and a redox dye added to 96-well microplates that contain mitochondrial substrates or inhibitors precoated and dried into the wells. The MitoPlate S-1™ has a triplicate repeat of a set of 31 substrates. Mitochondrial function is assayed by measuring the rates of dye reduction from electrons flowing into and through the electron transport chain from substrates whose oxidation produces NADH (e.g., L-malate) or FADH2 (e.g., succinate). The electrons donated to complex 1 or complex 2 travel to the distal portion of the electron transport chain where a tetrazolium redox dye (MC) acts as a terminal electron acceptor and changes from colorless to a purple formazan upon reduction. All 96 assays in the MitoPlate are run concurrently, and each assay provides different information because each substrate follows a different metabolic route using different transporters to enter the mitochondria, and then different dehydrogenases to produce NADH or FADH2. The MitoPlate S-1™ can also be used to assess the activity and specificity of substrate transport inhibitors, dehydrogenase inhibitors, or electron transport chain inhibitors. A second assay plate, the MitoPlate I-1™, provides another assessment of mitochondrial function by measuring the sensitivity of mitochondrial electron flow to a set of 22 diverse inhibitors titrated at 4 dilutions. The I-1 plates can be run using any of the NADH or FADH2 producing substrates, each providing additional information. Using these new assays we show that the mitochondria from different cell types exhibit different functional properties. This new technology will assist efforts to understand how mitochondria change in cell models of human disorders that have a mitochondrial basis.

Mitochondrial signature in human ovarian cancer tissue: involvement of cAMP pathway, SIRT3, OPA1, DRP1 and PHB2 proteins

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Abstract

Ovarian cancer (OC) is the most lethal gynaecological cancer for lack of early symptoms, effective diagnostic approach and relapse of the disease. Apoptosis resistance is one of the key points in OC development. Mitochondria play a critical role in apoptosis and have been proposed as target for therapy in OC [1, 2]. We evaluated in OC tissues cAMP/PKA signalling, and proteins like SIRT3, OPA1, DRP1 and PHB2, which are involved in mitochondrial dynamics and morphology, strictly related to apoptosis [3].

Ovarian tissue from fifteen controls and fifteen patients affected by OC were collected. Twenty-seven biochemical parameters were analyzed in homogenate such as enzymatic activities, mtDNA content, mitochondrial number and morphology. Statistical analysis were performed by Student' t-test, Mann-Whitney's and principal component analysis (PCA).

We found in OC tissues activation of cAMP/PKA pathway and increased SIRT3 protein level, both associated with augmented mitochondrial biogenesis, as revealed by increased PGC1α and TFAM protein levels, mtDNA content and mitochondrial number. Furthermore, loss of DRP1 function and increased OPA1 and PHB2 levels were found in OC, leading to altered mitochondrial dynamics and structure as highlighted by decreased diameter of crista junction.

The presented data show that ovarian cancer is characterized by a mitochondrial signature associated with resistance to apoptosis. In addition, the PCA of the biochemical parameters analyzed, allows to classify ovarian cancer patients in a distinct group from control subjects and endorses the effective molecular signature.

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Effect of obethicholic acid on liver mitochondria in a model of secondary biliary cirrhosis

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INTRODUCTION: Cholestasis occurs in many chronic human diseases including primary biliary cholangitis (PBC), primary sclerosing cholangitis, allograft rejection, iatrogenic obstruction of bile ducts and biliary atresia. Mitochondrial dysfunction and redox balance alteration are critical features in the pathogenesis of chronic cholestasis. Obeticholic acid (OCA) is a semi-synthetic bile acid analogue which has been recently approved for the treatment of PBC. However, the mechanism of OCA on secondary cholestasis are not known. The present study aimed at investigating the impact of OCA treatment in a model of secondary cholestasis, pointing out its effect on liver mitochondria.

METHODS: Rats were randomly distributed in three groups: sham operated (SHAM), bile duct ligation (BDL) and BDL treated with OCA (BDL+OCA). Rats were sacrificed after 3 weeks of surgery. We analysed serum alkaline phosphatase (ALP) as marker of cholestasis, and liver histology. Oxygen uptake and Respiratory Control Index (RCI), membrane potential, and permeability transition pore (mPTP) opening were determined on freshly isolated liver mitochondria.

RESULTS: OCA treatment was associated to a limited increase of serum ALP and less hepatic injury in BDL-operated rats. The BDL group showed a reduction of Complex I-linked state 3 respiration (and consequently of RCI) with respect to SHAM; however, this impairment was also observed in the BDL+OCA group. Nevertheless, the collapse of mitochondrial membrane potential reported in BDL was absent in BDL+OCA group. H_2O_2 production rate was significantly reduced in liver mitochondria from BDL+OCA rats as compared to BDL group. Finally, the opening of mPTP was induced by a low Ca^{2+} concentration in BDL with respect to SHAM: this effect was limited by OCA treatment.

DISCUSSION: Rats that undergo bile duct ligation exhibit liver mitochondria dysfunction, which leads to bioenergetics impairment and over-production of reactive species. In fact, chronic cholestasis is associated with a reduction of respiratory function, as well as collapse of membrane potential, which lead to increased hydrogen peroxide production and mPTP opening. OCA treatment limits the damaging effects of chronic cholestasis on hepatocytes, preserving mitochondrial function and preventing severe liver damage.

Proteomics-driven analysis reveals a dysregulation of a key mitochondrial protein in Short-Term Type-1 Diabetes Mellitus

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Type-1 Diabetes (T1D) is a metabolic disorder characterized by marked hyperglycemia that induces end-organ damages; it has been proposed that mitochondrial dysfunction plays a key role in T1D pathogenesis [1]. Here, employing an experimental model of T1D, we investigated by proteomic analysis the total lysate fractions of control and diabetic animals during the early phase of T1D. Among the proteins that showed a statically significant variation, we reported the overexpression of the mitochondrial Inhibitory Factor 1 (IF_1) [2] in the diabetic condition. This increment was positively correlated with the augmented level of its mRNA in the diabetic condition, in which a general derangement of the bioenergetic parameters and ROS levels was found. Next, we showed that the IF_1 increase in T1D condition induced a marked decrement of mitochondrial ATP hydrolase activity, while the ATP synthase activity was unaffected. As it has been reported that HIF-1α is a master regulator of IF_1 expression, we investigated, in our experimental conditions, its mRNA and protein levels. Interestingly, we found a significant increase of the HIF-1α protein content in the T1D, while no difference was reported in the mRNA level. Overall, our data strongly suggest a critical role of IF_1 during the early phase of T1D.

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Development of treatment strategies for MPAN, a sub-type of Neurodegeneration with Brain Iron Accumulation (NBIA)

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MPAN (Mitochondrial membrane Protein Associated Neurodegeneration) belongs to a family of rare and lethal neurodegenerative diseases named NBIA (Neurodegeneration with Brain Iron Accumulation), due to the recurrent observation of iron deposits in the brain. Characterized by parkinsonism, general dystonia and optic atrophy, MPAN is defined by pathogenic variants in the gene *C19orf12*, which encodes for a protein localizing to mitochondria, ER, and MAM. So far, C19orf12 has been suggested to be involved in autophagy initiation by promoting the formation of autophagosomes. To better understand the molecular mechanisms underlying the pathology of MPAN and to develop new treatment strategies for the disorder, we investigated the role of C19orf12 in the cell and performed a drug screening in a *Drosophila* model of MPAN. The involvement of C19orf12 in autophagy was assessed by immunocytochemistry. Results showed that compared to control cells, fibroblasts isolated from MPAN patients lacking C19orf12 generate fewer autophagosomes and are unable to remove depolarized mitochondria, thus confirming the predicted role of C19orf12 in the initial steps of autophagy and suggesting an involvement of C19orf12 in mitophagy. Having established a link between the absence of C19orf12 and the impairment of autophagy, we screened 153 regulators of autophagy in an unpublished *Drosophila* model of MPAN. The model, generated by downregulating the two orthologues of *C19orf12*, shows a lethal phenotype as flies are unable to eclose from pupae. 14 of the screened compounds were able to rescue the phenotype. Their therapeutic efficacy will be further evaluated in a published *Drosophila* model of MPAN, showing clear signs of neurodegeneration, and in neurons differentiated from patient-derived iPS cells, currently being established in our lab. The most promising drug will be selected for a clinical trial.

Increased Levels of cAMP by the Calcium-Dependent Activation of Soluble Adenylyl Cyclase in Parkin-Mutant Fibroblasts

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Abstract

Parkin-mutant (Pt) fibroblasts, exhibiting defective mitochondrial respiratory/OxPhos activity, showed a significant higher value of basal intracellular level of cAMP, as compared with normal fibroblasts (CTRL) [1,2]. cAMP is a major second messenger regulating mitochondrial metabolism, and it strictly interlocked with calcium homeostasis. Specific pharmacological inhibition/activation of members of the adenylyl cyclase and of the phosphodiesterase families, respectively, as well as quantitative reverse transcription polymerase chain reaction (RT-qPCR) analysis, indicate that the higher level of cAMP observed in Pt fibroblasts can contribute to a higher level of activity/expression by soluble adenylyl cyclase (sAC) and to low activity/expression of the phosphodiesterase isoform 4 (PDE4). As Ca^{2+} regulates sAC, we performed quantitative calcium-fluorimetric analysis, showing a higher level of Ca^{2+} in the both cytosol and mitochondria of Pt fibroblasts as compared with CTRL. Most notably, inhibition of the mitochondrial Ca^{2+} uniporter decreased, specifically the cAMP level in PD fibroblasts. All together, these findings support the occurrence of an altered mitochondrial Ca^{2+}-mediated cAMP homeostasis in fibroblasts with the parkin mutation.

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Iron availability regulates cancer related skeletal muscle wasting

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Iron is both an essential nutrient required for a plethora of cellular functions and a dangerous catalyst of free radical reactions if in excess or miscompartmentalized. While it is known that iron metabolism is strongly disrupted in cancers as seen with the high prevalence of iron-deficiency anaemia among cancer patients, little is known about the consequence of a low iron availability on muscle mass maintenance. Given the crucial role of iron containing proteins in energy metabolism, we speculated that decreased iron availability might contribute to skeletal muscle atrophy by altering mitochondrial function. Recently, we found that altered systemic iron metabolism is a signature of the wasting process in several *in vivo* models of cancer related muscle wasting, as reflected by a depletion of transferrin receptor levels coupled to an upregulation of ferroportin in skeletal muscle, leading to decreased cytosolic iron level. Coherently, we found profound alterations in mitochondrial function such as a drastic decreased in the activity of the iron sulfur cluster protein aconitase. Interestingly, the reduction of iron availability by several means (siTfR and selective chelators) triggered skeletal myotube atrophy *in vitro*, while iron ionophore or supplementation was sufficient to prevent and even rescue myotube atrophy induced by different cancer cell lines. Finally, intravenous injection of iron in mice bearing C26 colon-carcinoma reduced atrophy compared to the controls. Taken together these results suggest that dysregulated iron homeostasis contributes to cancer related muscle wasting and we are currently investigating the patwhays whereby iron controls skeletal muscle mass.

The human SLC38A9 transceptor: insights into the transport side and structure/function relationships.

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The amino acid transceptor SLC38A9 belongs to the SLC38 family that includes eleven members some of which are well characterized in cell systems and are historically classified in systems A and N according to transport mode and amino acid(s) recognized as substrate(s). Recently, the lysosomal amino acid transporter SLC38A9 has been deorphanized revealing its intriguing function as transceptor, i.e. a transporter with a receptor function: the protein is responsible for coupling amino acid sufficiency into lysosomes to mTORC1 recruitment to the lysosomal membrane for regulating cell growth and metabolism. The receptor function, relies on the presence of a 111 amino acids N-terminal tail that interacts with lysosomal RAG proteins upon amino acid translocation [1]. To characterize the transport side, the hSLC38A9 was produced in *E.coli*, purified and reconstituted in liposomes. In this model, Gln and Arg revealed to be the preferred substrates; a cooperative behavior and regulation by cholesterol was showed for both substrates. By combining bioinformatics to site-directed mutagenesis, two binding sites for cholesterol (CARC/CRAC) were described. hSLC38A9 revealed to be competent for Gln efflux, while Arg seems to play only a regulatory function from the intralysosomal side. Accordingly, bioinformatics and inhibition experiments showed that Gln and Arg may interact with different sites of hSLC38A9. A deletion mutant of the N-terminal tail was produced and transport of Gln was assayed revealing that the receptor domain is not involved in the intrinsic transport function of hSLC38A9.

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Electrophysiological characterization of channel formed by F-ATP synthases

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The permeability transition (PT) defines an increase in inner mitochondrial membrane permeability to ions and solutes leading to a disruption of mitochondrial function and eventually to cell death. PT is mediated by the opening of a large conductance channel, the mitochondrial permeability transition pore (PTP), whose molecular nature is highly debated [1]. A recent hypothesis posits that it originates from a conformational change occurring on the F-ATP synthase after Ca^{2+} binding; evidence supporting this hypothesis was obtained by monitoring PTP currents after reconstruction of purified proteins in planar lipid bilayer experiments [2] or with patch-clamp measurements on isolated mitoplasts [3].

Here, we measured channel activity in mitoplasts obtained after genetic ablation of F-ATP synthase subunits f and from ρ0 cells lacking mtDNA and therefore F-ATP synthase subunits a and A6L. Our preliminary results indicate that the basic features of the PTP are conserved in ρ0 cells while PTP channel activity is altered in f-subunit knockout mitoplasts, with lower maximal conductance and very few openings. Further electrophysiological investigations will be required to elucidate how the energy-conserving F-ATP synthase can be turned into the energy-dissipating PTP.

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Molecular identification and functional characterization of a novel glutamate transporter in yeast and plant mitochondria.

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The importance of glutamate is well established in human as well as in plants and *fungi* where mitochondria play a key role in its metabolism[1,2]. Glutamate is transported inside and/or out from mitochondria by proteins localized in the inner mitochondrial membrane.

The genome of *S. cerevisiae* encodes 35 members of the mitochondrial carrier family (MCF) and that of *A. thaliana* 58 MCF members. Here two members of this family, Ymc2p from *S. cerevisiae* and BOU from Arabidopsis, have been characterized. These proteins were overproduced in bacteria and reconstituted into liposomes. Their transport properties and kinetic parameters demonstrate that Ymc2p and BOU transport glutamate. Transport catalyzed by both carriers was saturable, inhibited by mercuric chloride and dependent on the proton gradient across the proteoliposomal membrane. The growth phenotype of *S. cerevisiae* cells lacking the genes *ymc2* and *agc1*, which encodes the only other *S. cerevisiae* carrier capable to transport also glutamate, was fully complemented by expressing Ymc2p, Agc1p or BOU. Furthermore, mitochondria isolated from wild-type, *ymc2*Δ and *agc1*Δ strains, but not from the double mutant *ymc2*Δ*agc1*Δ strain, swell in isosmotic ammonium glutamate showing that glutamate is transported together with a H+. It is proposed that the primary function of these proteins is to import glutamate into the mitochondrial matrix for energy production, C1 metabolism and protein synthesis.

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Function and dysfunction of human riboflavin transporters

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Riboflavin, otherwise known as vitamin B2, is an essential dietary component and represents the precursor of flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD), the redox enzymatic cofactors required for mitochondrial terminal metabolism and for the functionality of mitochondrial respiratory chain. Protein folding, ROS production and defense, as well as redox epigenetics also depend on cellular supply of FAD [1].

FAD formation in different cells starts from riboflavin uptake, which occurs via specialized carrier-mediated processes which are supported by three specific members of the solute carrier family 52 (SLC52A), identified and named respectively RFVT1, RFVT2 and RFVT3. Alterations of RFVTs have been correlated with rare inherited neuro-muscular disorders, some of which treatable with high doses of the vitamin [2].

To better study structure-function relationships in these human diseases we produced the human RFVT2 transporter by over-expression in *E. coli* and we reconstituted the purified protein in proteoliposomes for transport assay.

We also pointed our attention on a profound alteration of flavin cofactor homeostasis in human colorectal [3] and some other types of cancer, which are accompanied by dysregulation of RFVTs expression. Changing the level of RFVTs expression as a possible mean to reprogram flavoproteome will be discussed.

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Post-translational analysis of VDAC1 revealed a marginal role of kinase Nek1 in the phosphorylation of the pore

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The NIMA (Never In Mitosis gene A) related kinase 1, also known as Nek1, participates in the response to the DNA damage after UV exposure or oxidative stress, promoting the cell cycle arrest by phosphorylation of Chk proteins [1]. In addition, mutations in Nek1 gene were recently associated to the neuromuscular disease Amyotrophic Lateral Sclerosis (ALS) [2]. Beyond its role in the cell cycle regulation, it has been demonstrated that Nek1 localizes in the outer mitochondrial membrane (OMM) where it interacts with the Voltage-Dependent Anion selective Channel (VDAC) isoform 1, the main pore of the OMM [3]. VDAC1 is directly involved in the control of metabolic flux across the OMM and actively participates in the regulation of apoptosis; not coincidentally, VDAC1 plays a crucial role in neurodegeneration and, particularly, in ALS [4]. It has been proposed that Nek1 specifically phosphorylates the serine 193 of VDAC1 [3] and this could have a strong impact for apoptosis regulation and, possibly, for the mitochondrial dysfunction typical of ALS. To clearly understand the relationship between Nek1 and VDAC1, the gene encoding the kinase was inactivated in the haploid cells HAP1 and the level of phosphorylation of VDAC1 was analyzed by high-resolution mass spectrometry, using the wild-type cells as a control. Our results indicate that no phosphorylation of VDAC1 serine 193 was found in wild-type cells, while the serine 104 resulted significantly phosphorylated in the same sample, with a ratio of 1:100. This result suggests the presence of a different phosphorylation pathway of VDAC1 than those proposed in [3]. Furthermore, no remarkable changes were found in Nek1$^{-/-}$ cells, indicating that VDAC1 is not a downstream target of Nek1. Overall, our results indicate that the involvement of Nek1 in ALS is independent from VDAC1.

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Baracca Alessandra
Barile Maria
Bellanti Francesco
Bernardi Paolo
Cannino Giuseppe
Capitanio Nazzareno
Carrer Andrea
Cela Olga
Console Lara
Dao Pauline
De Pinto Vito
De Rasmo Domenico
Della Sala Gerardo
Ferretta Anna
Fiorito Veronica
Forte Elena
Foti Salvatore
Francia Francesco
Galber Chiara
Giuffrè Alessandro
Gnoni Antonio
Gorgoglione Ruggiero
Hierro-Yap Carolina
Indiveri Cesare
Iuso Arcangela
Ivontsin Leonid
Laquatra Claudio
Leone Piero
Lippe Giovanna
Liuzzi Francesca
Lopriore Piervito
Luévano Martínez L.A.
Mashkovtseva Elena
Mazza Tiziano
Napolitano Ottavio
Nowikovsky Karin
Pacelli Consiglia
Palese Luigi Leonardo
Palmieri Luigi
Piccoli Claudia
Pittalà Maria G.G.
Porcelli Vito
Rossiello Michela
Russo Silvia
Saletti Rosaria
Scalise Mariafrancesca
Schiavone Marco
Schoepp-Cothenet Barbara
Scrima Rosella
Signorile Anna
Smolina Natalia
Solaini Giancarlo
Stocco Anna
Tamma Mirko
Tatti enrico
Taurino Federica
Titova Viktoriia
Tolomeo Maria
Tonazzi Annamaria
Tucci Francesco
Urbani Andrea
Vidyasagar Naik Bukke
Wyart Elisabeth
Zancani Marco
Zuhra Karim