





54<sup>th</sup> National Meeting of the Italian  
Society of Biochemistry and  
Molecular Biology  
(SIB)

23<sup>rd</sup> - 27<sup>th</sup> September 2009  
Città Universitaria - Catania



# Scientific Programme

## Wednesday 23<sup>rd</sup> September 2009

Città Universitaria, Dipartimento di Matematica - Via S. Sofia 64, Viale A. Doria, 6

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- 08:30 Registration of SIB attendants (Aula CUS)  
09:00-13:30 Meeting of SIB groups (Dipartimento di Matematica, rooms 2, 3, 126, 127, 128)  
09:00-11:00 Gruppo Sviluppo, Differenziamento e Apoptosi (Dipartimento di Matematica, room 128)  
11:30-13:30 Gruppo Biochimica della Nutrizione (Dipartimento di Matematica, room 128)  
09:00-11:00 Gruppi Biochimica Cellulare ed Immunologia Biochimica (Dipartimento di Matematica, room 3)  
11:30-13:30 Gruppo Nucleotidi, Acidi Nucleici e Genomi (Dipartimento di Matematica, room 3)  
09:00-11:00 Gruppo di Neurochimica (Dipartimento di Matematica, room 127)  
11:30-13:30 Gruppo Membrane e Bioenergetica (Dipartimento di Matematica, room 127)  
09:00-11:00 Gruppo Ammine Biogene (Dipartimento di Matematica, room 126)  
09:00-13:30 Simposio congiunto Gruppi "Proteine" ed "Enzimologia e regolazione metabolica": Evolution and regulation of the enzymatic function (Dipartimento di Matematica, room 2)

*Chairpersons:* MENICO RIZZI (Novara)  
MARIA ANTONIETTA VANONI (Milano)

- 09:00 GIANFRANCO GILARDI (University of Turin)  
Going beyond nature? The making of new enzymes by directed evolution  
09:30 ALESSIO PERACCHI (University of Parma)  
Catalytic DNA: shedding light on substrate binding and catalysis by a small deoxyribozyme  
10:00 FABRIZIO CHITI (University of Florence)  
The birth of the enzymatic function along the folding pathway  
10:30 Presentation selected from submitted abstracts  
11:00 *Coffee break*  
11:30 MARIA DI GIROLAMO (Consorzio Mario Negri Sud)  
The evolution of macro-domains as target-recognition sites within enzymes that regulate ADP-ribosylation  
12:00 NADIA RAFFAELLI (University Polytechnic of Marche)  
Linking enzymatic function to transcription regulation: the case of NAD biosynthesis  
12:30 STEFANIA HANAU (University of Ferrara)  
X-ray escaping conformational changes affecting the enzymatic function  
13:00 Presentation selected from submitted abstracts  
13:30 *Lunch*  
14:00-15:00 **Poster Session 1**  
17:00 Opening Ceremony Aula Magna, Palazzo Centrale Università di Catania (Piazza Università)  
Welcome Addresses  
ANTONINO RECCA (Rector of the University of Catania)  
GIUSEPPE RONSISVALLE (Dean of the Faculty of Pharmacy, University of Catania)  
ANTONIO DE FLORA (SIB President)  
ANNA MARIA GIUFFRIDA STELLA (Meeting Honorary President)  
ANGELO VANELLA (Meeting President)  
18:00 ANTONINI LECTURE "Emilia Chiancone" (University "La Sapienza" of Rome)  
Dps proteins, an efficient means to detoxify iron and protect DNA in the bacterial response to oxidative stress  
19:00 *Cocktail*

## Thursday 24<sup>th</sup> September 2009

Città Universitaria, Dipartimento di Fisica, Aula Magna - Via S. Sofia 64, Viale A.Doria 6, Catania

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### 08:30-11:00 **Symposium A**

*Biochemistry and biotechnology of cell engineering*

*Chairpersons:* CESARE BALDUINI (Pavia)

MAURO MAGNANI (Urbino)

- 08:30 GIUSEPPE ASTORI (Cell Therapy Unit, Cardiocentro Ticino, Lugano)  
What you must know if you would like to enter in the cell therapy arena
- 09:00 PAOLA ROMAGNANI (Excellence Center for Research, Transfer and High Education Denothe, University of Florence)  
Toward the identification of a human renal progenitor system
- 09:30 MAURO MAGNANI (University of Urbino)  
Engineering red blood cells for the delivery of drugs and contrasting agents
- 10:00 Presentation selected from submitted abstracts
- 10:30 *Coffee break*

### **Removal of posters Session 1**

### 11:00-13:30 **Symposium B**

*Molecular mechanisms of neurodegenerative diseases*

*Chairpersons:* ANNA MARIA GIUFFRIDA STELLA (Catania)

TOMMASO RUSSO (Napoli)

- 11:00 Agata Copani (University of Catania)  
Beta-amyloid: not just toxic anymore
- 11:30 GIUSEPPE ROTILIO (University "Tor Vergata" of Rome)  
New insights into the role of oxidative stress in ageing and neurodegeneration
- 12:00 GIORGIO LENA Z (University of Bologna)  
Mitochondrial Complex I and neurodegeneration
- 12:30 VINCENZO NICOLETTI (University of Catania)  
Glycating conditions affect aggregation and toxicity of amyloidogenic peptides
- 13:00 GIANFRANCESCO GORACCI (University of Perugia)  
Brain low molecular weight phospholipases A2 (sPLA2): role in neuronal functions and in neurodegenerative diseases
- 13:30 *Lunch*

### 14:00-15:00 **Poster Session 2**

### 15:00-17:00 **Symposium C**

*micro-RNA: Novel players of gene regulation*

*Chairpersons:* GIUSEPPE MACINO (Roma)

IRENE BOZZONI (Roma)

- 15:00 GIUSEPPE MACINO (University "La Sapienza" of Rome)  
The micro RNA world
- 15:30 ANA EULALIO (Max-Planck Institute for Infection Biology, Berlin)  
Mechanisms of micro RNA-mediated gene silencing
- 16:00 IRENE BOZZONI (University "La Sapienza" of Rome)  
Role of small non coding RNAs in the physiopathology and therapy of Duchenne Muscular Dystrophy
- 16:30 LUIGI NALDINI ("S. Raffaele" Hospital of Milan)  
Exploiting and antagonizing microRNA regulation for therapeutic applications
- 17:00 *Coffee break*

17:30-20:00 **Symposium D**

***Epigenetic modifications in health and disease***

*Chairpersons:* LORENZO CHIARIOTTI (Napoli)  
FILIBERTO CIMINO (Napoli)

- 17:30 PAOLA CAIAFA (University "La Sapienza" of Rome)  
Epigenetics: poly(ADP-ribosyl)ation of PARP-1 regulates genomic methylation patterns
- 18:00 GIOACCHINO NATOLI (European Institute of Oncology, of Milan)  
The epigenome and the control of inflammatory gene expression
- 18:30 NICOLETTA LANDSBERGER (University of Insubria, Varese)  
Molecular genetics of Rett Syndrome: when epigenetic signals go unrecognized
- 19:00 LORENZO CHIARIOTTI (University "Federico II" of Naples)  
Epigenetics of human behaviour
- 20:00 **Removal of posters Session 2**
- 21:30 *Party, wine and cheese*  
(Città Universitaria Viale A. Doria, 6; Via S. Sofia 64, Catania)

**Friday 25<sup>th</sup> September 2009**

Città Universitaria, Dipartimento di Fisica, Aula Magna, Via S. Sofia 64, Viale A.Doria 6, Catania

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08:30-11:00 **Symposium E**

***Glycobiology of human diseases***

*Chairpersons:* ALESSANDRO PRINETTI (Milano)  
BRUNO VENERANDO (Milano)

- 8:30 JIN-ICHI INOKUCHI (Tohoku Pharmaceutical University, Japan)  
Insulin resistance as a membrane microdomain disorder
- 9:00 ANTONIO ROSSI (University of Pavia)  
Glycosaminoglycan sulfation and skeletal dysplasias
- 9:30 GIUSEPPE CAMPO (University of Messina)  
Hyaluronan: double role in inflammation
- 10:00 CARLA EMILIANI (University of Perugia)  
Acidic glycohydrolases localization and regulation: pathological implications
- 10:30 Presentation selected from submitted abstracts
- 11:00 *Coffee break*
- 11:30-13:30 **Symposium F**
- Biochemical mechanisms of metabolic diseases and their vascular complications***
- Chairpersons:* PATRIZIA GALLETTI (Napoli)  
BRUNO BARBIROLI (Bologna)
- 11:30 GIOVANNI SOLINAS (University of Fribourg, Switzerland)  
Pro-inflammatory pathways in obesity-related disorders
- 12:00 MASSIMO FEDERICI (University "Tor Vergata" of Rome)  
Proteases at the interface of metabolic diseases and vascular inflammation
- 12:30 DIEGO INGROSSO (Second University of Naples)  
Molecular mechanisms of hyperhomocysteinemia as a cardiovascular risk factor
- 13:00 FRANCESCO PAOLO MANCINI (University of Sannio)  
The dietary antioxidant resveratrol and cardiovascular disease
- 13:30 MAURO MACCARRONE (University of Teramo)  
Endocannabinoid metabolism and glucose transport

- 14:00 *Lunch*
- 14:00-15:00 **Poster Session 3**
- 15:00-18:00 **Symposium G**  
*Nitric oxide and Carbon monoxide in biology and medicine*  
*Chairpersons:* RICHARD ROMAN (Milwaukee)  
 GIOVANNI SCAPAGNINI (Campobasso)
- 15:00 NADER G. ABRAHAM (New York Medical College, USA)  
 Nitric oxide-carbon monoxide interaction and oxidative stress in obesity and diabetes
- 15:40 MAURIZIO BRUNORI (University "La Sapienza" of Rome)  
 Nitrite reduction, a function emerging from a pre-aerobic past
- 16:05 EMILIO CLEMENTI (University of Milan)  
 The role of nitric oxide and its control of mitochondrial dynamics in differentiating myoblasts  
*Chairpersons:* FERDINANDO PALMIERI (Bari)  
 HISANORI SUZUKI (Verona)
- 16:40 FRANCISCA RODRIGUEZ (University of Murcia, Spain)  
 Carbon monoxide and Nitric Oxide interactions: implications for renal pathophysiology
- 17:00 ROBERTO MOTTERLINI (Italian Institute of Technology, Genoa)  
 Carbon monoxide-releasing molecules: vasodilatory and anti- ischemic properties
- 17:20 GIOVANNI LI VOLTI (University of Catania)  
 NO/CO crosstalk in oxidative stress and organ protection
- 17:40 SHINYA YOSHIKAWA (University of Hyogo, Japan)  
 X-ray structures of CO, NO and cyanide derivatives of bovine heart cytochrome c oxidase
- 18:30-19:30 Assemblea Soci e Premio Medaglia SIB
- 19:00 **Removal of posters Session 3**
- 21:30 *Social dinner*

## Saturday 26<sup>th</sup> September 2009

Città Universitaria, Dipartimento di Fisica, Aula Magna, Via S.Sofia 64, Viale A.Doria 6, Catania

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- 09:00-11:30 **Symposium H**  
*Basic biochemistry and biology of vascular function*  
*Chairpersons:* MARIO ALBERGHINA (Catania)  
 ITALIA DI LIEGRO (Palermo)
- 9:00 PATRICIA D'AMORE (Harvard Medical School, USA)  
 Role of vascular endothelial growth factor (VEGF) in the adult
- 9:30 JOHN H. WALKER (University of Leeds, UK)  
 Phospholipase A2 enzymes in the endothelial cells: novel targets for treating cardiovascular disease and cancer
- 10:00 DOMENICO RIBATTI (University of Bari)  
 The role of inflammatory cells in angiogenesis
- 10:30 ITALIA DI LIEGRO (University of Palermo)  
 Neurons and astrocytes shed extracellular membrane vesicles containing angiogenic factors
- 11:00 LUCA MUNARON (University of Turin)  
 Calcium signaling and control of angiogenesis progression
- 11:30 *Coffee break*
- 12:00 **Poster Session 4**
- 13:00 *Lunch*
- 14:00 **Removal of posters Session 4**

## COMMITTEES

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# **Antonini Lecture**

**Emilia Chiancone**

Department of Biochemical Sciences, "Sapienza" University of Rome, Piazzale A. Moro, 5 - 00185 Rome

**DPS PROTEINS, AN EFFICIENT MEANS TO DETOXYIFY IRON AND PROTECT DNA  
IN THE BACTERIAL RESPONSE TO OXIDATIVE STRESS**



## **DPS PROTEINS, AN EFFICIENT MEANS TO DETOXYFIFY IRON AND PROTECT DNA IN THE BACTERIAL RESPONSE TO OXIDATIVE STRESS**

**Emilia Chiancone**

Department of Biochemical Sciences, "Sapienza" University of Rome, Piazzale A. Moro, 5 - 00185 Rome

Since 1992, when the first member of the family was described in *Escherichia coli*, Dps proteins (DNA-binding protein from starved cells) have acquired an important role within the complex machinery used by bacteria to protect DNA and the other macromolecules under oxidative stress conditions. The protective role of Dps proteins is exerted by means of two distinct mechanisms: a physical and a chemical one. The first relates to the capacity to bind DNA without apparent sequence specificity. Although this property has given the name to the family, later studies showed that not all Dps proteins bind DNA as this capacity depends on the presence of specific structural elements that allow the establishment of electrostatic interactions with the negatively charged DNA backbone. In contrast, the chemical mechanism is related to the activity of a highly conserved ferroxidase center and therefore is operative in all members of the family. The identification of this center in *Listeria innocua* Dps has contributed to the assignment of Dps proteins to the ferritin superfamily, which is characterized by a multimeric shell-like assembly endowed with iron oxidation/storage capacity. Thus, Dps proteins are dodecamers built with 23 symmetry similarly to ferritins that are assembled from 24 subunits with 432 symmetry. The ferroxidase center of Dps proteins has distinct structural and mechanistic properties: it is not embedded in a four-helix bundle, but is located at the interface of two subunits related by two-fold symmetry. Moreover, while the physiological iron oxidant used by ferritins is molecular oxygen, Dps proteins use hydrogen peroxide with the production of water. This peculiarity endows Dps proteins with the unique capacity to detoxify concomitantly the bacterial cytoplasm from Fe(II) and hydrogen peroxide. Thus, Dps proteins reduce the production of hydroxyl radicals via Fenton chemistry significantly, a peculiarity that is particularly advantageous in pathogenic bacteria. The experimental evidence of the above affirmations will be provided.



## Symposia



**Simposio congiunto Gruppi “Proteine”  
ed “Enzimologia e regolazione metabolica”:  
Evolution and regulation of the enzymatic function**



**GOING BEYOND NATURE? THE MAKING OF NEW ENZYMES BY DIRECTED EVOLUTION**

**Gianfranco Gilardi**

University of Turin

**Abstract not submitted**

## CATALYTIC DNA: SHEDDING LIGHT ON SUBSTRATE BINDING AND CATALYSIS BY A SMALL DEOXYRIBOZYME

Alessio Peracchi

Dipartimento di Biochimica e Biologia Molecolare, Università di Parma

Nucleic acid catalysis is a key biochemical phenomenon, thought to have preceded protein catalysis during the course of evolution and crucial even for extant organisms [1]. While all the nucleic acid enzymes found in vivo are made of RNA (ribozymes), it is well appreciated that DNA, too, can efficiently perform molecular recognition and catalysis [2]. Assessing the functional features of catalytic DNAs (deoxyribozymes) in comparison with natural ribozymes can yield insights into nucleic acid catalysis and provide a better comprehension of the nature of DNA and RNA as macromolecules. We study the 8-17 deoxyribozyme, one of the simplest and most common DNA enzymes discovered to date. This catalyst can be designed to recognize and cleave specific RNA targets (e.g. viral mRNAs) and is being actively studied as a gene therapy agent and as a biosensor [3, 4]. We have dissected the catalytic mechanism of this enzyme [5, 6] and, through systematic mutagenesis, we have identified those residues that are most crucial for function, providing suggestions on their potential roles [7]. By exploiting fluorescence spectroscopy, we have highlighted the interplay between metal-induced folding and activity. Finally, we have addressed the issue of substrate recognition by introducing locked nucleic acid (LNA) monomers into the substrate-binding domains of the catalyst. Such modified nucleotides not only stabilize binding of the enzyme to its RNA substrate, but also greatly accelerate the annealing to structured substrate sequences [8]. While helping to outline the principles upon which DNA catalysis relies, our results have direct practical implications for the biotechnological applications of DNAzymes and of nucleic acids in general. References 1) Cech, T. R. (2009) Crawling out of the RNA world. *Cell* 136, 599-602 2) Peracchi, A. (2005) DNA catalysis: potential, limitations, open questions. *Chembiochem* 6, 1316-1322 3) Baum, D. A. and Silverman, S. K. (2008) Deoxyribozymes: useful DNA catalysts in vitro and in vivo. *Cell. Mol. Life Sci.* 65, 2156-74 4) Liu, J. and Lu, Y. (2006) Fluorescent DNAzyme biosensors for metal ions based on catalytic molecular beacons. *Methods Mol. Biol.* 335, 275-88 5) Ferrari, D. and Peracchi, A. (2002) A continuous kinetic assay for RNA-cleaving deoxyribozymes, exploiting ethidium bromide as an extrinsic fluorescent probe. *Nucleic Acids Res.* 30, e112 6) Bonaccio, M., Credali, A. and Peracchi, A. (2004) Kinetic and thermodynamic characterization of the RNA-cleaving 8-17 deoxyribozyme. *Nucleic Acids Res.* 32, 916-925 7) Peracchi, A., Bonaccio, M. and Clerici, M. (2005) A mutational analysis of the 8-17 deoxyribozyme core. *J. Mol. Biol.* 352, 783-94 8) Donini, S., Clerici, M., Wengel, J., Vester, B. and Peracchi, A. (2007) The advantages of being locked. Assessing the cleavage of short and long RNAs by locked nucleic acid-containing 8-17 deoxyribozymes. *J. Biol. Chem.* 282, 35510-8

## THE BIRTH OF THE ENZYMATIC FUNCTION ALONG THE FOLDING PATHWAY

**Francesco Bemporad, Fabrizio Chiti**

Dipartimento di Scienze Biochimiche, Università di Firenze, Viale Morgagni 50, 50134 Firenze

Structural flexibility within the native state of a normally folded protein is known to be required for enzyme catalysis and for the interaction of a protein with its biological interactors. Moreover, a significant fraction of eukaryotic proteins, normally referred to as intrinsically disordered proteins (IDPs), appear to be unfolded or contain unstructured regions. For these reasons biological activity of conformational states distinct from fully folded structures could be more common than previously thought. By applying a procedure that allows the recovery of enzymatic activity to be monitored in real time, we show that a partially folded state populated transiently during folding of the acylphosphatase from *Sulfolobus solfataricus* (Sso AcP) is enzymatically active. The structure of this conformational state has been determined using the phi-value analysis and applying MD simulations restrained by the experimentally observed phi values. This structural characterization reveals a native-like topology within the enzymatically active partially folded state in the presence of a structurally heterogeneous active site. It also shows that enzymatic activity is possible, despite the structural disorganisation of the catalytic site, because the remainder of the structure acts as a scaffold and the substrate-binding and catalytic residues remain located within a limited region of space. The analysis of the aggregation process of Sso AcP into amyloid-like fibrils has also incidentally revealed the presence of aggregated species, forming early in the process and developing subsequently into amyloid-like species, in which the individual protein molecules present in the aggregated species maintain enzymatic activity. This occurs again in the presence of a native-like structure with local structural distortions. These results extend the spectrum of biological functions carried out in the absence of a folded state to include enzyme catalysis and cast the basis for studying the dynamics undergone by an enzyme during catalysis.

## THE EVOLUTION OF MACRO-DOMAINS AS TARGET-RECOGNITION SITES WITHIN ENZYMES THAT REGULATE ADP-RIBOSYLATION

Maria Di Girolamo

Consorzio Mario Negri Sud S. Maria Imbaro (Chieti) 66030, ITALY

The macro domain is an evolutionarily conserved globular protein domain of about 25 kDa that was originally identified in the C-terminus of the histone variant, macroH2A. The macro domain is of ancient origin, and is encoded by the genomes of many bacteria and archaea; it is found in about 200 proteins across all organisms, from thermophiles to human. The best-studied bacterial macro domain is the protein Af1521 from *Archaeoglobus fulgidus*, and it has an enzymatic phosphatase activity that can hydrolyze ADP-ribose-1-phosphate, a metabolite produced by cyclic phosphodiesterases during tRNA splicing. The human genome contains nine genes encoding macro domain-containing proteins. The phosphatase activity is not conserved among the human macro domain-containing proteins; instead most have evolved to bind a variety of NAD metabolites, including monomers and polymers of ADP-ribose. We have shown that some macro domains can interact with ADP-ribosylated proteins. ADP-ribosylation is a reversible posttranslational modification that can modulate the function of a target protein. The enzymes that catalyze this reaction in mammalian cells are either pathogenic bacterial toxins or endogenous cellular mono- and poly-ADP-ribosyltransferases. For cellular mono-ADP-ribosylation, both the enzymes and their targets have largely remained elusive, mainly due to a lack of specific techniques to study this reaction. We have used macro domains as selective baits for high affinity purification of mono-ADP-ribosylated proteins, which can then be identified by mass spectrometry. In addition to providing a new method for the identification of ADP-ribosylated proteins in intact cells, these experiments demonstrate that macro domains can interact with ADP-ribosylated proteins, thus suggesting that they can serve as protein-interaction modules. In line with this, an interaction between the mammalian histone macroH2A1 and PARP1 has been reported. PARP1 is the founding member of the poly-ADP-ribosyltransferase family, and this interaction leads to the inhibition of PARP1 auto-modification. Thus, while the roles of the mammalian macro domains remain to be defined, they appear to serve to mediate interactions with other proteins, including the PARPs themselves, thus representing a way to influence both poly-ADP-ribosyltransferase and mono-ADP-ribosyltransferase activities through a regulatory mechanism. The evolution of macro domains as sites for target recognition represents a further level of regulation of ADP-ribosylation pathways. 1) Allen M. D., A. M. Buckle, S. C. Cordell, J. Lowe and M. Bycroft: The crystal structure of AF1521 a protein from *Archaeoglobus fulgidus* with homology to the non-histone domain of macroH2A. *J Mol Biol*, 330, 503-11 (2003) 2) Neuvonen M. and T. Ahola: Differential activities of cellular and viral macro domain proteins in binding of ADP-ribose metabolites. *J Mol Biol*, 385, 212-25 (2009) 3) Karras G. I., G. Kustatscher, H. R. Buhecha, M. D. Allen, C. Pugieux, F. Sait, M. Bycroft and A. G. Ladurner: The macro domain is an ADP-ribose binding module. *EMBO J*, 24, 1911-20 (2005) 4) Corda D. and M. Di Girolamo: Functional aspects of protein mono-ADP-ribosylation. *EMBO J*, 22, 1953-8 (2003) 5) Dani N., A. Stilla, A. Marchegiani, A. Tamburro, S. Till, A. G., Ladurner, D. Corda and M. Di Girolamo: Combining affinity purification by ADP-ribose-binding macro-domains with mass spectrometry to define the mammalian ADP-ribosyl-proteome. *Proc. Natl. Acad. Sci. USA* 106, 4243-8 (2009) 6) Nusinow D. A., I. Hernandez-Munoz, T. G. Fazzio, G. M. Shah, W. L. Kraus and B. Panning: Poly(ADP-ribose)polymerase 1 is inhibited by a histone H2A variant, MacroH2A, and contributes to silencing of the inactive X chromosome. *J Biol Chem*, 282, 12851-9 (2007)

## LINKING ENZYMATIC FUNCTION TO TRANSCRIPTION REGULATION: THE CASE OF NAD BIOSYNTHESIS

Nadia Raffaelli

Department of Molecular Pathology and Innovative Therapies, Biochemistry Section, Università delle Marche, Ancona, Italy

Enzymes are able to modulate gene expression in several ways. Modifying enzymes indirectly influence gene expression either acting on chromatin or shuttling to and from the nucleus where they alter the activity of their target transcription factors. Other enzymes exert their regulatory functions directly at the promoter of genes, acting as both enzymes and coactivators, with both activities implicated in regulating target transcription factor function. In addition, transcription factors themselves might be endowed with intrinsic enzymatic activities. In eubacteria, coupling an enzymatic activity directly to a transcription factor is a widespread means of modulating gene expression. Notably, many eubacterial transcriptional regulators combine the DNA binding domain to an enzymatic domain which allows them to catalyze a key step in a biosynthetic pathway and regulate transcription of genes in that pathway. As a result, cellular metabolism directly influences gene expression. This presentation will focus on the description of two eubacterial transcriptional regulators of NAD biosynthesis, as examples of proteins emerging via fusion of a DNA binding domain with metabolic enzymes, thus providing a direct link between metabolism and transcription. In recent years the NAD biosynthesis machinery consisting of various *de novo*, synthesis salvage and recycling pathways, has been elucidated in the majority of eubacteria, highlighting a substantial variability in different species, that parallels the variety of bacterial habitats and lifestyles. The importance of maintaining NAD homeostasis in a variety of growth conditions is reflected by the existence of various regulatory strategies for the transcriptional control of NAD metabolism. In Enterobacteriaceae both the *de novo* biosynthetic pathway and recycling routes from nicotinamide and nicotinic acid are controlled by the NadR protein, that operates as a NAD-dependent transcriptional repressor. This regulator is also endowed with two ATP-dependent enzymatic activities involved in nicotinamide riboside recycling (1,2), thus representing a sophisticated multifunctional regulator/enzyme complex, able to modulate NAD synthesis in response to its cellular levels. A completely different regulatory strategy is used by Nudix-related transcriptional regulators (NrtRs), members of a novel family of transcription factors recently identified through comparative genomics analyses (3). In a broad range of bacteria, NrtRs act as repressors of various NAD biosynthetic genes depending on the bacterial species. Their de-repression is triggered by the accumulation of NAD degradation products, likely interpreted by the cell as depletion of the NAD pool, which needs to be replenished. NrtRs contain a DNA binding domain fused with a Nudix domain homologous to ADP-ribose pyrophosphatase (ADPRP), a Nudix hydrolase that catalyzes ADP-ribose (ADPR) hydrolysis to AMP and ribose 5-phosphate (Rib-P). ADPR is one of the products of NAD-consuming enzymes; its *in vitro* binding to the Nudix domain of NrtRs promotes dissociation of NrtR-DNA complexes, leading to derepression of transcription (4). Notably, some NrtRs are active ADPRP, thus contributing themselves to NAD production by providing Rib-P for PRPP synthesis upon ADPR hydrolytic cleavage. Most of NrtRs, however, have lost the catalytic activity, but retained the ability to bind, and hence to be regulated by ADPR. (1) Raffaelli N, Lorenzi T, Mariani PL, Emanuelli M, Amici A, Ruggieri S and Magni G (1999), *J Bacteriol*, 181, 5509 (2) Kurnasov OV, Polanuyer BM, Ananta S, Sloutsky R, Tam A, Gerdes S.Y. and Osterman AL (2002) *J Bacteriol*, 184, 6906 (3) Rodionov DA, De Ingeniis J, Mancini C, Cimadamore F, Zhang H, Osterman AL and Raffaelli N (2008). *Nucleic Acids Res* 36, 2047 (4) Huang N, De Ingeniis J, Galeazzi L, Mancini C, Korostelev YD, Rakhmaninova AB, Gelfand MS, Rodionov DA, Raffaelli N and Zhang H (2009) *Structure*, in press

## X-RAY ESCAPING CONFORMATIONAL CHANGES AFFECTING ENZYMATIC FUNCTION

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Overlays between the X-ray structures of 6-phosphogluconate dehydrogenase (6PGDH) alone and in binary complexes with either 6PG or coenzymes result in a r.m.s.d. of 0.22 Å for C alpha and 0.6-0.7 Å for all atoms, suggesting that ligand binding does not modify enzyme conformation. Nevertheless, much evidence exists on significant conformational changes induced, for instance, by 6PG. The enzyme is a homodimer but symmetry is disrupted in the ternary complex. In fact, while apo-enzyme is able to bind 2 NADP, in the presence of 6PG only 1 NADP/dimer is bound. In the presence of 4-phospho-erythronate (4PE) the coenzymes Kd decrease by two orders of magnitude, suggesting other conformational changes during the formation of the dehydrogenation transition state. The complete catalysed reaction in the forward direction consists in dehydrogenation, decarboxylation and enol-cheto tautomerism. Kinetic studies evidence that conformational change in the ternary complex, preceding chemical steps, is rate limiting. A similar conformational change occurs in the reverse reaction, i.e. reductive carboxylation of ribulose-5-phosphate (Ru5P). We find that low 6PG concentrations activate the reverse reaction by changing the conformational change rate. These data agree with the previous observation that 6PG activates decarboxylation of 3-keto 2 deoxy 6-phosphogluconate (a reaction intermediate analogue) and indicate that full catalytic competence is attained by one or more conformational changes, resulting in an asymmetric dimer as a single functional unit. Formation of binary and abortive ternary complexes have been studied in *T. brucei* wt and mutant 6PGDHs, where the replaced residues are either K185 or E192, the residues mainly involved in the acid-base catalytic mechanism. Also, 6PGDH was mutated in either H188 or C372, which are near to the 6PG binding site. K185 replacement by either H or R, and E192 mutation to Q, indicate that 6PG binding causes a change in the protonation state of the two residues, resulting in a net transfer of 1 H<sup>+</sup> from K185 to E192. Furthermore, 6PG binding is accompanied by a release of 0.5 H<sup>+</sup>, which could be related to a conformational change instead. Measurement of the H<sup>+</sup> released by the H188 and C372 mutants shows a linear relationship between the N° of released H<sup>+</sup> and enzyme activity. Furthermore, in the presence of 6PG in the wt, cysteines reactivity dramatically decrease, this stabilizing effect being impaired in the mutants, with the initial rate of cysteines modification in the enzyme-6PG complex, correlating to the mutant specific activity. These data mean that despite X-ray not detecting any difference between free and 6PG bound enzymes, substrate binding induces a conformational change, resulting in the release of H<sup>+</sup> and a reduction in cysteines reactivity, and that this change is required for full catalytic competence. ITC characterization of the binary and ternary complexes show anomalous E192Q behaviour, suggesting a slow exothermic event accompanying the formation of the ternary complex with NADP and 4PE, identified with an association of dimers to tetramers. Thus, the wt oligomerization state was investigated, and tetramer was found to be associated with the ternary complexes enzyme-4PE-NADP(H), and with the binary complex enzyme-NADPH. An overall reaction mechanism model has been created, taking account of dimer-tetramer equilibrium. A final consideration is that, despite several pieces of evidence for conformational changes related to 6PGDH catalytic activity, crystallographic data only depict a single conformation. The only exception, giving support to the dimer-tetramer association, are the data on abortive ternary complexes in *L. lactis* 6PGDH, showing asymmetric units composed of three subunits, and with only one active site occupied by NADP and by either the product Ru5P or the transition state analogue 4-phospho-erythronohydroxamate.

**Symposium A:  
Biochemistry and biotechnology of cell engineering**



## WHAT YOU MUST KNOW IF YOU WOULD LIKE TO ENTER IN THE CELL THERAPY ARENA

**Giuseppe Astori, Viviana Lo Cicero, Sabrina Soncin, Daniel Sürder, Mauro Gola  
Gianni Soldati and Tiziano Moccetti**

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Recent findings in biology, biotechnology and medicine have led to the development of therapies where the stem cells are the “active substances”. In controlled clinical trials, has been demonstrated that stem cells have properties for treating or preventing diseases. Cells are administered with the aim to restoring, correcting or modifying physiological functions by exerting principally a pharmacological, immunological or metabolic action. The European Regulation 726/2004 on “advanced therapy medicinal products (ATMPs)” has entered in force on December, 30, 2008. The production of an ATMP often requires very specific expertise, which goes beyond the traditional pharmaceutical field and covers areas bordering on other sectors such as biotechnology and medical devices. The manufacture of an ATMP should be in compliance with the principles of good manufacturing practice (GMP) in respect of medicinal products and investigational medicinal products for human use. This means that stringent acceptance criteria for raw materials used in the production should be strictly defined. Furthermore, preclinical studies should define in particular cell identity, safety, potency and dose-finding. Finally, the ATMP release criteria must ensure cell viability, product sterility, absence of adventitious agents, and low endotoxin level. We report the experience of the Cell Therapy Unit of the Cardiocentro Ticino, the first and only cell factory accredited in Switzerland for the production of ATMPs.

## TOWARD THE IDENTIFICATION OF A HUMAN RENAL PROGENITOR SYSTEM

**Paola Romagnani**

Excellence Center for Research, Transfer and High Education DENOTHE, University of Florence, Italy

In most adult epithelia the process of wounding to replace the dead cells is maintained through the presence of stem cells. Recently, we identified in adult human kidneys a population of renal progenitors selectively localized at the urinary pole of the Bowman's capsule and characterized by the co-expression of the surface markers CD133 and CD24. CD24+CD133+ renal progenitors exhibit self-renewal potential, and regenerate tubular structures in mice affected by acute renal failure. More recently, we demonstrated that CD24+CD133+ cells are a hierarchical population of renal progenitors which can also replace podocytes through their division and migration along the Bowman's capsule towards the glomerular tuft in response to podocyte injury. Accordingly, treatment of mice affected by focal segmental glomerulosclerosis with CD24+CD133+ renal progenitors regenerated glomerular structures and reduced functional renal damage. These results support the concept that the adult human kidney contains a stem cell compartment which can regenerate both tubular as well as glomerular epithelial cells.

# ENGINEERING RED BLOOD CELLS FOR THE DELIVERY OF DRUGS AND CONTRASTING AGENTS

**Mauro Magnani**

Dep. Biomolecular Sciences University of Urbino, Italy

Erythrocytes, also known as Red Blood Cells (RBC), are typically used in transfusion medicine to replace lost blood in patients that underwent different kind of medical treatments as well as in accidents resulting in blood loss. In addition to these well know uses, RBC are experiencing a number of new applications both as therapeutics as well as diagnostic agents. Most of these applications are possible thanks to the peculiar properties of these cells of being opened and resealed without affecting their main properties and in vivo circulation as well as to a technology, that we have invented and patented (US patent 6,139,836; EU patent 882448; Japan patent 123228), to perform the procedure in the clinic, with minimal amounts of patient blood. The biomedical use of these processed RBC include the possibility of engineering the same by the addition of drugs, biologics and/or nanomaterials. These constructs are a new armamentarium available to the physicians for the release of drugs in circulation, for targeting drugs to selected sites in the body, or for in vivo diagnostic procedures based on magnetic and/or optical methods. Autologous human RBC loaded with corticosteroid analogues have been used in the treatment of Cystic Fibrosis, Crohn disease, Ulcerative Colitis and COPD patients, and we have documented so far the benefits of this new technology as well as the absence of drug side effects in over 2,500 treatments. Based on these results the E.M.E.A. has granted the designation of "Orphan Drug" to "Dexamethasone Sodium Phosphate for encapsulation in human erythrocytes for treatment of Cystic Fibrosis" (Orphan Drug Designation EMEA/OD/039/04-EU/3/04/230). The encapsulation of superparamagnetic nanoparticles within RBC has leads to the generation of new biomimetic constructs that now permits the use of these nanomaterials in vivo avoiding their rapid sequestration and their accumulation in unwanted districts (PCT WO 2008/003524 A3). Similarly, the encapsulation of infrared fluorescent agents into RBC has opened the way to the measurement of vasomotion in the human retinal vasculature suggesting a possible correlation with retinal edema (Macula 2009 meeting in NYC). In summary, the newly developed drug delivery method via erythrocytes is a technology platform which could be used in a wide range of applications and opens to unlimited new therapeutic approaches in terms of drugs to be delivered and pathologies that could be treated. Furthermore, the same system has been adapted to deliver contrasting agents within the body permitting the improvement of actual fluorangiographic procedures and the imaging by Magnetic Resonance (MRI) and MPI. A spin off company (EryDel S.p.A.) has been recently founded and financed by venture founds to bring these applications to the market and to implement the clinical applications of our technology.



**Symposium B:  
Molecular mechanisms of neurodegenerative diseases**



## BETA-AMYLOID: NOT JUST TOXIC ANYMORE

**M.L. Giuffrida 1, F. Caraci 1, G. Molinaro 2, S. Cataldo 3, B. Pignataro 3, V. Bruno 2, P. De Bona 4  
G. Pappalardo 4, F. Nicoletti 2, E. Rizzarelli 5, A. Copani 1, 4**

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The self-association of  $\beta$ -amyloid (A $\beta$  1-42) monomers into soluble oligomers is thought to be required for neurotoxicity in Alzheimer's disease (AD) (Walsh and Selkoe, *J. Neurochem.* 2007). The function of monomeric A $\beta$ (1-42) has been unknown until recently (Giuffrida et al., *J. Neurosci.* 2009). We have demonstrated that nanomolar concentrations of monomeric A $\beta$ (1-42) are able to support the survival of developing neurons under conditions of trophic deprivation, and to protect mature neurons against excitotoxic death. The neuroprotective activity of A $\beta$ (1-42) monomers is mediated by the activation of the phosphatidylinositol-3-kinase pathway, and involves the stimulation of IGF-1 receptors. Interestingly, monomers of A $\beta$ (1-42) carrying the arctic mutation (E22G) associated with familial AD lack any neuroprotective activity. Our data suggest that the self-assembly of A $\beta$ (1-42) monomers into aggregates might contribute to the overall neuronal loss of the AD brain by depriving neurons from a physiological rescuing mechanism.

## NEW INSIGHTS INTO THE ROLE OF OXIDATIVE STRESS IN AGEING AND NEURODEGENERATION

**Giuseppe Rotilio**

Department of Biology, University of Rome "Tor Vergata"

New insights into the role of oxidative stress in ageing and neurodegeneration Giuseppe Rotilio and Maria Rosa Ciriolo Department of Biology, University of Rome "Tor Vergata". The involvement of free radicals has been studied extensively as the primary biochemical mechanism of oxidative stress in several patho/physiological cellular processes. Recently, data supporting a complementary hypothesis for oxidative stress in disease that can occur without free radicals are emerging. This hypothesis is currently defined as the "redox hypothesis", but should be more correctly indicated as the "thiol redox hypothesis". In fact, it suggests that oxidative stress occurs as a consequence of disruption of thiol redox circuits, which normally function in cell signaling and physiological regulation. Many proteins contain redox-sensitive thiols, and reactions of thiol systems occur largely by nonradical two-electrons transfers that are controlled by the thioredoxins (Trx), glutathione (GSH) and cysteine. Trx and GSH systems are maintained under stable, but nonequilibrium conditions, due to a continuous oxidation of cell thiols. Redox-sensitive thiols are critical for signal transduction, transcription factor binding to DNA, receptor activation, and other processes. Because of the nonequilibrium conditions in the thiol pathways, aberrant generation of nonradical oxidants at rates comparable to normal oxidation may be sufficient to disrupt protein function. Identification of specific thiol control pathways, in particular those coupled with phosphorylation processes, could be of great importance to develop interventional strategies to restore normal redox control and protect against oxidative stress in aging, cancer and neurodegeneration. Here, we summarize recent data obtained in our laboratory on the involvement of these pathways in the control of key metabolic enzymes and transcription factors that may be relevant to biochemical mechanisms of ageing and neurodegeneration.

## MITOCHONDRIAL COMPLEX I AND NEURODEGENERATION

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MITOCHONDRIAL COMPLEX I AND NEURODEGENERATION Complex I (NADH Coenzyme Q oxidoreductase) is the major point of entry of electrons into the mitochondrial respiratory chain. The huge size (45 subunits in mammals) and the presence of several (eight) delicate iron-sulphur clusters make this enzyme particularly vulnerable to attack by reactive oxygen species (ROS); at the same time the enzyme is also recognized as a major producer of ROS particularly under conditions of slow electron flow such as due to high membrane potential (respiratory State 4), regulatory state (phosphorylation of subunits), presence of inhibitors or of mutations in either nuclear or mitochondrial DNA affecting its subunits. Complex I has been found to be altered in several pathological states including mitochondrial encephalomyopathies, Parkinson's disease and others. Moreover a great number of pesticides and insecticides that can be in contact with humans are Complex I inhibitors. Elucidation of the mechanism of Complex I inhibition and of ROS generation is mandatory to understand the pathogenesis of these diseases. Studies in our laboratory exploiting Complex I inhibitors have elucidated the mechanism of Coenzyme Q (CoQ) reduction in the consecutive delivery of 2 electrons by centre N2 to CoQ with the intermediate of the semiquinone (SQ) form; inhibitors preventing reduction of CoQ to SQ maintain N2 in the reduced state thus favouring delivery of one electron to oxygen to form superoxide, whereas inhibitors preventing SQ reduction do not generate ROS. Complex I is now recognized to be associated with Complex III (ubiquinol cytochrome c reductase) in such a way that electrons are channelled through bound CoQ between the two enzymes; this supramolecular association not only represents a kinetic advantage but is also required to prevent disassembly of Complex I. We have demonstrated that lipid peroxidation is able to dissociate supramolecular association. We have also shown in a mitochondrial fraction enriched with Complexes I and III that when the complexes are dissociated into the individual enzymes the production of ROS is much increased. This observation explains the discrepancies usually observed on ROS generation between Complex I isolated and in the native membrane. The hypothesis can be advanced that an initial oxidative stress due to mitochondrial DNA mutations, anoxia or other reasons may destabilize the I-III supercomplex, thus inducing a vicious circle further destabilizing Complex I and inducing further ROS generation, with subsequent loss of oxidative phosphorylation finally leading to cell death; such a mechanism might be involved in the pathogenesis of some forms of neurodegeneration.

## GLYCATING CONDITIONS AFFECT AGGREGATION AND TOXICITY OF AMYLOIDOGENIC PEPTIDES

**Vincenzo Giuseppe Nicoletti**

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Proteins show an intrinsic propensity to misfolding that is influenced by the amino acid composition, and can be accelerated by certain environmental conditions such as increased temperature, high or low pH, agitation, oxidative agents, transition metals, or elevated glucose. Glycation is the well-described non-enzymatic Maillard reaction of reducing sugars with protein side chains, lipids, or nucleic acids to form Schiff base. Complex multistep reactions and rearrangements during long-term incubation produce various compounds termed advanced glycation end products (AGEs). AGEs were found to induce conformational changes especially to long-living proteins. This contributes to the onset of several diseases, including diabetic complications, renal failure, inflammation, atherosclerosis, cancer, neurodegenerative disorders and more generally age related diseases. AGEs can be recognized by the multiligand receptor for AGEs (RAGE) that has been therefore implicated in the pathogenesis of such diseases. Alzheimer's disease (AD), Parkinson's disease (PD), Huntington's disease (HD), amyotrophic lateral sclerosis (ALS) and prion diseases show common cellular and molecular mechanisms including protein misfolding and aggregation, a process termed amyloidogenesis. The amyloid aggregates usually consist of elongated, unbranched protein fibrils deposits containing misfolded proteins with a beta-sheet conformation, and represent the end stage of a molecular cascade of several steps. Increasing evidences now support the view that spontaneous aggregation into small soluble oligomeric (protofibrillar) assemblies during earlier steps, is more directly tied to pathogenesis than the insoluble deposits themselves. Consequently, there is current emphasis in understanding the microenvironmental conditions that favour the initial oligomerization. Glycation plays a prominent role in the conversion of a protein from its native structure to the amyloid and toxic state. This process can be accelerated by free radicals and certain transition metals, particularly Cu(II) and Zn(II). Toxic activity is enhanced by the interaction with RAGE, that functions as a signal transducer for cell impairment. A full understanding of the mechanism of protein misfolding and cross-linking as well as specific receptor signalling is helpful in designing new pharmacological/chemical tools against amyloidogenesis and related diseases.

## **BRAIN LOW MOLECULAR WEIGHT PHOSPHOLIPASES A2 (sPLA2): ROLE IN NEURONAL FUNCTIONS AND IN NEURODEGENERATIVE DISEASES**

**V. Nardicchi, E. Biagioni Angeli, M. Ferrini, E. Chiricozzi and G. Goracci**

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sPLA2s are characterized by having a relatively low molecular weight (13-18 kDa) and are currently indicated as “secretory” or “secreted” PLA2 because they are synthesized with a N-terminal signal peptide and some of them were isolated from mammalian extracellular fluids. The released sPLA2 may bind to surface receptors and the N-type has been identified in neurons. Binding to pre-synaptic nerve terminals of snake sPLA2 is neurotoxic and induces influx of Ca<sup>2+</sup> causing a massive release of neurotransmitters (Rigoni et al. 2007). A similar effect can be caused by sPLA2 released by astrocytes or microglia activated by inflammatory mediators (Sun et al. 2005). In addition to their extra-cellular functions, mammalian sPLA2 participate to cell signaling contributing to the generation of lipid mediators as eicosanoids, lysophospholipids and PAF together with cPLA2 and iPLA2. The relative contribution of sPLA2 to these processes is still largely unknown. Recent observations have attracted the interest to other roles of sPLA2 in intracellular mechanisms. Few years ago, it has been demonstrated the presence of type IIA sPLA2 (GIIA) in rat brain cortex mitochondria (Macchioni et al. 2004). The same study demonstrated that another isoform (GV) is localized in the nuclei of cultured cells. More recently, GV has been detected in the nuclei of rat cerebral cortex (Nardicchi et al. 2007). Other sPLA2 isoforms are present in neural cells as GIB, GIII and GX but no information is available on their sub-cellular location and functions. Endogenous GIIA appears to be involved in neuritogenesis because we have obtained evidence that this isoform accumulates into growth cones and neurite tips when PC12 cells are induced to differentiate into neuron-like cells by NGF Biagioni Angeli et al., 2009). The variety of sPLA2s isoforms, having similar structures and properties and potentially coexisting in the nervous tissue, complicates the assignment of specific functions to each of them or the attribution of specific roles in the onset and/or the aggravation of neurodegenerative disease. This task is even more complex if it is considered that other types of PLA2s (cPLA2, iPLA2 and PAF-AH) are also present in neural cells and a cross-talk between them within the same cell or between different cell types has been hypothesized and very likely takes place. The discovery of GIIA isoform in the mitochondria of neural cells and furthermore its release in the in energy-deficient conditions opened new perspectives (Macchioni et al., 2004). Indeed, the relationship between mitochondrial dysfunctions and neurodegenerative diseases is well documented even if the mechanisms are still debated. Although each neurodegenerative disease has a separate etiology with distinct morphological characteristics, they may also share the same terminal neurochemical common processes. Recently, it has been demonstrated a correlation between apoptotic cell death, induced by ROS or RNS generation, and increased activation of PLA2, most likely mitochondrial GIIA (Chiricozzi et al, 2009). Biagioni Angeli E., Nardicchi V., Ferrini M., Bianconi M. and Goracci G. (2009) *J. Neurochem.* 110 (Suppl. 1), 94 Chiricozzi E., Fernandez-Fernandez S., Almeida A., Bolanos J. P. and G. Goracci (2009) *J. Neurochem.* 110 (Suppl. 1), 20 Macchioni L., Corazzi L., Nardicchi V., Mannucci R., Arcuri C., Porcellati S., Sposini T., Donato R., and Goracci G. (2004) *J Biol Chem* 279, 37860-37869. Nardicchi V., Macchioni L., Ferrini M., and Goracci G. (2007) *Biochim Biophys Acta* 1771, 1345-1352. Rigoni M., Pizzo P., Schiavo G., Weston A. E., Zatti G., Caccin P., Rossetto O., Pozzan T., and Montecucco C. (2007) *J Biol Chem* 282, 11238-11245. Sun G. Y., Xu J., Jensen M. D., Yu S., Wood W. G., Gonzalez F. A., Simonyi A., Sun A. Y., and Weisman G. A. (2005) *Mol Neurobiol* 31, 27-42. Supported by Fondazione Cassa di Risparmio di Perugia C.P. 2008.021.321



**Symposium C:  
micro-RNA: Novel players of gene regulation**



## THE MICRO RNA WORLD

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Over the past few years, a new and surprisingly abundant class of RNA regulatory genes known as microRNAs (miRNAs) has been found to confer a novel layer of genetic regulation in cells. miRNAs comprise a large family of 22-nucleotide single-stranded RNAs that silence gene expression by binding to target mRNAs. miRNA-mRNA binding usually involves strong base-pairing between the 5' end of a miRNA and its target complementary sequence in the 3'-untranslated region (3'UTR) of an mRNA, while additional base pairings can also contribute to the binding. miRNA binding appears to result in translational repression and, in some cases, degradation of cognate mRNAs, causing partial or full silencing of the respective protein-coding genes. We now know that hundreds of distinct miRNA genes control a range of physiological processes in almost all eukaryotes, including development, growth, differentiation and metabolism. miRNAs are currently estimated to comprise 1–5% of animal genes, making them one of the most abundant classes of gene regulators. This discovery introduces a new set of evolutionary mechanisms that has the potential to have profoundly influenced phenotypic complexity and diversity during animal phylogeny.

## MECHANISMS OF MICRO RNA-MEDIATED GENE SILENCING

**Ana Eulalio (1,2) and Eliza Izaurralde (2)**

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MicroRNAs are evolutionarily conserved small noncoding RNAs that regulate gene expression at the post-transcriptional level by base-pairing to partially complementary sequences in the 3' UTRs of target mRNAs. Control of gene expression by miRNAs plays an important role in a broad range of biological processes including development and cellular differentiation, and has also been implicated in several human disorders such as cancer and cardiovascular diseases. Although much is known about their biogenesis and biological functions, the mechanism(s) by which miRNAs silence gene expression in animal cells remains controversial. To elucidate how miRNA-mediated silencing is accomplished, we screened an RNA interference library for suppressors of miRNA-mediated regulation in *Drosophila melanogaster* cells. In addition to known proteins required for miRNA biogenesis and function, the screen identified the p-body component GW182 and the decapping activator Ge-1 as being required for silencing by miRNAs. We have functionally characterized the role of these proteins in the context of the miRNA pathway. Furthermore, our results indicate that translational repression and accelerated mRNA degradation are independent effects of miRNA action that together determine the extent of silencing, in a target-specific manner.

## **ROLE OF SMALL NON CODING RNAS IN THE PHYSIOPATHOLOGY AND THERAPY OF DUCHENNE MUSCULAR DYSTROPHY**

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microRNAs are recognized as important regulators of gene expression in the differentiation commitment of several cell types and have been shown to occupy very high hierarchical positions in the cascade of regulatory events controlling cell specification. Moreover, deregulated miRNA expression was associated to a large variety of human diseases. Duchenne Muscular Dystrophy (DMD) is a severe genetic disorder caused by mutations in the dystrophin gene. The disruption of the Dystrophin-Associated Protein Complex (DAPC) at the muscle membrane, due to dystrophin deficiency, represents the primary event that leads to the disease pathogenesis. However, the downstream events that contribute to the disease progression are still not completely elucidated and are likely to be the cause of DMD heterogeneity. Even though a cure is not yet available, several different therapeutic strategies are nowadays entering human experimentation. In particular, exon skipping has been proven to be very powerful in restoring dystrophin expression and conferring benefit in animal models. We have applied the use of antisense RNAs in order to modify the splicing of dystrophin frame shifting mutations in order to rescue the correct reading frame of the transcript. In the last years we have demonstrated the effectiveness of this approach both in human DMD myoblasts and in the mouse mdx animal model. More recently, taking advantage of a controlled rescue of dystrophin synthesis through exon skipping in mdx mice, we discovered that molecular circuitries, important for muscle differentiation and tissue integrity, are controlled directly by dystrophin through epigenetic control of a specific class of miRNAs.

# EXPLOITING AND ANTAGONIZING MICRORNA REGULATION FOR THERAPEUTIC APPLICATIONS

**Luigi Naldini**

"S. Raffaele" Hospital of Milan

**Abstract not submitted**

**Symposium D:  
Epigenetic modifications in health and disease**



# EPIGENETICS: POLY(ADP-RIBOSYL)ATION OF PARP-1 REGULATES GENOMIC METHYLATION PATTERNS

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DNA methylation is the only epigenetic modification that occurs on eukaryotic DNA. 5-methylcytosine is considered to be the fifth base of DNA as - through its non-random distribution along the genome - it expands the genetic information encoded by the sequence of the four bases in DNA into the realm of epigenetics. This epigenetic code constitutes part of the epigenetic chromatin modifications that control gene expression patterns allowing DNA to perform its function timely. In fact, the genome methylation pattern is characterized by the presence of methylated cytosines on bulk DNA, whereas the unmethylated residues are mainly located within particular regions termed CpG islands (CGIs). The CGIs represent 1-2% of genomic DNA and are generally located in the 5' promoter regions of housekeeping genes, sometimes overlapping the coding region to variable extents (usually the first exon). Although their sequence is enriched in CpG dinucleotides, which are the best substrates for DNA methyltransferase activity, the CGIs are mainly unmethylated and the associated genes are actively transcribed; transcription is inhibited when these regions undergo methylation. Changes in this "normal" pattern can be observed in cancer cells where typical events are aberrant hypermethylation of housekeeping gene promoters and widespread genome hypomethylation. The molecular mechanisms behind these cancer-related changes in DNA methylation patterns are not well understood. Two questions are particularly important: (i) how CpG islands are protected from methylation in normal cells, and how this protection is compromised in cancer cells, and (ii) how the genome-wide demethylation in cancer cells occurs. The latter question is especially intriguing since, so far, no DNA demethylase enzyme has been found. We hypothesize that the right nuclear balance between unmodified and PARylated poly(ADP-ribose) polymerase 1 (PARP-1), which depends on the dynamics of PARPs/PARG activity, is the key to maintain genomic methylation pattern. According to our data, decreased or increased levels of PARylated PARP-1 are responsible for diffuse hypermethylation or hypomethylation of DNA, respectively. In our model, polymers present on PARP-1 interact noncovalently with DNA methyltransferase1 (Dnmt1), preventing its enzymatic activity. In the absence of PARylated PARP-1, Dnmt1 is free to methylate DNA; if, in contrast, high levels of PARylated PARP-1 persist, Dnmt1 will be stably inhibited, preventing DNA methylation. CTCF, an ubiquitous abundant nuclear protein with different functions, has been included as an important player in Dnmt1/PARP interaction as CTCF, which exists in PARylated form, is by itself capable of activating PARP-1 automodification. Recent data showed that poly(ADP-ribosyl)ation controls important genes as Dnmt1, p16 and Igf2 so that a deregulation of PARP-PARG activities could induce tumorigenesis. Now a new question emerges: does PARylated PARP-1 introduce an epigenetic mark on chromatin? PARylated PARP-1 could mark those DNA sequences that must be maintained in a non methylated state in normal cells and directly prevent Dnmt1 access to these sequences.

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## THE EPIGENOME AND THE CONTROL OF INFLAMMATORY GENE EXPRESSION

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The antimicrobial response and both normal and pathological inflammation involve coordinated changes in the expression of several hundreds of genes, which in large part are direct targets of the NF- $\kappa$ B/Rel family of transcription factors. Products of the activated genes act at different levels, including leukocyte migration, polarization and activation, microbial killing and tissue repair. Apart from directly binding and activating, among others, genes encoding chemokines, inflammatory cytokines and antimicrobial proteins, NF- $\kappa$ B/Rel transcription factors contribute to the response by controlling feed-forward transcriptional loops in which an essential coregulator necessary for downstream gene activation (or repression) is induced by NF- $\kappa$ B and cooperates with it in transcriptional control. Some of these coregulators act to overcome or modify the barrier imposed by chromatin to transcription activation. In fact, the epigenome of macrophages, professional cells of the innate immune system, undergoes striking rearrangements in the very first hours after a microbial challenge. Our experimental approach involves the integration of epigenomic analyses in primary mouse cells of the innate immune system and gene knockout studies in order to clarify the specific role and fine mechanism of action of inflammatory transcriptional coregulators.

## **MOLECULAR GENETICS OF RETT SYNDROME: WHEN EPIGENETIC SIGNALS GO UNRECOGNIZED**

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Rett Syndrome (RTT) is a devastating X-linked neurodevelopmental disorder found primarily in females. With an incidence of 1:10,000 girls, RTT represents the second cause of profound mental disability in females. Patients with classic RTT suffer from a broad array of phenotypes, affecting almost any component of the central and autonomic nervous systems that include impaired social behavior and communication abilities, loss of motor skills and the development of stereotypic hand movements, epileptic and respiratory crisis. Mutations in the epigenetic MECP2 transcriptional repressor are the primary cause of Rett syndrome but are also found in patients affected by learning disability, neonatal encephalopathy, autism and mental retardation therefore making RTT paradigmatic for the study of autism spectrum disorders. Although, there is no effective therapy to prevent/treat RTT symptoms, the recently discovered disease reversibility in mice suggests that there are therapeutic possibilities. However, since slight perturbations in MeCP2 levels are deleterious for brain, gene therapy is not a valid approach. One alternative is to identify proteins or pathways that suppress MeCP2 dysfunction phenotypes and can be therapeutically modulated. Recent findings suggest that MeCP2 phosphorylation is important for neuronal maturation and connectivity. The disruption of this process or of the involved kinases might lead to neural-specific pathologies. Accordingly, our groups have significantly contributed to demonstrate the existence of two kinases, CDKL5 and HIPK2, involved in MeCP2 regulation. Importantly CDKL5 works in the same molecular pathway of MeCP2 and causes several forms of mental retardation, including RTT, and Hipk2-null mouse model shows neurological phenotypes.

## EPIGENETICS OF HUMAN BEHAVIOUR

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Recent research has demonstrated that complex epigenetic mechanisms, which regulate gene activity without altering the DNA code, have long-lasting effects within mature neurons and, thus, may affect human behaviour. By the same way, epigenetic alterations in brain have been found to be the underlying cause of many neurological and psychiatric disorders. DNA methylation patterns are sculpted during development and it has been a long held belief that they remain stable after birth in somatic tissues. DNA methylation is instead dynamic later in life in neurons and thus potentially responsive to different environmental stimuli throughout life; it has been hypothesized a mechanism linking the environment early in life and long-term epigenetic programming of behaviour. However such kind of studies are very difficult to be performed because epigenetic changes unlike genetic variations, that can be detected even in blood samples, are tissue-specific and thus analyses should be conducted directly on specific areas of the brain. On these bases, we have investigated whether alteration of DNA methylation in brain may be associated with human suicidal behaviour. We analyzed, by three independent quantitative methods, the DNA methylation degree at BDNF gene which is involved in processes related to neuronal plasticity and connectivity including anxiety-like behaviours. This study was performed on samples of brain tissue obtained from the Wernicke's area of suicide completers and control subjects who died for other causes. Wernicke's area has been chosen for its critical involvement with human language and with associative and integrative functions, consistently with several findings of neurocognitive alterations in suicide attempters. For each of the study subjects the drug history, toxicological findings, genetic polymorphisms and possible pre-existing psychopathological conditions were evaluated. The results of our high resolution DNA methylation analyses performed on 77 subjects will be discussed.

**Symposium E:  
Glycobiology of human diseases**



## INSULIN RESISTANCE AS A MEMBRANE MICRODOMAIN DISORDER

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Caveolae are a subset of membrane microdomains (lipid raft) particularly abundant in adipocytes. Critical dependence of the insulin metabolic signal transduction on caveolae/microdomains in adipocytes has been demonstrated. These microdomains can be biochemically isolated with their detergent insolubility and were designated as detergent resistant microdomains (DRMs). Gangliosides are known as structurally and functionally important components in microdomains. We demonstrated that increased GM3 expression was accompanied in the state of insulin resistance in mouse 3T3-L1 adipocytes induced by TNF $\alpha$  and in the adipose tissues of obese/diabetic rodent models such as Zucker *fa/fa* rats and *ob/ob* mice(1). We examined the effect of TNF $\alpha$  on the composition and function of DRMs in adipocytes and demonstrated that increased GM3 levels result in the elimination of insulin receptor (IR) from the DRM while caveolin and flotillin remain in the DRMs, leading to the inhibition of insulin's metabolic signaling(2). These findings are further supported by the report that mice lacking GM3 synthase exhibit enhanced insulin signaling(3). To gain insight into molecular mechanisms behind interactions of IR, caveolin-1 (Cav1) and GM3 in adipocytes, we have performed immunoprecipitations, cross-linking studies of IR and GM3, and live cell studies using fluorescence recovery after photobleaching (FRAP) technique. We found that (i) IR form complexes with Cav1 and GM3 independently; (ii) in GM3-enriched membranes the mobility of IR is increased by dissociation of the IR-Cav1 interaction; (iii) the lysine residue localized just above the transmembrane domain of the IR  $\beta$ -subunit is essential for the interaction of IR with GM3. These evidence substantiate that insulin resistance in adipocytes is caused by dissociation of the IR-Cav1 complex by the interactions of IR with GM3 in microdomains(4). In addition, our data substantiate a novel diagnostic strategy for metabolic disorders by measuring the circulating levels of GM3(5). In this symposium, I demonstrate a new concept "Metabolic diseases, such as type 2 diabetes, are a membrane microdomain disorder caused by aberrant expression of gangliosides" and propose the new therapeutic strategy "membrane microdomain ortho-signaling therapy". References: 1. Tagami S et al. , Ganglioside GM3 participates in the pathological conditions of insulin resistance. *J Biol Chem* 277, 3085–92, 2002. 2. Kabayama K et al., TNF $\alpha$ -induced insulin resistance in adipocytes as a membrane microdomain disorder: involvement of ganglioside GM3. *Glycobiology* 15, 21–29, 2005 3. Yamashita et al., 2003. Enhanced insulin sensitivity in mice lacking ganglioside GM3. *Proc Natl Acad Sci U S A* 100, 3445-59, 2003. 4. Kabayama K et al., Dissociation of the insulin receptor and caveolin-1 complex by ganglioside GM3 in the state of insulin resistance. *Proc Natl Acad Sci. USA* 104, 13678-83, 2007. 5. Sato T et al., Circulating Levels of Ganglioside GM3 in Metabolic Syndrome: A Pilot Study. *Obesity Research & Clinical Practice* 2, 231-38, 2008.

## GLYCOSAMINOGLYCAN SULFATION AND SKELETAL DYSPLASIAS

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Proteoglycans (PGs) are important macromolecules of the extracellular matrices composed of a core protein covalently linked to different glycosaminoglycans (GAGs) including chondroitin sulfate (CS), dermatan sulfate, heparan sulfate (HS) and keratan sulfate (KS). Sulfation is the most relevant modification of GAG chains. Mutations in the enzymes coding for modifications of HS chains result mainly in lethality due to defects in development. Defect in KS sulfation results in macular corneal dystrophy an autosomal recessive disorder causing opacities in the cornea. Altered CS sulfation either due to defects in the sulfate activation pathway or in specific chondroitin sulfotransferases results in skeletal phenotypes. Generalized GAG undersulfation caused by defects in the sulfate activation pathway either linked to the intracellular sulfate transporter (SLC26A2) or to the cytosolic bifunctional enzyme involved in PAPS synthesis (PAPS synthase 2) results in different chondrodysplasias. Defects in PAPS synthase 2 have been reported only in a Pakistani family with spondyloepimetaphyseal dysplasia and in the brachymorphic mouse. Mutations in the SLC26A2, a widely distributed sulphate transporter, cause four different recessive chondrodysplasias: recessive multiple epiphyseal dysplasia, diastrophic dysplasia (DTD), atelosteogenesis type 2 and achondrogenesis 1B. To complement the study in the patients, we generated the first *Slc26a2* knock-in mouse (*dtd* mouse) characterized by growth retardation, skeletal dysplasia and joint contractures, thereby recapitulating essential aspects of the DTD phenotype in humans. To get new insight on how macromolecular sulfation affects endochondral bone growth we studied PG sulfation and chondrocyte proliferation in the growth plate of the *dtd* mouse. For PG sulfation analysis we isolated by manual microdissection the growth plate from tibial sections; disaccharides, released from chondroitin sulfate PGs after digestion with chondroitinase ABC, were analysed by RP-HPLC. Results demonstrated a significant undersulfation of *dtd* growth plates compared to those of wild-type animals. As a consequence of chondroitin sulphate undersulfation reduced chondrocyte proliferation was detected in the proliferative zone of the tibial growth plate of mutant mice compared to wild-type animals. Skeletal phenotypes result also from defect in chondroitin sulfotransferases that transfer sulphate on specific position of the galactosamine moiety. Patients with mutations in the chondroitin-4-sulfotransferase have never been reported, but the chondroitin-4-sulfotransferase knock-out mouse has a strong skeletal phenotype. Mutations in the chondroitin-6-sulfotransferase (also known as carbohydrate sulfotransferase 3, CHST3) have been reported in a phenotypic spectrum including Larsen syndrome, humero-spinal dysostosis and spondyloepiphyseal dysplasia Omani type. Chondroitin sulfate disaccharide analysis in dermal fibroblasts from two patients with CHST3 mutations showed markedly decreased 6-O-sulfation, confirming functional impairment of CHST3, but enhanced 4-O-sulfation. When compared to SLC26A2 deficiency, CHST3 deficiency has a similar or more pronounced effect on joint formation and articular homeostasis, but a less severe effect on endochondral growth of skeletal elements. The phenotypic difference might be ascribed either to different functions of the 4-sulfation versus 6-sulfation on chondroitin sulfate or to the degree of sulfation of other GAGs or proteins, affected in SLC26A2 but not in CHST3 deficiency. It appears therefore that the elucidation of sulfation processes, their associated biologic mechanisms and related clinical disorders is only just beginning and may yield in the future more biologically and clinically relevant information. Work supported by Telethon-Italy (grant #GGP06076) and the European Community (project LSHM-CT-2007-037471).

## HYALURONAN: DOUBLE ROLE IN INFLAMMATION

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Molecules in biological systems can often perform more than one function. High molecular weight (MW) hyaluronan (HA) produced by type B synovial cells is one of the main components of synovial fluid. Both HA concentration and MW usually decline in inflamed joints. Previous research suggested that HA degradation occurs in pathological conditions, probably because of HA chains depolymerization by reactive oxygen species. However, it was also reported that both low and high MW HA were produced during pathological conditions, with different roles. For instance, HA fragments induce the expression of genes involved in the inflammation processes, while high MW HA was reported to exert the opposite effects. When native endogenous HA is degraded in inflammation tissue, the molecular sizes of the degraded HA can exacerbate inflammation, cellular infiltration, migration and extracellular matrix degradation by inducing cytokines, chemokines, matrix metalloproteases, free radicals expression. The HA oligosaccharides are the key components in a vicious circle of inflammation or tumor metastasis. To exert functions, they may bind specific proteins such as Toll-like receptors (TLRS), nuclear factor kappaB (NF-kB) and CD44. We especially investigated the effect of HA interaction with these receptors. Firstly we found that the size of HA was able to modulate inflammation and apoptosis differently in unstimulated or lipopolysaccharide (LPS)-stimulated murine chondrocytes. High MW HA reduced NF-kB activation, pro-inflammatory cytokine expression, NO generation and apoptosis. In contrast, low MW HA exerted a slight inflammatory activity in unstimulated chondrocytes while it enhanced cytokine production in LPS-stimulated cells compared with cells treated with LPS alone. Medium MW HA did not exert any inflammatory activity in untreated cells and was unable to reduce inflammatory cytokines and apoptosis in LPS-stimulated chondrocytes. We hypothesized that HA via its carboxylic group interactions may interfere with the mechanism of NF-kB activation that in turn primes inflammation. In addition, we examined the effects of HA, at different MW, on the TLR-4 receptor modulation in murine chondrocytes, both stimulated and unstimulated with LPS. Also in this study the size of this polymer was able to modulate inflammation differently in unstimulated or LPS-stimulated cells. High MW HA was able, in LPS-stimulated chondrocytes, to inhibit not only TLR4 receptor, MyD88 and TRAF6 expression but also NF-kB activation, the increment in pro-inflammatory cytokines, iNOs and MMP-13. In contrast, low MW HA slightly enhanced TLR4 receptor, MyD88 and TRAF6 expression, NF-kB activation, pro-inflammatory cytokines, iNOs and MMP-13 activities in both LPS-stimulated/unstimulated cells. Also in this case medium MW HA did not exert any activity. We suggested, by means of a specific antibody targeting the TLR-4 receptor, that low MW HA stimulates TLR-4 receptor, while high MW HA masks TLR-4 receptor by preventing LPS stimulation. We also studied the effects of HA on the CD44 receptor modulation in articular mouse chondrocytes, both PMA-stimulated/unstimulated. In PMA-stimulated cells high MW HA was able to inhibit not only the CD44 receptor and PKC expression, but also NF-kB activation and the increment in pro-inflammatory cytokines, MMP-13, iNOS, and NO production stimulated by PMA. Instead, low MW HA exerted a significant inflammatory effect in unstimulated cells, while in stimulated cells it enhanced the PMA increment of NF-kB activation, CD44, PKC, TNF-alpha, IL-1beta, MMP-13, and iNOS expression. Medium MW HA had no effect. Low MW HA interacted with the CD44 receptor, as suggested by the use of a specific CD44 antibody. Surprisingly, high MW HA showed a protective effect also in PMA-stimulated chondrocytes plus the CD44 blocking antibody thus suggesting that its inhibitory effect on PMA-induced inflammation was exerted by directly reducing PKC activation.

## ACIDIC GLYCOHYDROLASES LOCALIZATION AND REGULATION: PATHOLOGICAL IMPLICATIONS

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In addition to the well known functions of gangliosides on cell surface as antigens and receptors for different molecules and bacterial toxins, these molecules play an important role in cell adhesion, transmembrane signalling, and protein trafficking. Quantitative and qualitative changes occur in ganglioside expression during the oncogenic transformation, where an altered expression of enzymes associated with ganglioside glycosylation has been observed. This is the case of the acidic glycohydrolases beta-hexosaminidase, beta-galactosidase, beta-glucosylceramidase, all involved in the monosialoganglioside GM3 metabolism. These glycohydrolases display altered expression and targeting in leukaemic cells. In previous studies, we found the acidic beta-hexosaminidase, as a mature form, associated to the extracellular side of plasma membrane and to the lysosomal membrane. This plasma membrane-associated form displays the same properties of the soluble lysosomal counterpart and is able to hydrolyze its natural substrate GM2 to GM3. In addition, several lines of evidence indicate that the enzyme activity at the cell surface can be extended also to neighbouring cells. These results suggest that membrane-associated forms translocate from the lysosomal membrane to the plasma membrane by an as yet unknown mechanism and reveal a new aspect of glycohydrolase biology. At the cell surface, these enzymes may play functional role by modulating GM3 ganglioside level and glycosphingolipid glycosylation. To go more insight the biological role of these membrane associated enzymes, studies were carried out in human cells overexpressing beta-hexosaminidase; in human peripheral blood T-lymphocytes either resting or in vitro activated; in blasts from peripheral blood of patients suffering from different forms of Chronic Lymphoid Leukaemia (CLL). We get evidence that the level of plasma-membrane associated beta-hexosaminidase, beta-galactosidase and beta-glucosylceramidase (but not of alpha-mannosidase, a glycohydrolase not involved in stepwise degradation of GM3) increases significantly in cells overexpressing beta-hexosaminidase. In T-lymphocytes GM3 represents the main ganglioside constituent of cell plasma membrane (72% of total ganglioside content) where it is mainly concentrated in glycosphingolipid-enriched microdomains and participate to a multimolecular signaling complex involved in T cell activation. We revealed the presence of glycohydrolases on the plasma membrane of human T-lymphocyte, where their activity progressively increases (up to 10 fold for beta-hexosaminidase) as a function of T-cell activation; this increase correlates with increased levels of GM3 and glycosphingolipid pattern modification. The increase in enzyme levels, as well as in GM3 level, does not correlate with a particular lymphocyte subpopulation, and take place in both CD4+ and CD8+ T-cell subpopulations. Data obtained from the analysis of CLL demonstrate that an impairment of T-cell activation pathway results in a decreased recruitment of glycohydrolases on plasma membrane, underlying a biological role of these enzymes at the cell surface. Acknowledgements Work supported by PRIN-COFIN grant and by Fondazione Cassa di Risparmio di Perugia, grant 2008.021.375 to C.E.

**Symposium F:  
Biochemical mechanisms of metabolic diseases  
and their vascular complications**



## PRO-INFLAMMATORY PATHWAYS IN OBESITY-RELATED DISORDERS

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Obesity is characterized by a chronic low-grade inflammation consisting of induction of pro-inflammatory signaling and gene expression in multiple tissues, and macrophages infiltration into adipose tissue. Research from different laboratories revealed that signal transducers typically involved in innate immune response are also playing a direct role in the control of metabolism in response to metabolic stress. As paradigm of the molecular interactions between inflammation and metabolism we have investigated the role of the c-Jun N-terminal Kinases-1 (JNK1) and phosphoinositide 3-kinase gamma (PI3K $\gamma$ ) in obesity, energy balance, and insulin resistance. c-Jun N-terminal Kinases (JNKs), which respond to several pro-inflammatory cytokines such as TNF-alpha and IL1, and metabolic stress such as ROS, ER-stress, and long-chain saturated fatty acid, are considered key players in the development of obesity-induced insulin resistance. Jnk1 $^{-/-}$  mice are resistant to diet-induced obesity and glucose intolerance, and acute inhibition of JNK improves insulin sensitivity in obese mice. To identify the role of JNK1 in obesity and insulin resistance we have investigated the role of glucotoxicity and lipotoxicity in JNK activation, the role of JNK activation in primary pancreatic islet, in primary hepatocytes, and by using bone marrow transplantation technology we studied the action of JNK1 in hematopoietic and non-hematopoietic compartments. Finally we proposed a model for the role of JNK1 in diet-induced glucose intolerance based on three different mechanisms: obesity resistance, interference with IRS signaling, and promotion of metabolic inflammation. The phosphoinositide 3-kinase gamma (PI3K $\gamma$ ) is a pro-inflammatory kinase highly expressed in hematopoietic cells and at lower level in endothelial cells, vascular myocytes, cardiomyocytes, and other non-hematopoietic cells. PI3K $\gamma$  plays a major role in innate immunity and inflammation and in particular in myeloid cells chemotaxis. Furthermore, PI3K $\gamma$  was also implicated in the effects of  $\beta$ -adrenergic signaling within the cardiovascular system. We have investigated the role of PI3K $\gamma$  in diet-induced obesity and insulin resistance. C57bl6/j mice bearing a targeted deletion at the PI3k $\gamma$  locus (PI3k $\gamma$  $^{-/-}$ ) and Wt controls were placed either on chow or high-fat diet for 16 weeks. No significant difference was observed between Wt and PI3k $\gamma$  $^{-/-}$  mice placed on chow diet, however when fed with high-fat diet PI3k $\gamma$  $^{-/-}$  mice show an obesity resistant phenotype and markedly improved glucose and insulin tolerance compared to Wt mice. Hyperinsulinemic euglycemic clamps show improved systemic insulin sensitivity in PI3k $\gamma$  $^{-/-}$  compared to Wt controls. Furthermore, PI3k $\gamma$  $^{-/-}$  mice display reduced levels of inflammatory markers. PI3K $\gamma$  is known to signal via a lipid kinase-dependent pathway and a kinase independent pathway via activation of cAMP phosphodiesterase. To identify the specific signaling mechanism involved in the metabolic phenotype of PI3K $\gamma$  KO mice we use mice expressing a catalytically inactive PI3K $\gamma$  that retain the kinase independent signaling, the PI3K $\gamma$ KD/KD mice. We could recapitulate obesity resistance, decreased metabolic inflammation, and improved glucose homeostasis in PI3K $\gamma$ KD/KD mice demonstrating that the metabolic action of PI3K $\gamma$  depends on its lipid-kinase activity. Our results show for the first time that the pro-inflammatory kinase PI3K $\gamma$  is a major signaling node in diet-induced obesity and insulin resistance, and suggest that compounds targeting PI3K $\gamma$  kinase activity might be valuable drug-candidates for obesity-related diseases. Altogether, our results support the concept that inflammation, energy balance, and glucose homeostasis are integrated at the molecular level, and that targeting pro-inflammatory signaling cascades activated by metabolic stress might be a valuable strategy to treat obesity-related diseases.

## PROTEASES AT THE INTERFACE OF METABOLIC DISEASES AND VASCULAR INFLAMMATION

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Diabetes and its vascular complications will be the most common health problem in the next decades both in western countries and in the third world. Although several intracellular pathways linking hyperglycemia and dyslipidemia to vascular damage have been identified, mechanisms explaining how metabolic toxicity trigger low-grade inflammation and ischemic disease are still elusive. We hypothesize that Transmembrane Proteases (TPs) act at the interface of metabolic cues and vascular homeostasis and serve to trigger, sense and amplify the low-grade inflammatory state that sustains the progression of cardiovascular disease. TPs are proteins anchored in the plasma membrane with their catalytic site exposed to the external surface of the membrane. TPs participate in extracellular proteolysis (degradation of extracellular matrix components, regulation of chemokine activity, release of membrane-anchored cytokines, cytokine receptors and adhesion molecules) and influence cell functions (growth, secretion of angiogenic molecules, motility). TPs generate intracrine, autocrine and paracrine signals with effects on inflammation, cell growth, migration and metabolism depending on substrates and cell/tissue context. The overall effect of a ligand/receptor dependent pathway may therefore depend on factors regulating ligand and receptor expression and factors regulating ligand and receptor shedding. Most of the extracellular regulated membrane proteolysis is due to enzymes of the ADAM family, particularly ADAM10/12/15/17. Their shedding activity is finely regulated by endogenous inhibitors called TIMPs (Tissue inhibitor of Metalloproteinase, 1/2/3/4), being TIMP-3 the most effective on all the ADAMs. Interestingly, TIMP3 acts also as a modulator of angiogenesis/vasculogenesis, cell migration/proliferation, ischemia/reperfusion (I/R) injury and innate immune system activation. Experimental and clinical evidences suggest that the TPs may be involved in the development of vascular diseases, playing a key role in the activation of four major pathways, i.e. Inflammation, Glucose Toxicity, Oxidative Stress, Vascular/Hemodynamic Remodelling. Our data point out a downregulation of Timp3 expression as a common element for development of concomitant defects causing insulin resistance, inflammation, unsystematic angiogenesis and cell growth. Timp3 is the major known regulator of ectodomain shedding and it belongs to a family of endogenous inhibitors of metalloproteinases (MMPs) but retains 2 different biological activities crucial in both atherosclerosis and inflammation: a. to control MMP-2 and MMP-9, which are overexpressed in atherosclerotic plaques and allow migration of VSMC in neointima hyperplasia and restenosis lesions. b. to inhibit enzymes of the ADAM (A Disintegrin And Metalloprotease) family, therefore regulating the ectodomain shedding process and the network of inflammatory signals that may be generated in a cell- or tissue-specific manner. We and others suggested that the ectodomain shedding regulation may represent a relevant Timp3 function to slow the development of metabolic and vascular diseases. These findings can support the hypothesis that both chronic or acute modifications of the TIMP-3 related pathways might play a critical role in coupling subclinical inflammatory state to impaired glucose metabolism creating a common soil for the development of diabetes and atherosclerosis. Our data point to a downregulation of Timp3 expression as a common element for development of concomitant defects causing insulin resistance, inflammation, unsupervised angiogenesis and cell growth. These findings can support the idea that both chronic (genetically linked) or acute (metabolically linked) modifications of the TIMP-3 related pathways might play a critical role in coupling subclinical inflammatory state to impaired glucose metabolism creating a common soil which might predispose to the development of diabetes and atherosclerosis.

# MOLECULAR MECHANISMS OF HYPERHOMOCYSTEINEMIA AS A CARDIOVASCULAR RISK FACTOR

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Hyperhomocysteinemia (HHcy) is a cardiovascular risk factor (CVRF), mainly but not exclusively relevant to stroke, both in the general population and in high CV mortality subsets, such as end stage renal disease patients, affected with high HHcy prevalence (1, 2). Despite the epidemiological evidence, the mechanism of homocysteine (Hcy) toxicity is still uncertain. It has been proposed that Hcy is actually a surrogate CVRF, if not an innocent bystander. In other words alterations of other products of sulfur amino acid metabolism, such as Hcy thiolactone or H<sub>2</sub>S, could be responsible for CV damage, high Hcy being just "a red flag in a disrupted circuit". HHcy is accompanied by a significant rise of both the intracellular and circulating concentrations of its precursor S-adenosylhomocysteine (AdoHcy), which is a strong inhibitor of S-adenosylmethionine-dependent methyltransferases. Protein and DNA methylation have been found markedly reduced in hyperhomocysteinemic uremic patients (3, 4). Under these conditions, DNA hypomethylation is able to induce altered biallelic expression of pseudoautosomal and imprinted genes. These effects are partially reversed upon Hcy-lowering therapy. AdoHcy increase is also accompanied by Adenosine reduced availability, with potential vascular effects. From a clinical perspective, it has been shown that AdoHcy is a better biomarker of atherosclerosis than Hcy itself (5). HHcy induces endothelial toxicity and it has been suggested that Hcy lowering measures are most effective toward early, rather than advanced, atherosclerotic plaques. It has been proposed that formation of homocysteinylated proteins (Hcy-proteins) is a potential mechanism of Hcy vascular damage ("molecular target hypothesis"). Circulating Hcy is covalently bound to proteins, in particular serum albumin, to form various Hcy-protein adducts. Various Hcy-proteins have been isolated and characterized (6). Moreover, it has been demonstrated that plasma levels of Hcy-proteins are significantly higher, in uremic hyperhomocysteinemic patients, than in healthy controls and only partially susceptible to reversal upon therapy (7). This suggests the possible role of Hcy-proteins in the mechanism of Hcy toxicity. More recently, data from our laboratory showed that treatment with Hcy-albumin, within the circulating concentration range detected in the uremic milieu, induces an increase of monocyte adhesion and the production of specific adhesion molecules involved in the genesis of endothelial damage. Furthermore, a number of cytokines and chemokines, involved in the activation and recruitment of monocytes at endothelial surface level, as well as in the endothelial damage generation, are significantly upregulated. These results, as a whole, support the interpretation that Hcy-proteins are neither a byproduct of HHcy, nor a mere marker of chronic exposure to elevated plasma Hcy, but rather a direct effector of early inflammatory modifications leading to the development of atherosclerotic lesions. 1. Hodis HN, Mack WJ, Dustin L, Mahrer PR, Azen SP, Detrano R, Selhub J, Alaupovic P, Liu CR, Liu CH, Hwang J, Wilcox AG, Selzer RH; BVAIT Research Group. *Stroke* 40:730, 2009. 2. de Ruijter W, Westendorp RG, Assendelft WJ, den Elzen WP, de Craen AJ, le Cessie S, Gussekloo J. *Brit Med J*. Jan 8;338:a3083, 2009. 3. Ingrosso D, Cimmino A, Perna AF, Masella L, De Santo NG, De Bonis ML, Vacca M, D'Esposito M, D'Urso M, Galletti P, Zappia V. *Lancet* 361:1693, 2003. 4. Ingrosso D, Perna AF. *Biochim Biophys Acta*. 2008 Dec 30. PubMed PMID: 19245874. 5. Liu C, Wang Q, Guo H, Xia M, Yuan Q, Hu Y, Zhu H, Hou M, Ma J, Tang Z, Ling W. *J Nutr*. 138:311-5, 2008 (see also comm by Wagner C, Koury MJ. *J Nutr*. 138:980, 2008.) 6. Jakubowski H, Perla-Kaján J, Finnell RH, Cabrera RM, Wang H, Gupta S, Kruger WD, Kraus JP, Shih DM. *FASEB J*. 23:1721, 2009. 7. Perna AF, Satta E, Acanfora F, Lombardi C, Ingrosso D, De Santo NG. *Kidney Int*. 69:869, 2006.

## THE DIETARY ANTIOXIDANT RESVERATROL AND CARDIOVASCULAR DISEASE

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Resveratrol is a plant antibiotic belonging to the class of phytoalexins and present at relatively high concentrations in red wine because it is produced by grapes. The latter fact, together with its documented cardioprotective effect, has been pointed to as the most likely explanation of the so-called "French Paradox". The protective effect of resveratrol on the cardiovascular system is mainly related to its potent antioxidant activity and its potential of inhibiting oxidative stress that is known to promote atherogenesis due to several mechanisms including lipoprotein oxidation. In addition, there is plenty of *in vitro* and *in vivo* experimental evidence to support this longevity-enhancing effect of resveratrol: this polyphenol, in fact, regulates nitric oxide production, ameliorates endothelial function, reduces smooth muscle cell proliferation, inhibits platelet aggregation, reduces inflammation, and protect mice from diet-induced obesity and metabolic diseases. Finally, resveratrol can also regulate gene expression. Among several mechanisms, we demonstrated that resveratrol protects the activity of the transcription factor Peroxisome Proliferator Activated Receptor-alpha (PPARalpha), a nuclear receptor that has a powerful anti-atherosclerotic action due to its ability to regulate intracellular and extracellular lipid metabolism, cholesterol trafficking and inflammatory response. Endothelial progenitor cells might play an important role in the resistance to atherogenesis because of their potential to repair damaged endothelium and to promote ischemia-induced angiogenesis. We demonstrated that red wine and resveratrol improve endothelial progenitor cells number and function in *ex vivo* and *in vivo* settings. Additionally, we provided evidence that the increase of the number of circulating endothelial progenitor cells is associated with increased blood flow and neovascularization in hindlimb ischemia. In conclusion, *in vitro*, *ex vivo*, and *in vivo* experimental data powerfully support the beneficial protective effects of resveratrol and moderate consumption of red wine in cardiovascular disease. However, the lack of randomized controlled clinical trials, together with insufficient epidemiological studies, does not warrant the use of pure resveratrol or moderate consumption of red wine to reduce the risk of atherosclerotic diseases.

## ENDOCANNABINOID METABOLISM AND GLUCOSE TRANSPORT

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Endocannabinoids bind to cannabinoid, vanilloid and peroxisome proliferator-activated receptors. The biological actions of these lipids are controlled by key agents responsible for their synthesis, transport and degradation, forming all together an “endocannabinoid system (ECS)”. In the past few years, evidence has been accumulated for a role of the ECS in regulating food intake and energy balance, both centrally and peripherally. In line with this, endocannabinoids like anandamide have been recognized as modulators of glucose transport in adipocytes, and up-regulation of the ECS in the gastrointestinal tract has been shown to have a potential impact on inflammatory bowel diseases. In this lecture, the main features of endocannabinoid metabolism will be presented, in order to put in a better focus the role of endocannabinoid signalling in glucose transport, and its relevance for obesity, cardiovascular pathologies and gastrointestinal diseases. Also the central and peripheral pathways that underlie these effects will be discussed, as well as the possible exploitation of ECS components as novel drug targets for therapeutic intervention in eating disorders.



**Symposium G:  
Nitric oxide and Carbon monoxide in biology and medicine**



## **NITRIC OXIDE-CARBON MONOXIDE INTERACTION AND OXIDATIVE STRESS IN OBESITY AND DIABETES**

**Nader G. Abraham**

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HO-derived CO and NOS-derived NO and other gaseous in the body participate in important physiological function and in metabolic events. The interaction between CO and NO on mitochondrial function including Krebs cycle and cytochrome oxidase, carbohydrate metabolism, amino acids metabolism (H<sub>2</sub>S) and cGMP will be discussed. Since NO was first identified in vascular system in late 1980s, we will focus first the interaction of CO and NO on endothelium functions. We examined the effect of interaction of HO-1 gene expression and NOS protein during an increase in oxidative stress in vascular dysfunction models including obese/diabetic models. We hypothesized that Upregulation of HO-1-derived CO reduces oxidative stress and increases eNOS, peNOS via an increases in pAMPK-pAKT cross talk in obese diabetic mice. Obese, ob, lean mice were divided into groups comprising lean, lean-inducers of HO-1, L-4F-treated, ob, ob-L-4F-treated. Food intake, blood glucose levels, CO, eNOS, pAMPK-pAKT and insulin receptor phosphorylation. Subcutaneous fat tissue (SAT) and visceral adipose tissue (VAT) were determined by MRI. The effect of HO-1 expression-derived CO and bilirubin on adiponectin levels and adipogenesis was also examined in human adipocytes stem cells. Both SAT and VAT global volumes decreased in ob-L-4F treated animals. Decreased levels of eNOS, peNOS, pAKT and pAMPK in vascular endothelium in ob mice were reversed by HO-1 expression. Upregulation of HO-1 produced adipose remodeling and increases peNOS levels in adipocytes. The anti-obesity effects of HO-1-derived CO, bilirubin are manifest by a decrease in visceral fat content with reciprocal increases in adiponectin and crosstalk between pAMPK and pAKT. The increase in HO-1-mediated cross talk in pAMPK-pAKT resulted in regulation of NO bioavailability. This presentation will also attempts to summarize the recent advances in understanding of mechanism whereby various gas exert bioregulation via multidisciplinary including genomic approaches and strategies.

## **NITRITE REDUCTION, A FUNCTION EMERGING FROM A PRE-AEROBIC PAST**

**M. Brunori, N. Castiglione, G. Giardina, I. Pecht, S. Rinaldo and F. Cutruzzolà**

Department of Biochemical Sciences, Sapienza-University of Rome

In eukaryotes, small amounts of nitrite exert cytoprotection from ischemia/reperfusion-related tissue damage in vivo, presumably via reduction to nitric oxide (NO) under anaerobic conditions and inhibition of mitochondrial function. Several hemoproteins have been involved in this protective mechanism, starting with deoxymyoglobin's and deoxyhemoglobin's capability to reduce nitrite; in spite of recent results, some aspects of the process are still problematic. In facultative aerobic bacteria, such as *Pseudomonas aeruginosa*, nitrite is reduced to NO by specialized heme-containing enzymes called cd1 nitrite reductases. Details of the catalytic mechanism have been recently clarified, based on the specific role of the unusual d1-heme and its unique kinetic properties. Available data support the hypothesis that the nitrite based reactions of contemporary eukaryotes are a vestige of earlier bacterial biochemical pathways. The bottom line is the significance for cellular homeostasis of nitrite, previously considered physiologically irrelevant.

## **THE ROLE OF NITRIC OXIDE AND ITS CONTROL OF MITOCHONDRIAL DYNAMICS IN DIFFERENTIATING MYOBLASTS**

**Emilio Clementi, Serena Pisoni, Sestina Falcone, Maria Teresa Bassi, Luca Scorrano  
Salvador Moncada, Clara De Palma**

Università di Milano; IRCCS E. Medea, Bosisio Parini; University of Geneva; University College London

Mitochondrial fission and fusion processes participate to cell adaptation to changing metabolic needs contributing to development and function of tissues. We found an unsuspected relationship between generation of the key muscle messenger nitric oxide (NO) and inhibition of mitochondrial fission, acting as one essential determinant of myogenic differentiation. We observed that elongation of mitochondrial network and generation of NO occurred in parallel in differentiating primary myogenic precursors cells. Blockade of NO synthesis with L-NAME inhibited myogenic differentiation and the formation of mitochondrial network. L-NAME acted selectively on fission and enhanced activity and translocation to mitochondria of the fission promoting GTPase Drp1. The dominant-negative Drp1 K38A mutant reversed the effects of L-NAME on both fission and myogenesis, establishing a causal relationship between the two processes. Fissioned mitochondria displayed altered bioenergetics and rendered cells susceptible to apoptotic stimuli. All the effects of L-NAME were mimicked by inhibiting guanylate cyclase and prevented by administration of NO or its physiological messenger cyclic GMP. These results establish that regulation of mitochondrial fission and bioenergetics by NO plays a central function in myogenesis.

## **CARBON MONOXIDE AND NITRIC OXIDE INTERACTIONS: IMPLICATIONS FOR RENAL PATHOPHYSIOLOGY**

**Francisca Rodriguez, Miguel G. Salom, Bernardo Lopez, Moises Hernández  
Isabel Hernández, Francisco J. Fenoy**

Departamento de Fisiología. Universidad de Murcia, Spain

Heme oxygenase (HO) is the rate-limiting enzyme in heme catabolism that leads to biliverdin, free iron and carbon monoxide (CO) production. Renal HO activity is driven by heme oxygenase-1 (HO-1) and heme oxygenase-2 (HO-2) isoenzymes, which are both expressed in vascular and tubular structures. HO-1 expression is low under basal conditions, but increases greatly in tubules and renal vessels in response to a variety of stressors stimuli. HO-2 is constitutively located in renal interlobar arteries and tubular structures, and it is responsible for the bulk of renal total HO activity in normal conditions. The Heme-HO system is believed to be involved in the regulation of renal hemodynamics and excretory function. HO-derived CO influences the reactivity and tone of the renal vasculature, and it also reduces the renal autoregulatory responses induced by increases in perfusion pressure. Metalloporphyrins, which inhibit endogenous HO activity, decrease total renal and medullary blood flow, lower the internal diameter of many arteries, and potentiate the pressure-induced vasoconstrictor response of renal arteries. Furthermore, renal CO affects transport in the thick ascending limb of Henle, whereas HO inhibition attenuates the pressure-natriuresis response in normal rats. Nitric oxide (NO), a product of L-arginine metabolism by NO synthase (NOS) isoforms, is a major contributor to renal vasodilatory mechanisms, and is also a key modulator of renal excretory function. The heme-HO and the L-arginine-NOS pathways interact at multiple sites and the functional impact of these interactions is often difficult to predict. For instance, CO inhibits NOS activity and reduces NO-dependent vasodilatory mechanisms. In contrast, NO decreases HO activity, promotes HO-1 protein expression, and interferes with the ability of CO to produce vasodilation. These interactions are functionally significant in renal physiology because several studies have shown that NOS inhibition enhances the CO-induced vasodilation and also the vasoconstriction caused by HO inhibitors. The latter case is likely due to the fact that NO synthesis inhibition is followed by increases in renal CO production both in vivo and in vitro. Overall, it appears that the renal vasodilatory mechanisms mediated by the HO-CO system are particularly facilitated when NO levels are low. In addition to the contribution of HO activity to renal physiological function, overexpression of HO-1 is believed to ameliorate renal damage in pathological conditions through its antioxidant and cytoprotective properties. In particular, HO-1 induction has a protective effect on ischemic renal failure, that seems to be partially mediated by decreasing the excessive production of peroxynitrite observed during ischemia. This renoprotective effect of HO-1 induction has also been reported in experimental models where NO bioavailability is diminished and oxidative stress is elevated, such as angiotensin-dependent hypertension or type-1 diabetes mellitus.

## CARBON MONOXIDE-RELEASING MOLECULES: VASODILATORY AND ANTI-ISCHEMIC EFFECTS

Roberto Motterlini

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Heme oxygenase-derived carbon monoxide (CO) serves as signaling mediator in a wide array of physiological functions to the extent that the beneficial effects observed when small amounts of CO gas are administered to mammalian organisms may be exploited for therapeutic purposes. In this context, the development of carbon monoxide-releasing molecules (CO-RMs) represents a pharmaceutical stratagem for the safe delivery of CO in the treatment of various pathological disorders. Transition metal carbonyls and boranocarbonates have been identified as ideal scaffolds for the synthesis of water-soluble compounds that release controlled amounts of CO within biological systems. Specifically, CORM-3 (ruthenium tricarbonyldichloro glycinate) and CORM-A1 (sodium boranocarbonate), which possess different chemical reactivities and kinetics of CO release, have been tested in various models of disease (1, 2, 3). The results collected to date indicate that CO-RMs are pharmacologically active as they exert vasodilatory, anti-ischemic and anti-inflammatory effects and can protect tissues against oxidative stress (4, 5, 6). Although the mechanism of action of CO-RMs remains to be fully elucidated, we have proposed that a dynamic interaction of CO with specific intracellular metal centers may be the common denominator for the diversified beneficial effects mediated by this gaseous molecule. Circumstantial evidence points to mitochondria as plausible, and perhaps, preferential targets of the signals transduced by CO (7, 8, 9). Thus, CO-RMs may help to identify cellular components that are responsive to CO and facilitate the therapeutic delivery of this gas in a safe, measurable and controllable fashion. 1. Motterlini R, Clark JE, Foresti R, Sarathchandra P, Mann BE, and Green CJ. Carbon monoxide-releasing molecules: characterization of biochemical and vascular activities. *Circ Res* 90: E17-E24, 2002. 2. Clark JE, Naughton P, Shurey S, Green CJ, Johnson TR, Mann BE, Foresti R, and Motterlini R. Cardioprotective actions by a water-soluble carbon monoxide-releasing molecule. *Circ Res* 93: e2-e8, 2003. 3. Motterlini R, Sawle P, Bains S, Hammad J, Alberto R, Foresti R, and Green CJ. CORM-A1: a new pharmacologically active carbon monoxide-releasing molecule. *FASEB J* 19: 284-286, 2005. 4. Motterlini R, Mann BE, and Foresti R. Therapeutic applications of carbon monoxide-releasing molecules (CO-RMs). *Expert Opin Investig Drugs* 14: 1305-1318, 2005. 5. Motterlini R. Carbon monoxide-releasing molecules (CO-RMs): vasodilatory, anti-ischemic and anti-inflammatory activities. *Biochem Soc Trans* 35: 1142-1146, 2007. 6. Alcaraz MJ, Guillen MI, Ferrandiz ML, Megias J, and Motterlini R. Carbon monoxide-releasing molecules: a pharmacological expedient to counteract inflammation. *Curr Pharm Des* 14: 465-472, 2008. 7. Boczkowski J, Poderoso JJ, and Motterlini R. CO-metal interaction: vital signaling from a lethal gas. *Trends Biochem Sci* 31: 614-621, 2006. 8. Taille C, El-Benna J, Lanone S, Boczkowski J, and Motterlini R. Mitochondrial respiratory chain and NAD(P)H oxidase are targets for the antiproliferative effect of carbon monoxide in human airway smooth muscle. *J Biol Chem* 280: 25350-25360, 2005. 9. Lancel S, Hassoun SM, Favory R, Decoster B, Motterlini R, and Neviere R. Carbon monoxide rescues mice from lethal sepsis by supporting mitochondrial energetic metabolism and activating mitochondrial biogenesis. *J Pharmacol Exp Ther* 1329: 641-648, 2009.

## NO/CO CROSSTALK IN OXIDATIVE STRESS AND ORGAN PROTECTION

**G. Li Volti 1, F. Salamone 2, F. Cappello 3, F. Galvano 1, A. Mangiameli 2, G.A. Marino 3, A. Vanella 1**

1 Dpt. Biol. Chem., University of Catania, 2 Dpt. Int. Med., University of Catania, 3 Dpt. Exp. Med., University of Palermo

The aim of the present research was to elucidate if hepatoprotective effects of silibinin, a flavonolignan, occurs via inhibition of mitochondrial damage and NF $\kappa$ B signaling. Db/db mice (n=16) were divided in two groups. The first (n=8) was fed a methionine-choline deficient (MCD) diet and was treated with silibinin (20 mg/g/daily for 4 weeks); the second (n=8) was fed an MCD diet and was treated with vehicle. Serum samples and liver specimens were used for metabolic assessment and molecular studies. A significant decrease of HOMA-IR and of serum ALT was observed in db/db + MCD mice treated with silibinin compared to vehicles. Histological score was significantly decreased in the silibinin group compared with vehicles. Electron microscopy showed enlarged mitochondria characterized by abnormal cristae arrangement in the vehicle group. Silibinin restored mitochondrial organization. Finally, NF $\kappa$ B activation was markedly increased in db/db + MCD mice as demonstrated by increased binding activity of nuclear p50 and p65 subunits. Silibinin significantly inhibited p50 and p65 binding activity. This study confirms that silibinin has an hepatoprotective and insulin-sensitizing action.

## X-RAY STRUCTURES OF CO, NO AND CYANIDE DERIVATIVES OF BOVINE HEART CYTOCHROME C OXIDASE

Shinya Yoshikawa

Department of Life Science, University of Hyogo

Cytochrome c oxidase (CcO) is the terminal oxidase of mitochondrial and prokaryotic cell respiration, which reductively converts molecular oxygen (O<sub>2</sub>) to two water molecules coupled with a proton pumping process. CcO contains four redox active metal sites, hemes a and a<sub>3</sub>, CuA and CuB. The two metal sites, heme a<sub>3</sub> and CuB together form the O<sub>2</sub> reduction site. Electrons for O<sub>2</sub> reduction are transferred from cytochrome c in the positive side space to the O<sub>2</sub> reduction site via CuA and heme a. The protons used for water formation are transferred from the negative side space via two proton transfer pathways known as the D- and K-pathways. Time-resolved resonance Raman spectroscopy showed that in the process of complete reduction of O<sub>2</sub> by fully reduced CcO, the initial intermediate is the oxygenated form and the second intermediate is the oxide-bound form where the formal oxidation state of Fe is 3 oxidation equivalent higher than ferrous Fe. These results indicate that the bound O<sub>2</sub> is reduced to the oxide level in the transition from the initial intermediate to the second intermediate. The transition is a one-step four-electron reduction process. The X-ray structural analyses have shown that the cuprous CuB has trigonal planar coordination geometry, suggesting that the cuprous CuB is a poor electron donor as well as a poor ligand acceptor. Although Tyr244, which is covalently bound to one of the three histidine ligands to CuB, is a possible electron/proton donor to O<sub>2</sub> at Fea<sub>3</sub>, the interaction between the hydroxyl group of Tyr244 and the bound O<sub>2</sub> is sterically blocked. These X-ray results are consistent with the expected stability of the O<sub>2</sub>-bound form demonstrated by resonance Raman analyses. However, the X-ray structures alone do not provide any further clues for elucidation of the mechanism of the O<sub>2</sub> reduction. Furthermore, intermediate species between the oxygenated form and the oxide-bound form are not detectable in the O<sub>2</sub> reduction process under normal enzymatic turnover conditions, as described above. Therefore, the structural and functional analyses of the O<sub>2</sub> reduction site using respiratory inhibitors as probes are needed to provide insight into the mechanism of O<sub>2</sub> reduction catalyzed by this enzyme. X-ray structures of the fully reduced bovine CcO showed that CO was bound either at ferrous Fea<sub>3</sub> or at cuprous CuB in the O<sub>2</sub> reduction site under light depending on the temperature. A bent end-on binding of NO to ferrous Fea<sub>3</sub>, 2.5 Å apart from the cuprous CuB suggests essentially no interaction between CuB and NO. These X-ray structures of the O<sub>2</sub>-analogue derivatives suggest that CuB controls O<sub>2</sub> supply to Fea<sub>3</sub> but does not donate electrons directly to O<sub>2</sub> at Fea<sub>3</sub>. In the CN- derivative of the fully reduced CcO, a water molecule was hydrogen-bonded to both carbon atom of the CN- at Fea<sub>3</sub> and OH of Tyr244 at the upper end of a proton transfer pathway for water-formation. Tyr244 is covalently bound to His242 ligated to CuB. The network, CuB-His240-Tyr244-H<sub>2</sub>O could donate two electrons from CuB and OH of Tyr244 to O<sub>2</sub> at Fea<sub>3</sub>. Thus, introduction of H<sub>2</sub>O at Tyr244 could trigger a four-electron reduction of the bound O<sub>2</sub> in essentially one step by two electrons from the network and two electrons from Fea<sub>3</sub>. Either NO or CO binding to Fea<sub>3</sub> giving the low-spin state induced the conformational change in a helix between the two hemes to decrease a water-capacity of the water channel placed in the bottom half of the proton pumping pathway of the bovine enzyme. Thus, binding of O<sub>2</sub>, a low-spin forming ligand, would decrease the water-channel capacity to facilitate effective blockage of proton back-leak, before proton pumping process, driven by the electron transfer from heme a to the bound O<sub>2</sub>, starts.



**Symposium H:  
Basic biochemistry and biology of vascular function**



## **ROLE OF VASCULAR ENDOTHELIAL GROWTH FACTOR (VEGF) IN THE ADULT**

**Patricia A. D'Amore**

Schepens Eye Research Institute and Harvard Medical School Boston, MA USA

VEGF is a well-known angiogenic factor whose role in development and vascular pathology is well documented. The persistent expression of VEGF in virtually all adult tissues, in the absence of active angiogenesis, suggests an alternative function for VEGF in the adult. We hypothesize that VEGF in the adult plays a role as a survival factor for vascular and non-vascular cells. To test this hypothesis, we neutralized VEGF systemically by adenoviral expression of the soluble VEGF receptor 1 (soluble Flt1; sFlt1). Systemic neutralization led to vessel non-perfusion in several organs that was accompanied by the presence of microthrombi and significant tissue dysfunction. We also identified a number of non-vascular VEGF receptor expressing cells. In the choroid plexus, systemic VEGF neutralization led to capillary non-perfusion as well as breakdown of the ependymal ventricular lining with resulting leukoencephalopathy. This phenotype was due to the unexpected express of VEGFR2, the primary VEGF signaling receptor, by the ependymal cells. In the eye, there was significant death of the photoreceptors that occurred in the absence of microvascular pathology. Rather, we found that photoreceptors express VEGFR2 and that the supporting Muller cells produce both VEGF and VEGFR2. Using siRNA to knockdown VEGF expression in cultured Muller cells, we demonstrate an autocrine role for VEGF in the Muller cell survival. Culture of Muller cells and isolated photoreceptors in the presence of VEGF neutralization anti-sera revealed a paracrine role for Muller cell-derived VEGF in the survival of photoreceptors. Finally, we shown that secretion of the soluble isoforms of VEGF by the retinal pigment epithelium is necessary for the integrity and stability of the underlying choriocapillaris. These observations indicate that VEGF expression in the adult plays an important role in the survival of both vascular and non-vascular cells. Furthermore, in light of the wide spread use of systemic anti-VEGF therapy for in cancer therapy and intravitreal anti-VEGF in the treatment of the wet form of macular degeneration and in diabetic microangiopathies, we recommend that anti-VEGF therapies be used with caution and with close attention to possible unexpected side effects.

## **PHOSPHOLIPASE A2 ENZYMES IN ENDOTHELIAL CELLS: NOVEL TARGETS FOR TREATING CARDIOVASCULAR DISEASE AND CANCER**

**A.F. Odell and J.H. Walker**

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Hydrolysis of glycerophospholipids by phospholipase A2 enzymes results in the generation of free fatty acids and lysophospholipids. In the endothelial cell the release of arachidonic acid (AA) is particularly important since it is the precursor of prostacyclin which is important as an inhibitor of platelet activation and as a vasodilator. Calcium-dependent cytosolic phospholipase A2-alpha has a central role in the release of AA in response to cellular activation. We have demonstrated an unusual and novel mode of regulation of cPLA2 activity by sequestration at the Golgi apparatus. Understanding this sequestration at the molecular level could lead to the development of novel drugs that might reduce blood pressure and decrease blood clotting by reactivating endothelial cell cPLA2. In a second area of study we have shown that the activities of cPLA2 and a calcium-independent PLA2 are linked to the regulation of cell cycle progression of endothelial cells. This could argue for these enzymes as novel targets for preventing unwanted angiogenesis in particular in cancer and macular degeneration.

## **THE ROLE OF INFLAMMATORY CELLS IN ANGIOGENESIS**

**Domenico Ribatti**

Department of Human Anatomy and Histology, University of Bari Medical School, Bari, Italy

Angiogenesis is a complex and highly orchestrated process leading to the formation of new blood vessels from pre-existing capillaries and venules. This process occurs in different conditions, such as embryo development and post-natal tissue growth, inflammation, and cancer. Both structural cells and inflammatory cells in the different tissues are involved in the mechanisms of endothelial cell proliferation, migration and activation, through the production and release of a large spectrum of pro-angiogenic mediators. These may create the specific microenvironment that favours an increased rate of tissue vascularization. In this presentation, I will focus on the inflammatory cell component of the angiogenic process. I will consider the contribution of inflammatory cells to normal and inflammation-associated angiogenesis. The role of these elements will be discussed in the context of angiogenesis related to tumor development. As angiogenesis is the result of a net balance between the activities exerted by positive and negative regulators, I will also provide information on some antiangiogenic properties of inflammatory cells that may represent targets for a potential pharmacological manipulation of the angiogenic process in chronic inflammation as well as in cancer.

## NEURONS AND ASTROCYTES SHED EXTRACELLULAR MEMBRANE VESICLES CONTAINING ANGIOGENIC FACTORS

**Gabriella Schiera, Patrizia Proia\*, Alessandra Lo Cicero, Giovanni Savettieri\*\* and Italia Di Liegro**

Dip. Scienze Biochimiche, \*DISMOT, \*\*Dip. Neuroscienze Cliniche, Università degli Studi di Palermo, Palermo, Italy

In the last few years we have been using a three-cell type in vitro model of blood-brain barrier (BBB), set in our laboratory, for investigating the events involved in the establishment and maintenance of BBB and the underlying molecular mechanisms. Brain capillary endothelial cells (BCECs), co-cultured with astrocytes and neurons, have been found to form a monolayer with permeability properties similar to those of the natural BBB. The cells indeed undergo a significant lowering of the paracellular flux of compounds like dopamine and labeled sucrose (1-4), and an increase of the transendothelial electrical resistance (TEER). Moreover, BCECs cultured with astrocytes and/or neurons, or fed with astrocyte- and/or neuron-conditioned media produce a larger amount of occludin (a critical protein of the tight junctions, TJs) and tend to localize it at the cell periphery, thus suggesting formation of tight junctions (TJs) and confirming formation of a barrier (1-4). Since in parallel studies we discovered that oligodendroglioma cells shed extracellular vesicles (5), and that these vesicles contain angiogenic as well as proapoptotic factors, we investigated whether also neurons and/or astrocytes can influence BCEC behaviour by releasing angiogenic factors via extracellular vesicles. The results of these analyses demonstrated that all kinds of brain cells actually release extracellular membrane vesicles. Moreover, the vesicles contain VEGF, FGF-2, and TGFbeta, all of which were already known to be produced in the brain and to affect endothelial cells. On the basis of immunofluorescence, as well as of western blot analyses, we concluded that the three factors are present in different relative amounts in vesicles released by either astrocytes or neurons (6-7). To clarify the pathway of secretion of these angiogenic factors, as well as their fate after vesicle release, we tried to integrate alternative approaches. First of all, we labeled vesicles by feeding producing cells with radioactive methionine, and analyzed the fate of radioactivity when the labeled vesicles are added to unlabeled cells. These experiments showed that labeled proteins from vesicles are found in the receiving cells. Second, we cloned the coding portions (ORFs) of mRNAs encoding angiogenic factors into the pEGFP-N2 plasmid, and transferred the plasmids into mammalian cells, in order to produce fluorescent recombinant proteins. We are now selecting stably transfected fluorescent cell lines that will be used to study the fate of the corresponding proteins during the process of secretion and/or shedding of extracellular vesicles. Finally, the effects of membrane vesicles, isolated from cell-conditioned media, on BCECs in culture, were directly analyzed. The results of these latter analyses showed that vesicles prepared from neuron- and astrocyte-conditioned media have a stabilizing effect on the barrier properties of the endothelial cells, while vesicles released by the endothelial cells themselves do not seem to contribute at all. REFERENCES 1. Savettieri G. et al., 2000, *NeuroReport* 11: 1081-4. 2. Cestelli A. et al., 2001, *J. Controll Rel* 76: 139-47. 3. Schiera G. et al, 2003, *J Cell Mol Med* 7: 165-70. 4. Schiera G. et al., 2005, *J Cell Mol Med* 9: 373-9 5. D'Agostino S. et al., 2006, *Int J Oncol* 29: 1075-85.. 6. Schiera G et al. 2007, *J Cell Mol.Med* 11: 1384-94. 7. Proia P. et al. 2008, *Int J Mol Med* 21: 63-7.

## CALCIUM SIGNALING AND CONTROL OF ANGIOGENESIS PROGRESSION

Luca Munaron

Department of Animal & Human Biology University of Torino

Intracellular calcium, ( $\text{Ca}^{2+}$ )<sub>i</sub>, controls virtually all cell functions, mainly through its ability to regulate a broad number of enzymes. Highly diverse extracellular agonists, including hormones, neurotransmitters and growth factors trigger changes in ( $\text{Ca}^{2+}$ )<sub>i</sub> to transduce information for cell proliferation, differentiation, death, and motility in physiological and pathological conditions (Berridge et al., 2003). ( $\text{Ca}^{2+}$ )<sub>i</sub> signaling plays a key role in different phases of angiogenesis progression: the best known proangiogenic peptides, VEGF and bFGF, increase intracellular calcium in endothelial cells (ECs) through complex signaling pathways involving arachidonic acid (AA) and nitric oxide (NO) metabolism (Munaron 2004, 2006, 2008). Interestingly proangiogenic ( $\text{Ca}^{2+}$ )<sub>i</sub> signals in ECs usually display peculiar properties: they last for minutes and are spatially restricted to the peripheral regions (lamellipodia) without invading the nuclear area (Tomatis et al., 2007); they are due to a calcium entry from the extracellular medium through the opening of calcium-permeable channels sensitive to the antitumoral, antimetastatic and antiangiogenic drug CAI (Antoniotti et al, 2003; Fiorio Pla et al., 2008). The cytosolic localization of ( $\text{Ca}^{2+}$ )<sub>i</sub> signals affects the transcriptional pattern of specific endothelial genes and could be highly relevant from the biological point of view (Feske et al., 2001). In the last years, several lines of evidence led to suggest that angiogenesis in tumors could differ from neovascularization in normal tissues both for the mechanisms underlying the process and for the properties of ECs (Carmeliet, 2005). We have recently shown that AA/NO-activated sustained  $\text{Ca}^{2+}$  entry is not restricted to normal vascular endothelium, but it is also present in human ECs derived from tumoral tissues. In particular, it is critical for progression through the early phases of in vitro tubulogenesis of breast tumor-derived human endothelial cells (BTECs), and it is down regulated during the reorganization of ECs and tubule maturation (Fiorio Pla et al., 2008). Even if proangiogenic calcium entry in ECs could be an universal event, it may differ between normal and tumor-derived tissues: some changes could occur in the expression (and membrane targeting) of the calcium channels involved, in their intracellular regulation, and in the downstream calcium-dependent machinery. The identity of calcium channels activated by proangiogenic factors is still elusive, but several members of the transient receptor potential (TRP) superfamily of channels are expressed and functional in ECs and are potential candidates: interestingly some components of TRPC and TRPV subfamilies are activated by AA and NO and therefore could be relevant players in the angiogenic process. Antoniotti et al. *J Cell Physiol* 2003 197(3):370-8. Berridge MJ et al. *Nat Rev Mol Cell Biol.* 2003 4(7):517-29 Carmeliet P. *Nature.* 2005 438(7070):932-6 Feske S et al. *Nat Immunol.* 2001 2(4):316-24 Fiorio Pla A et al. *Mol Cancer Res.* 2008;6:535-45 Munaron L et al. *Technol Cancer Res Treat* 2008;7:335-340 Munaron L. *Recent Patents Anticancer Drug Discov* 2006;1:105-119 Munaron L et al. *Curr Med Chem* 2004;11:1533-1543 Mottola A et al. *Faseb J* 2005;19:2075-2077 Tomatis C et al. *Cell Calcium* 2007; 41(3):261-9.



## Poster Sessions



## Enzymes

**Poster session:  
23/09/2009 (h. 14.00-15.00)**



## **UNRAVELLING ALDOLASE C MOONLIGHT FUNCTIONS: A PROTEOMIC VIEW**

**E. Imperlini 1,2, S. Spaziani 1,3,4, A. Alfieri 3, A. Mancini 2,4, P. Buono 1,2,3,4,  
S. Orrù 1,2,3 and F. Salvatore 1,4**

1 CEINGE, Napoli; 2 Fondazione SDN, IRCCS, Napoli; 3 DISIST Univ. Parthenope, Napoli; 4 DBBM, Univ. Federico II, Napoli

Aldolase C (AldC) is expressed in different areas of the human brain, with a maximum expression occurring in a stripe-like pattern in the Purkinje cells of cerebellum. Its peculiar distribution suggests that new functions could be assigned to this enzyme (1). Furthermore, AldC could display a ribonucleolytic activity, being able to regulate the stability of light neurofilaments mRNA, essential elements for the maintenance of the differentiated state of neurons (2). Aim of this project was to investigate moonlight functions of AldC in order to clarify its specific role in the cerebellum by functional proteomic experiments to define the AldC interactors. We immunoprecipitated endogenous AldC with its putative protein interactors from healthy adult mouse cerebellum; mass spectrometry-based proteomic procedures allowed us to identify DEAD-box RNA helicase DDX1, a protein involved in many aspects of RNA metabolism, as AldC protein partner. 1. Journal of Neurocytology 2001, 30:957 2. Brain Res 2007, 1139:15

## **ADENINE PHOSPHORIBOSYLTRANSFERASE (APRT) DEFICIENCY: A NEW GENETIC MUTATION WITH RECURRENT RENAL STONE DISEASE IN A KIDNEY TRANSPLANTED PATIENT**

**V. Micheli, F. Massarino, G. Jacomelli, M. Bertelli, M.R. Corradi, A. Guerrini, A. Cucchiara, J.L. Ravetti  
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APRT deficiency is a rare inborn error inherited as an autosomic recessive trait, causing accumulation of adenine and its poorly soluble metabolites 8-OH-adenine and 2,8-OH adenine (DHA). A 48-year-old male patient with renal failure and lithiasis was kidney transplanted, underwent renal colics soon after, and DHA crystals were identified in kidney biopsy by X-ray diffraction. APRT activity was undetectable in lysate and intact erythrocytes; plasma and urine contained considerable amounts of DHA and adenine. APRT in the patient's asymptomatic brother was 23% of normal in lysate, and normal in intact erythrocytes, with no DHA in plasma and urine. Homozygous C>G substitution at -3 in the splicing site of exon 2 (IVS2 -3 c>g), never described before, was found in the patient's genomic DNA; his brother was heterozygote for such mutation. Studies in cDNA, reverse-transcribed from RNA, showed normal sequence transcript in the brother, and no amplification at all in the patient. The newly identified mutation associated with virtually absent APRT activity is seemingly responsible for a splicing alteration leading to incorrect gene transcription (truncated protein or no protein at all).

## **BACILLUS SUBTILIS NADA AND NADB: THE QUINOLINATE SYNTHASE “COMPLEX”**

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The first two steps in NAD de novo biosynthesis lead to the production of quinolinate starting from L-aspartate, dihydroxyacetonephosphate and an oxidant (oxygen, fumarate or quinones in vitro) catalyzed by NadB (L-aspartate oxidase) and NadA (quinolinate synthase). These two enzymes are absent in mammalian; therefore they are considered ideal targets for the development of novel therapeutic agents. Catalytically active recombinant *B. subtilis* NadA was obtained following purification in strictly anaerobic conditions and used to determine relevant biochemical properties, including the characterization of its Fe/S center. Studies on recombinant *B. subtilis* NadB showed that its properties are similar to those of the *E. coli* enzyme. The existence and properties of the putative NadA/NadB multienzymatic complex was investigated using both biochemical and proteomic approaches. The former included evaluation of the effect of each enzyme on selected biochemical properties of the other; the proteomic approach involved affinity chromatography using each protein immobilised and evaluating binding of the partner protein under various experimental conditions.

## **BIOCHEMICAL CHARACTERIZATION OF CATALASE ISOLATED FROM THE PSYCHROPHILIC EUBACTERIUM PSEUDOALTEROMONAS HALOPLANKTIS**

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Micro-organisms living in the Antarctic sea grow at extreme low temperatures and represent an ideal tool to study oxidative stress defence since this particular environment leads to a rise of the reactive species of oxygen. Under this regard, we report a study on catalase (CAT), an enzyme decomposing H<sub>2</sub>O<sub>2</sub>, isolated from the Antarctic eubacterium *Pseudoalteromonas haloplanktis* (Ph). PhCAT doesn't exhibit the classical Michaelis kinetic because high substrate concentration provoked an inhibitory effect; hence both the theoretical and the observed K<sub>m</sub> and V<sub>max</sub> values were considered. PhCAT was affected by either Na-azide, a non-competitive inhibitor (K<sub>i</sub> 34.7 mM), and β-mercaptoethanol, an inactivator (IC<sub>50</sub> 1.3 mM). The maximum activity of PhCAT was reached at 25°C. In the 0-25°C interval a value of 10.6 kJ/mol for the energy of activation of reaction was calculated. Regarding the heat stability, a T<sub>m</sub> of 47°C was found following 10 min exposition of PhCAT at temperatures between 0 and 90°C. These results, together with the achievement of the putative 3D model of PhCAT, indicate that this enzyme is endowed with a significant structural flexibility, mainly localized at the active site.

## **CK2 AND GSK3 PHOSPHORYLATION ON S29 CONTROLS ATAXIN-3 NUCLEAR UPTAKE**

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Ataxin-3, the protein responsible for spinocerebellar ataxia type 3 (SCA3) has been demonstrated to possess a ubiquitin hydrolase activity. A bioinformatic analysis showed the presence, on ataxin-3 sequence, of a number phosphorylation sites for casein kinase 2 (CK2) and glycogen synthase 3 (GSK3). Mass spectrometry phosphomapping of in vitro phosphorylated AT-3 confirmed phosphorylation by both kinases at Ser 29. S29A mutant was produced by substituting phosphorylated serine with alanine and its subcellular localization and proteolysis were investigated through confocal microscopy and sub-cellular fractionation in COS7 transfected cells. S29 was found to be relevant for nuclear import of AT-3, since S29A mutant, while not showing any relevant difference with wild-type in proteolysis and mitochondrial localization, localized inside the nucleus to a much lower extent than the wild-type. In keeping with this finding, S29D mutant was found to behave like the wild-type protein. Finally, treatment of COS-7 cells overexpressing wild-type AT-3Q6 with both CK2 and GSK3 inhibitors strongly inhibited nuclear uptake, showing that both kinases are involved in AT-3Q6 subcellular sorting.

## **EFFECTS OF THE LACK OF RHODANESE RHDA IN AZOTOBACTER VINELANDII**

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The rhodanese-like proteins in vitro catalyse the transfer of a sulfur atom from thiosulfate to cyanide, with formation of thiocyanate, but the wide distribution of these proteins corroborates the hypothesis that they evolved distinct physiological functions. Among the rhodanese-like proteins of *Azotobacter vinelandii*, RhdA contains an active-site motif not common found in other rhodanases. Phenotypical characterisation of an *A. vinelandii* mutant strain in which *rhdA* was deleted evidenced inactivation of enzymes containing labile Fe-S. In the present work, further characterization of the mutant strain revealed that glutathione was significantly lower in the mutant compared to that of the wild-type strain, in line with the increased general levels of reactive oxygen species found in the mutant strain. Moreover, we found that the expression of *ahpC*, a member of the OxyR regulon, was significantly induced in the mutant strain. These results provided experimental evidence that an internal oxidative stress problem occurred in *A. vinelandii* cells lacking RhdA, thus corroborating the hypothesis that RhdA has a role in maintaining cellular redox balance.

## **ENZYMES FOR CAPTURING GREENHOUSE GASES: USE OF IMMOBILIZED CARBONIC ANHYDRASE TO DEVELOP A BIOREACTOR SUBTRACTING CARBON DIOXIDE FROM MIXED GAS STREAMS**

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Carbon dioxide can be decrease from the emissions of fossil fuels in a bioreactor by using carbonic anhydrases (CA, EC4.2.1.1) zinc metalloenzymes catalyzing the reversible hydration of CO<sub>2</sub> and the dehydration of HCO<sub>3</sub><sup>-</sup>. Pre-requisite to develop a bioreactor is an effective enzyme immobilization on insoluble supports. Nylon represents an attractive matrix because of its chemical and physical properties. CA isoenzyme II, purified from a low cost source such as bovine erythrocytes, was immobilized on nylon+ membrane and esterase activity was tested by monitoring the hydrolysis of p-nitrophenyl acetate in relation to: a) time, b) storage temperature (4°C, RT or -20°C), c) storage condition (wet or dried). After an initial decrease, the residual activity (up to 50% of the initial one) remained stable for more than 2 years. The initial reduction of activity could likely be due to enzyme leakage from membrane during washing. The optimal storage temperature was 4°C. At 4°C air-dried membranes showed 10-20% preservation of esterase activity with respect to wet membranes. Overall results indicate that CAII is an attractive tool to build an enzyme-based reactor for CO<sub>2</sub> capture.

## **EXPRESSION OF MOUSE ACID SPHINGOMYELINASE (MASM) IN THE METHYLOTROPHIC YEAST PICHIA PASTORIS**

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Niemann-Pick disease is a lysosomal storage disorder resulting from an inherited deficiency of acid sphingomyelinase ASMase [EC 3.1.4.12] which catalyzes the hydrolysis of sphingomyelin (SM) in ceramide and phosphoryl choline. The enzymatic defect causes SM accumulation within the cells of the monocyte-macrophage system. The defective enzyme results from different point mutations in the SMDP1 gene on chromosome 11, in turn exiting into a protein with low activity or even completely inactive. Enzyme replacement therapy (ERT) is a possible approach for the treatment of lysosomal storage diseases. In ERT the purified fully active recombinant enzyme is administered by intravenous infusion to individuals affected by the storage disease. In this study the cDNA coding for the mature form of mASM was cloned in the methylotrophic yeast *Pichia pastoris*. The recombinant mASM was expressed as secreted protein and purified by a combination of ammonium sulphate precipitation, anion exchange and affinity chromatography. The purified recombinant protein showed a molecular weight of 72 kDa, was recognized by an anti-ASM antibody and hydrolyzed physiological and synthetic substrates.

## HIGH RESOLUTION CRYSTAL STRUCTURE OF HAEMOPHILUS INFLUENZAE NAD NUCLEOTIDASE (NADN)

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*Haemophilus influenzae*, is a bacterium responsible for significant morbidity and mortality in young children. The encapsulated types and the non-typeable strains, are responsible for a wide range of human localized diseases, and cause meningitis. For its growth, has an absolute requirement of Nicotinamide Adenine Dinucleotide (NAD). By relying on extracellular NAD, *Haemophilus influenzae* shows a unique strategy to sustain the request of NAD(P) for cell viability. Indeed, the bacterium carries out a periplasmic degradation of the dinucleotide to adenosine and nicotinamide riboside, which then diffuse across the cell membrane and are transformed back to NAD in the cytoplasm. The nucleotidase responsible for periplasmic NAD degradation, is a zinc dependent enzyme termed NadN. We have determined the 1.3 Å resolution crystal structure of HiNadN in complex with adenosine. HiNadN consists of 640 residues organized into an N-ter and a C-ter  $\alpha/\beta$  domains, with two catalytically essential zinc ions located in the active site at the domains interface. The structural analysis reveals a striking conformational change on ligand binding and allows to identifying key residues for catalysis.

## HUMAN CYTOSOLIC FAD SYNTHETASE: MOLECULAR CHARACTERISATION AND KINETIC PROPERTIES.

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The primary role of riboflavin (Rf) in cell metabolism derives from its conversion into FMN and FAD, via Rf kinase and FAD synthetase (FADS). Different isoforms of FADS are encoded by FLAD1 gene in humans. hFADS2 is a soluble 490-amino-acid protein, lacking an extra-sequence of 97 amino acids present at the N-terminus of the putative mitochondrial hFADS1 (1,2). Purified recombinant hFADS2 shows typical flavoprotein absorbance spectra, with a main peak at 274 nm and two minor peaks at 370 and 450 nm. The bound cofactor is FAD. Holo-hFADS2 is in a folded state, as demonstrated by CD analysis. An apo-form prepared by KBr treatment shows no significant difference in the CD spectrum, thermal and chemical stability with respect to the holo-form. hFADS2 exhibits  $K_m$  for FMN and ATP in the  $\mu\text{M}$  range; the turnover number is exceedingly low ( $8.8 \times 10^{-2} \text{ s}^{-1}$ ), with FAD remaining enzyme-bound. The release of FAD is likely to be tightly controlled and presumably requires the interaction with the cofactor accepting-apo-protein. Acknowledgments: FIRB 2003, project RBNE03B8KK 1. Brizio et al. (2006) *Biochem. Biophys. Res. Commun.* 344:1008 2. Galluccio et al. (2007) *Protein Expr. Purif.* 52:175

## **HYALURONAN SYNTHESIS IS REGULATED BY ADENOSINE-ACTIVATED PROTEIN KINASE (AMPK) IN HUMAN AORTIC SMOOTH MUSCLE CELLS (SMC)**

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The glycosaminoglycan hyaluronan (HA) contributed to vessel thickening by inducing quiescent SMC to proliferate and migrate. Therefore HA synthesis inhibition could represent a new strategies to prevent-atherosclerosis. As the biosynthesis of HA precursors requires ATP, we hypothesized that AMPK, which is the main sensor of ATP:AMP, could control HA production. We found that the activation of AMPK in human aortic SMC inhibited HA synthesis whereas the production of other glycosaminoglycans were unchanged. The main HA synthetic enzyme in SMC is HAS2 that is located at plasma membrane level and we hypothesized that AMPK, through phosphorylations, could modify HAS2 activity. To demonstrate this issue, we transfected HAS2 and a constitutive active AMPK (caAMPK) in COS7 cells and found a strongly reduced HAS activity. Interestingly, a phosphatase pre-treatment completely restored the HA synthetic activity suggesting that HAS2 can be regulated by phosphorylation. As SMC proliferation and migration contributed to atherosclerosis development, we demonstrated that AMPK activation reduced cell growth and motility indicating that AMPK can be a candidate for vasoprotective drug design.

## **NEGATIVE REGULATION OF DIACYLGLYCEROL KINASE THETA MEDIATES ADENOSINE-DEPENDENT HEPATOCYTE PRECONDITIONING**

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In liver ischemic preconditioning, stimulation of adenosine A2a receptors (A2aR) prevents ischemia/reperfusion injury by promoting diacylglycerol (DAG) mediated activation of protein kinase C (PKC). Converting DAG to phosphatidic acid, diacylglycerol kinases (DGK) acts as terminator of DAG signalling and may play a role in hepatocyte ischemic preconditioning. Following ischemic preconditioning or A2aR activation, a decrease in DGK activity was associated with the onset of hepatocyte tolerance to hypoxia. CGS21680 stimulation of A2aR specifically inhibited DGK isoform theta by activating RhoAGTPase. Consistently, both down-regulation of DGK theta by siRNA or hepatocyte pre-treatment with the DGK inhibitor R59949 induced cell tolerance to hypoxia. The pharmacological inhibition of DGK was associated with the DAG-dependent activation of PKC delta and epsilon and of their downstream target p38 MAPK. Inhere we unveil a novel signalling pathway contributing to hepatocyte preconditioning, which through RhoA-GTPase, couples A2aR to the down-regulation of DGK. Such an inhibition is essential for the sustained accumulation of DAG required for triggering PKC-mediated survival signals.

## **NOVEL DRUGS FOR SCHIZOPHRENIA TREATMENT: THE MECHANISM OF HUMAN D-AMINO ACID OXIDASE INHIBITION**

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The human flavoprotein D-amino acid oxidase (hDAAO) in brain degrades the gliotransmitter D-serine, a glutamate co-agonist required for activation of NMDA receptors. At synapse, a reduced concentration of D-serine results in depression of glutamatergic neurotransmission and has been associated to schizophrenia onset. To shed light on the mechanisms of hDAAO regulation, we investigated the effect of small ligands on the enzyme stability/activity and on D-serine cellular concentration. Binding of FAD cofactor to hDAAO apoprotein ( $K_d = 8 \mu\text{M}$ ) yields to the holoenzyme form, having a “compact” tertiary structure. Chlorpromazine (CPZ), a drug used for the treatment of schizophrenia, competes with FAD for the binding to the apoprotein ( $K_d = 5 \mu\text{M}$ ): the hDAAO-CPZ complex is more sensitive to proteolysis and thermal denaturation than the free holoenzyme. The classical substrate-competitive inhibitor benzoate ( $K_d = 7 \mu\text{M}$ ) binds hDAAO increasing the affinity for FAD and thus the amount of the holoenzyme form. These results suggest that the use of a different strategy of hDAAO inhibition (substrate vs. cofactor competition) could differently affect the in vivo concentration of D-serine.

## **OVEREXPRESSION OF E. COLI OLIGOPEPTIDASE B CONFERS RESISTANCE TO INTRACELLULARLY ACTING ANTIBACTERIAL PEPTIDES**

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Several strategies have been developed by bacteria to limit the activity and increase resistance to antimicrobial peptides, widespread bactericidal molecules of innate immunity. In a screening for *E. coli* genes conferring decreased susceptibility to the proline-rich antimicrobial peptides, the gene coding for Oligopeptidase B (OpdB) was identified. In this study we investigated the involvement of this serine peptidase in resistance to the action of non-lytic, proline-rich and membranolytic alpha-helical antimicrobial peptides. We showed that recombinant OpdB can efficiently hydrolyze cationic peptides up to 30 residues in length, shortening them to inactive fragments. In addition, we found that OpdB overexpression in *E. coli* made the cells less susceptible to proline-rich peptides and to indolicidin but not to lytic peptides, and that the level of activity of the peptidase directly correlates with the degree of resistance. These results indicate that the peptide-degrading activity of OpdB may represent a novel bacterial mechanism of resistance towards those antimicrobial peptides that act intracellularly, after membrane penetration.

## **PURIFICATION AND CHARACTERIZATION OF TREHALASE FROM CHIRONOMUS RIPARIUS LARVAE**

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Trehalase is very important for insect metabolism, since trehalose is the main circulating sugar in these organisms. Specific inhibitors could control the vitality of insects potentially dangerous for men, domestic animals and harvest. We recently demonstrated that *C. riparius* larvae exposure to sublethal concentrations of insecticides has effects on trehalose catabolism. These results prompted us to consider trehalase as a target for the identification of newer and more specific insecticides. To develop new drugs, a purification and characterization of trehalase from insects is required. A crude homogenate of *C. riparius* larvae was subjected to a series of purification steps, based on solubilisation with the CHAPS, on ion exchange and on Con A affinity chromatography. The purification factor obtained was 1080. Gel filtration analysis indicated the enzyme to be a monomeric protein with a molecular weight of 67 KDa. Kinetic experiments showed a high affinity for trehalose ( $K_m = 0.48 \pm 0.03$  mM) and maximal activity at pH 6.5 ( $4.54 \pm 0.09$  U/mg). The substrate affinity is about 10-fold higher than that of porcine trehalase.

## **RENALASE: A NEW HUMAN FLAVOPROTEIN POSSIBLY INVOLVED IN THE REGULATION OF CARDIAC FUNCTION AND BLOOD PRESSURE**

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Renalase, a putative monoamine oxidase, has been recently identified by the group of G.V. Desir (1). It is considered to be a new renal hormone which is involved in the regulation of cardiac function and blood pressure by way of its assumed monoamino oxidase activity. The renalase protein contains a signal peptide for secretion (1-17 aa) overlapping a FAD binding region (3-42 aa) and an amino oxidase region (75-335 aa). The similarity to MAO A and B is only 13% and it is restricted to the first 80-100 aa. We have expressed the full-length human cDNA in *Escherichia coli* and purified the soluble protein. Here we report on its biochemical properties. We have demonstrated for the first time that indeed this renalase is a flavoprotein and shown that contains non-covalently bound FAD at variance with the MAO enzymes. Furthermore, our protein is devoid of catecholamine oxidase activity, thus casting doubts on the real substrate of this enzyme. 1. Xu J, Li G, Wang P, Velazquez H, Yao X, Li Y, Wu Y, Peixoto A, Crowley S, Desir GV., *J Clin Invest* (2005), 115(5):1275-80.

## STRUCTURAL BIOLOGY AND INHIBITION OF FLAVIVIRAL ENZYMES

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The Flavivirus genome consists of 11 kb (+)ssRNA molecule (+)ssRNA genome, decorated at its 5' UTR with a conserved cap I structure (N7meGpppA2'Ome), that is essential for mRNA stability and proper replication. The genome encodes for a 370 kDa polyprotein, which is processed into 3 structural proteins and 7 non structural proteins involved in replication (NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5). During viral replication proper translation requires capping of viral RNA and, among other enzymes, the function of the NS5 N-terminal domain (Methyltransferase, Mtase) is essential. Mtase transfers a methyl group from S-adenosyl-L-methionine to capped RNA. In order to characterize the series of enzymatic reactions that support capping, we analyzed the crystal structures of Wesselsbron virus MTase, in complexes with the AdoMet cofactor, with AdoHcy (product of the methylation reaction), with Sinefungin (one of the strongest MTases inhibitor), and with three different cap-analogues (GpppG, N7MeGpppG, N7MeGpppA). The cap analogue binding modes and our application of virtual docking methods in a successful search for antivirals are discussed.

## STUDY ON THE CYTOSOLIC RIBONUCLEASE INHIBITOR

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The cytosolic ribonuclease inhibitor (cRI) is a ubiquitous, evolutionarily conserved protein of about 50 kDa, a peculiar feature of this protein is the high cysteine content. cRI is a potent scavenger of ROS in vitro, the reaction induce intramolecular thiol-disulfide transition with a all-or none pattern, initiated by oxidation of highly reactive adjacent cysteine residues. It has been shown that oxidative stress induce the oxidized form of cRI in intact cells; on the other hand cRI depletion increases cell sensitivity to oxidative stress. This evidence strongly suggests a role for cRI in intracellular redox homeostasis. We have performed coimmunoprecipitation experiments searching for cytosolic proteins interacting with cRI. Flag eluted immunocomplexes, obtained from HeLa cells expressing a recombinant Flag-cRI, specifically reveal the presence of actin. In addition, pull-down analyses show that Flag-cRI interaction with purified actin is inhibited by thiol reagents. Immunofluorescence cytological analysis indicates that cRI and actin colocalize at specific structures. These results suggest that actin may interact with cRI in vivo in a redox dependent manner

## THE ACTIVITY OF ADENOSINE DEAMINASE TOWARDS 2',3'-SUBSTITUTED ADENOSINES: A MOLECULAR MODELING APPROACH

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Adenosine deaminase (ADA, EC 3.5.4.4) catalyzes a rapid and irreversible conversion of adenosine to inosine. The enzyme is also able to deaminate a wide range of structurally diverse purine nucleosides [1] and plays a key role in purine metabolic pathways as well as in mammalian immune system development. The sugar moiety plays a critical role in ADA binding and activation, determining the optimal conformation for enzyme recognition. In this respect, we have shown that substitutions at the 2'- and 3'-O-position are compatible with ADA activity,[2] while the compound with a methyl group in 1'-position was not a substrate of ADA. Modeling studies have been undertaken in order to explain above results with docking experiments within the three-dimensional structure of ADA. [1]. Santaniello, E. et al. In *Biocatalysis in the Pharmaceutical and Biotechnological Industries*; Patel, R. N. Ed.; CRC Press: Boca Raton 2007, pp 502-528. [2]. Ciuffreda P.et al. 2', 3'-Isopropylidene group, a molecular scaffold to study the activity of adenosine deaminase and adenylate deaminases on adenosine analogues modified in the ribose moiety. *Nucleos. Nucleot. Nucl.* 26, 1311-1313 (2007).

## THE CRYSTAL STRUCTURE OF HUMAN $\alpha$ -AMINO- $\beta$ -CARBOXYMUCONATE- $\epsilon$ -SEMIALDEHYDE DECARBOXYLASE SUGGESTS A REGULATORY LINK BETWEEN GLYCOLYSIS AND NAD SYNTHESIS

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The enzyme  $\alpha$ -amino- $\beta$ -carboxymuconate- $\epsilon$ -semialdehyde decarboxylase (ACMSD) is a zinc-dependent amidohydrolase that plays an important role in picolinic acid (PA), quinolinic acid (QA) and NAD homeostasis. Both PA and QA are key players in several physiological and pathological conditions affecting the central nervous system. As their reciprocal concentration needs to be tightly controlled, the modulation of ACMSD activity appears as a promising novel avenue for the treatment of neurological disorders, cerebral malaria amongst them. We report the 2.0 Å resolution crystal structure of human ACMSD in complex with the glycolytic intermediate 1,3-dihydroxyacetonephosphate (DHAP) that we discovered to be a potent enzyme inhibitor. Arg47, Asp291 and Trp191 appear as key residues for DHAP recognition in human ACMSD. Upon ligand binding a significant conformational change affects a strictly conserved Trp-Met couple that we propose as a major gating determinant controlling ligand admission in ACMSD. Our data shed light into ACMSD catalysis, may be used for the design of inhibitors of potential medical interest and suggest an intriguing regulatory link between NAD synthesis and glycolysis.

## **THE POWER OF THE COMBINED PROTEIN ENGINEERING APPROACH: EVOLUTION OF A GLYCINE OXIDASE FOR A NEW MECHANISM OF GLYPHOSATE RESISTANCE**

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Glyphosate, a broad-spectrum herbicide, is an unnatural aa that inhibits EPSP synthase in the shikimate pathway, an essential enzyme for biosynthesis of aromatic aa in plants and certain bacteria. The FAD-containing flavoprotein glycine oxidase (GO) catalyses the oxidative deamination of various amines and D-aa. GO is also active on glyphosate: the V<sub>max</sub> is similar to that for glycine but a 100-fold higher K<sub>m</sub> is evident. A docking analysis identified 11 residues at GO active site potentially involved in glyphosate binding. Site-saturation mutagenesis of these positions resulted in enzyme variants with an increased kinetic efficiency on glyphosate. The combination of substitutions that increase kinetic properties and protein stability/expression allowed the isolation of a mutant with a 200-fold increase in catalytic efficiency on glyphosate and a 15000-fold increase in specificity constant as compared to wt GO. Resolution of the 3D-structure of this GO mutant shows that the substitutions allow the formation of additional electrostatic interactions which favor glyphosate binding. This evolved GO mutant forms the basis of a novel mechanism of glyphosate tolerance in transgenic plants.

## **ZEBRAFISH AS A MODEL FOR THE STUDY OF THE POSSIBLE ROLE OF SIALIDASE NEU4 IN THE DEVELOPMENT OF VERTEBRATES**

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The zebrafish (*Danio rerio*) is considered a good model organism for vertebrate development since embryos are transparent and organogenesis is almost complete within 24h outside the mother's body. The fish embryos develop organs that are similar to those in humans. Gene expression can be studied by knock-down experiments using morpholino oligos (MO). Sialidases or neuraminidases are a family of enzymes that catalyze the removal of terminally linked sialic acid residues from glycoconjugates. Mammalian sialidases are implicated in lysosomal catabolism, as well as in regulation of cell differentiation, growth, adhesion and apoptosis. In order to study the role of sialidase neu4 in zebrafish development, we performed gene inactivation experiments using splice-inhibiting MO. neu4 is expressed already at early development stages and shows localized expression area corresponding to lens. MO-injected embryos show a range of developmental defects, including edema, tail malformations and altered circulation. A preliminary characterization of neu4 morphants was performed by ISH using different markers. Morphants show aberrant somite organization and altered intersomitic vessel assembly.

## INVESTIGATING THE STRUCTURAL PLASTICITY OF A CYP450: 3D STRUCTURE OF ERYK AND BINDING TO ITS PHYSIOLOGICAL SUBSTRATE

**\*L. C. Montemiglio, \*+C. Savino, \*+S. Gianni, \*B. Vallone**

\*Dept. of Biochem. Sciences "A. Rossi Fanelli", Univ. of Rome "Sapienza"; +Inst. of Molec. Biology and Pathology, CNR, Italy

The CYP450s family consists of heme-containing enzymes that catalyze oxidative metabolism of endogenous and xenobiotic compounds. Considerable efforts have been focused on the understanding of their mechanism of ligand selectivity and hydroxylation. We addressed the mechanism of recognition and binding of a P450 from *S. erythraea* (EryK), which catalyses one of the final steps of erythromycin biosynthesis, to its physiological substrate ErD. We determined the crystal structure of two different ligand free forms of EryK observing that, depending on the ionic strength of crystallization condition, either an "open" or a "closed" structures can be obtained. Furthermore, we performed kinetic studies to clarify the mechanism of substrate binding and observed that EryK binds its physiological substrate following a double exponential behaviour, indicative of a complex mechanism. The relative amplitudes of the two phases depend on the ionic strength. We conclude that EryK undergoes a pre-existing equilibrium between an open and a closed conformation, which bind the ligand with different affinities. Ionic strength can modulate the relative populations of "fast" and "slow" conformers.

## DUAL SUB-CELLULAR LOCALIZATION OF FAD1P IN *S. CEREVISIAE*

**M. Barile\*, T.A. Giancaspero\*, F. Bruni\*, M. Roberti\*, M. Caselle#**

\*DBBM "E. Quagliariello", Università di Bari, Italia, #Dipartimento di Fisica, Università di Torino, Italia

FAD synthetase is the last enzyme in the pathway that converts riboflavin into FAD. For many years it was assumed that FAD biosynthesis occurred only in the cytosol (1). A cytosolic localisation was also proposed for the product of *S. cerevisiae* FAD1 gene (2). However, using cell fractionation and activity measurements, we demonstrated the presence of FADS activity in yeast mitochondria (3). At the moment the protein responsible for FAD synthesis in *S. cerevisiae* mitochondria remains to be identified and characterised, as well as the mechanism by which a single gene FAD1 can generate distinct isoforms destined to different cellular compartments. Here we report experiments aimed to: i) definitively prove the existence of a yeast mitochondrial FADS; ii) study changing in its expression level under different conditions; iii) propose a model of generation of distinct cytosolic and mitochondrial isoforms. Acknowledgments: FIRB 2003, project RBNE03B8KK 1. McCormick et al., (1997) *Methods Enzymol.* 280 :407-13 2. Wu et al., (1995) *Mol. Cell. Biol.* 15 :264-71 3. Bafunno et al., (2004) *J. Biol. Chem* 279:95-102.

## **Proteins**

**Poster session:  
23/09/2009 (h. 14.00-15.00)**



## **BIOSILICA STRUCTURE FROM MARINE SPONGES: OPTICAL FIBER PROPERTIES OF SPICULES**

**Marco Giovine<sup>1,2</sup>, Andrea Camposeo<sup>3</sup>, Dario Pisignano<sup>3</sup> and Umberto Benatti<sup>2</sup>**

<sup>1</sup>AB Center, Genoa <sup>2</sup>University of Genova <sup>3</sup>National Nanotech Lab of CNR-INFM and ISUFI, Università del Salento

Siliceous sponge spicules are characterized by a large variety of dimensions and shapes, with an ultrastructure based on silica nanoparticles strictly packaged around an axial filament constituted by a family of proteins called silicateins. This biosynthesis scheme determines the production of a peculiar composite material with remarkable technological properties, like high flexibility and the amazing property to transmit light along its axis. Here, we have characterized the optical properties of the spicules of the sponge *R. racovitzae*. These spicules are able to transfer both red light and white light. In our experimental conditions it is evident that the light transmission properties of this biosilica structure is remarkably different from the commercial optical fiber. In the sponge case, in fact, light is widely diffused along spicule axis, while in the industrial fibers light is transported along the cable without appreciable loss of intensity. These results confirm that glass sponges spicules have specific optical characteristics, and the peculiar structure of this composite material is able to determine the different transmittance properties compared to the industrial ones.

## **CHARACTERIZATION OF A NEW FLUORESCENT IRREVERSIBLE LIGAND TO TRANSLOCATOR PROTEIN (18 KDA)**

**E. Da Pozzo <sup>1</sup>, S. Taliani <sup>2</sup>, S. Bendinelli <sup>1</sup>, M. Bellandi <sup>2</sup>, L. Rossi <sup>3</sup>, V. Gremigni <sup>3</sup>  
F. Da Settimo <sup>2</sup>, C. Martini <sup>1</sup>**

<sup>1</sup> Dept. of Psy.Neurob.Pharm.Biotech.; <sup>2</sup> Dept. of Med.Chem.; <sup>3</sup> Dept. of Hum.Morph.Apl.Bio., University of Pisa, Italy

The 18 kDa Translocator Protein (TSPO), a mitochondrial protein, is altered both in density and cellular localization for diseases, as cancer and neurodegenerations. For this reason, the evaluation of TSPO expression and distribution may represent a promising diagnostic marker. The development of irreversible ligands has proven invaluable in the characterization of cellular receptors. Moreover, novel imaging techniques offer the chance to visualize cell receptors by using fluorescent probes. The combining of the two techniques can offer advantages both in protein purification and characterization, and in protein cell visualization and density determination. In the present study, we carried on the design and the biological characterization of new fluorescent irreversible TSPO ligand. The ligand-TSPO binding parameters were evaluated using a model of two-step interaction, between receptor and ligand. Using the classical TSPO ligand radiolabelled 3H-Ro5-4864, kinetic studies were carried out to determine the irreversible binding characteristics. The new compound showed nanomolar affinity for TSPO. Further studies are on-going to visualize TSPO cell distribution.

## **CYSTATINS B IN ZEBRAFISH: STUDIES ON GENE EXPRESSION AND PROTEIN STRUCTURAL ORGANIZATION**

**Manuela Camerota, Angela Sangermano, Giovanni Iazzetti, Francesco Aniello and Rossella Di Giaimo**

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Cystatin B (CSTB) is an antiprotease involved in many human neurodegenerative diseases. Its cellular functions are still unclear because, although ubiquitous, its mutations affect only CNS. We have shown that, in vivo, rat CSTB has a toxic polymeric structure and interacts with many partners. We report here studies on CSTB gene organization and protein structure in *Danio rerio*. Zebrafish genome has 2 genes coding for CSTB on chromosome 24. An interesting difference between the proteins is a peculiar aa that is C (ZFCSTBC), as in mammals, or R (ZFCSTBR). While gene duplication is frequent in zebrafish, usually one copy is expressed. ZFCSTB genes are, instead, both transcribed with slightly different time-courses during development and tissue specificity in the adult. Preliminary in situ hybridization experiments show that brain signals are mainly in the cerebellum, as for mammals. The polymeric structures of ZFCSTB R and C proteins are both resistant to boiling in 1% SDS. ZFCSTBR shows stable aggregates also in presence of reducing agents, suggesting a role of this particular C residue in regulating structures. This might be a reason why this isoform is absent in higher organisms.

## **EUKARYOTIC VOLTAGE DEPENDENT ANION CHANNELS (VDAC): EXPRESSION AND FUNCTION OF MULTIGENIC FAMILY IN DROSOPHILA MELANOGASTER**

**F. Guarino\* F. Tomasello\* A. Guarnera\* S. Reina\* V. Specchia\*\* M.P. Bozzetti\*\* A. Messina\* V. De Pinto\***

\*Dep. of Chemical Sciences, University of Catania. \*\*Dep. of Biological and Environmental Science, University of Lecce.

Voltage Dependent Anion Channel (VDAC) are a family of pore-forming proteins of the mitochondrial outer membrane regulating permeability of metabolites between the cytosol and the mitochondrion. Complete sequencing of *Drosophila melanogaster* genome revealed the presence of three additional porin genes, whose function and significance was unknown. The expression of all isoforms were analyzed at mRNA and protein level in germinal tissues derived from male and female. The mRNA level of Porins transcripts is significantly higher in male tissues than female tissues. Analysis of the Porins protein expression revealed different pattern and localization. To study the functional property the coding sequences of Porins were cloned and the proteins over-expressed were reconstituted in artificial membrane. Porin2, Porin4 have pore-forming activity, but their property differ from that of the well characterized Porin1. In conclusion, different level of expression, localization and functional features of the new identified Porins isoforms of *Drosophila melanogaster* defined with this work may suggest the accomplishment of specialized function in various tissues or in different developmental stage

## **EVOLUTION OF THE PROTEOLYTIC ACTIVITY OF TRANSFERRINS.**

**Francesco Giansanti\***, **Loris Leboffe°**, **Fabio Polticelli°** and **Giovanni Antonini°+**

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Mammalian lactoferrin and hen ovotransferrin catalyze the hydrolysis of synthetic substrates, showing a substrate specificity similar to that of trypsin, whereas the rate is much lower. This catalytic activity is irreversibly inhibited by synthetic serine-protease inhibitors, suggesting the presence of a serine in the active sites of both proteins. Even though such catalytic sites have not been identified, pKa shift calculations indicate that there are several conserved serine residues of lactoferrin and ovotransferrin that may be involved. The biological meaning of such proteolytic activity is not clear, however it is possible that the proteolytic activity of both lactoferrin and ovotransferrin belongs to the common defensive properties. The observations reported here are of interest from an evolutionary point of view since it is likely that the nonimmune defensive properties of transferrins appeared early in evolution. In birds, the defensive properties of ovotransferrin remained joined to iron transport functions; in mammals, iron transport functions became peculiar of serum transferrin, while the defensive properties towards infections were optimised in lactoferrin.

## **IS THE N-TERMINAL DOMAIN OF MAMMALIAN VDAC1 ESSENTIAL FOR THE FUNCTIONING OF THE PORE-FORMING PROTEIN FAMILY?**

**Simona Reina<sup>1</sup>**, **Vanessa Palermo<sup>2</sup>**, **Cristina Mazzoni<sup>2</sup>**, **Francesca Guarino<sup>1</sup>**  
**Vito De Pinto<sup>1</sup>**, **Angela Messina<sup>1</sup>**

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Mitochondria play a key role in cellular metabolism due to their functions in ATP synthesis, regulation of cell redox status, cytosolic calcium homeostasis and cell signaling. Voltage Dependent Anion Channels (VDACs) are pore-forming proteins found in the outer mitochondrial membrane of all eukaryotes and constitute the major pathway by which metabolites are exchanged between the cytosol and mitochondria. In mammals three genes encode for VDAC. The knowledge of VDAC isoforms is mainly restricted to VDAC1 and VDAC2. VDAC3 has been poorly studied since it does not show pore-forming activity in cellular assays or in reconstitution experiment. In this work we investigated the effect of the substitution of the N-terminal sequence of the human VDAC3 with the homologous sequences of human VDAC1 and VDAC2. The activity of the chimeric proteins was monitored in the *Saccharomyces cerevisiae* strain BY4742 where the endogenous VDAC1 was deleted. Results obtained in complementation assay, chronological ageing and oxidative stress resistance measurements outline the importance of the N-terminal moiety of VDAC1 and VDAC2 in the function of the protein and of the whole mitochondria.

## **NITRATION IN PHYSIOLOGICAL CONDITIONS: ROLE OF NITRATED ALPHA-TUBULIN IN MICROTUBULE STABILITY**

**Simona Nonnis 1, Cristina Ronchi 1, Graziella Cappelletti 2, Lara Pagliato 1  
Armando Negri 1, Tedeschi Gabriella 1**

1 Università degli Studi di Milano, D.I.P.A.V., Milano 2 Università degli Studi di Milano, Dip. di Biologia, Milano

There are evidences that Tyr nitration is a physiological event implicated in biological processes modulated by NO. We showed previously that nitration of proteins, mainly cytoskeletal, occurs as a part of a physiological process in differentiating neurons and in adult brain. Here we investigate the role of nitration in microtubule rearrangement and dynamics. Studies on the association of nitrated proteins with the cytoskeletal fraction in differentiating neuronal cells following exposure to microtubule depolymerising treatments suggest that nitration correlates with the increased microtubule stability underlying the progression of neuronal differentiation.  $\alpha$ -Tubulin is one of the major targets of nitration during this process. In order to assess the role of this PTM in modulating tubulin assembly and stability, we have undertaken an in vitro study to selectively nitrate purified tubulin. Nitrated tubulin can assemble and microtubules show an increased stability to depolymerising agents. The same results were obtained by live cell imaging suggesting that nitration could play a novel functional role in the complex and dynamic organization of the cytoskeleton.

## **PRODUCTION AND PROPERTIES OF THE PUTATIVE MONOOXYGENASE DOMAIN OF HUMAN MICAL, A NOVEL FLAVOPROTEIN IMPLICATED IN CYTOSCHELETAL REMODELING**

**Gianluca Caprini and Maria Antonietta Vanoni**

Dipartimento di Scienze Biomolecolari e Biotecnologie, Università degli Studi di Milano.

MICALs form a family of conserved multidomain proteins that are conserved in animals. They participate in the transduction of signals initiated by semaphorins that result in cytoskeletal rearrangements associated with, e.g., vesicle trafficking, cell migration, cell-cell interactions, axon steering and pathfinding. The C-terminal calponine homology, LIM, Src3 homology domain recognition and coiled coil regions have been shown to mediate MICALs interaction with various proteins (e.g.:CasL, vimentin, Rab1, Plexin A, CRMP). The N-terminal domain is structurally related to FAD-linked monooxygenases and has been shown to be essential for MICAL function. The catalytic activity of MICAL monooxygenase-like domain (MICAL-MO) is not known. It may control cytoskeletal rearrangements by producing, under certain conditions, reactive oxygen species or by redox modifying small molecules or the side chain of specific proteins. To set the basis for the identification of MICAL-MO substrates/products we have produced and purified recombinant forms of the MO domain of human MICAL, which are being characterized by means of steady-state and pre-steady state kinetics and by equilibrium absorbance spectroscopy.

## **PYK2 REGULATES PLATELET ADHESION TO VWF**

**L. Cipolla, I. Canobbio, P. Tallarico, C. Balduini, M. Torti**

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Pyk2, nonreceptor protein tyrosine kinase, localizes in focal adhesions and is activated by a variety of extracellular stimuli. Pyk2 is primarily expressed in neuronal and haemopoietic tissues, suggesting a role in regulation of cellular morphology. We previously showed that platelet agonist von Willebrand factor (vWF) induces Pyk2 tyrosine phosphorylation and activation in human platelets and plays a role in the early signal transduction events activated by binding to glycoprotein Ib-IX-V(1). In the present work, we investigated the biological role of Pyk2 in platelet activation using Pyk2 knock-out mice (Pyk2 KO)(2). Pyk2 KO are viable and fertile, without overt impairment in development, behaviour and bleeding time. Immunoblotting analysis does not reveal Pyk2 in Pyk2 KO platelets, whereas expression of highly homologues of Pyk2, FAK, is not modified. Platelets from Pyk2 KO mice show normal aggregation at physiological concentration of vWF but reduced adhesion to vWF-coated surfaces in static conditions, demonstrating the importance of Pyk2 in regulating platelet adhesion. 1. Canobbio I., *Thromb Haemost.* 2002;87(3):509-17 2. Okigaki M., *Proc Natl Acad Sci USA.* 2003;100(19):10740-5

## **STRUCTURAL AND FUNCTIONAL CHARACTERIZATION OF THE NUCLEOTIDES BINDING DOMAINS OF MRP6 TRANSPORTER PROTEIN**

**Angela Ostuni, Maria Francesca Armentano, Rocchina Miglionico  
Maria Antonietta Castiglione Morelli, Faustino Bisaccia**

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MRP6 (Multidrug Resistance Protein 6) is a member of the ABC transporter family characterized by an additional N-terminal transmembrane associate domain (TMD0) as well as the domains TMD1 and TMD2 characteristic of many other ABC transporters [Borst, P. et al. (2000) *J. Natl. Cancer Inst.* 92, 1295-1302]. The human gene (ABCC6) maps on chromosome 16p13 and encodes a protein of the 1503 aminoacid residues. Mutations of ABCC6 gene cause pseudoxanthoma elasticum (PXE), an inherited disorder characterized by calcification of the elastic fibers in skin, arteries and retina [Uitto, J et al. (2001) *Trends Mol. Med.* 7, 13-17]. A lot of mutations has been found in the nucleotide binding domains (NBD1 and NBD2) that are critical for the ATP binding and hydrolysis [Ringpfeil F. et al. (2000) *Proc Natl Acad Sci U S A.* 97(11):6001-6]. In order to study the MRP6 transport mechanism and the substrate specificity, we have overexpressed the NBD domains in *E. coli*. CD spectroscopy shows the presence of alpha helical structures in both the NBD domains. Fluorescence experiments demonstrate that both the NBD1 and NBD2 domains bind the nucleotides ATP, ADP and AMP with different affinity

## **STRUCTURAL DETERMINANTS OF THE IMMUNOREGULATORY ACTIVITY OF MILK-DERIVED PEPTIDES: THE C-TERMINAL SEQUENCE OF BOVINE BETA-CASEIN**

**Stefania Iametti, A. Barbiroli, E. Ragg, P. Ferranti, O. Fierro, S. Favalli, H. Frokiaer, F. Bonomi**

DISMA, University of Milan; DSA, University of Naples; ISA-CNR, Avellino; LIFE, University of Copenhagen, Denmark

Peptides derived from digestive or cellular degradation of food proteins have been studied for they affect a number of biochemical responses in human, including systemic ones. Caseins are among the prime sources of the so called "bioactive peptides". The immunomodulating activity of synthetic peptides corresponding to the C-terminal sequence of bovine beta-casein was measured in a splenocyte proliferation assay, and compared with structural information from CD and NMR studies. Immunosuppressive activity required the minimal sequence (203-209) GPFPIIV, and was absent when L-Pro at P206 was substituted by D-Pro. This substitution results in loss of the structural features of this peptide, that cannot be relieved by introducing the arginine residue at position 202. However, peptides that include the hydrophobic stretch (194-201) YQQPVLGPV were active and structured, even when P206 was substituted by the structural homologues of proline, INP and THZ. These results point to a structure-function relationship in casein-derived peptides, and indicates that even the smallest peptides may have biological activities, once their structural identity is preserved.

## **SYNOVIAL FLUID PROTEIN PATTERN IN HORSE JOINT DISEASES**

**M. Tartaglia, L. Avellini, A. Gaiti, V. M. Masi, M. Pepe, F. Scoppetta, E. Chiaradia**

Dip. di Pat. Diag. e Clin. Vet. sez. Scienze Sperim. e Biotec. Applicate. – Università di Perugia - Perugia-Italy

The most common diseases that frequently affect horse diarthrodial articulations are Osteochondrosis (OC) and Osteoarthritis (OA). OC is a developmental disorder with multifactorial etiology characterised by the failure of endochondral ossification of the articular epiphyseal and physeal cartilage. This condition can be one of many causes of osteoarthritis, a progressive disorder that leads to joint destruction and severe impairment of mobility. The aim of this study was to analyse, with the proteomic approach, horse synovial fluid (SF) collected from healthy joints and affected by OC or OA in order to reach a better understanding of the pathogenesis of both diseases and to identify possible distinctive biomarkers. The 2D-gels were performed by using IPG strips, 17cm long, pH 4-7 and 9-16%T SDS-page, stained with colloidal Coomassie blue G-250 and were scanned using the Bio-Rad GS-800™ calibrated densitometer. The comparative analysis, performed with PD QUEST (BioRad, Hercules, CA), showed a different number of spots over and down expressed among the three groups of samples. Mass spectrometry analysis of these spots is in progress.

## TEMPERATURE-INDUCED RELAXATION OF THE TERTIARY STRUCTURE OF A RAT ODORANT-BINDING PROTEIN

**1 A. Scirè, 2 A. Marabotti, 3 M. Staiano, 4 L. Briand, 3 A. Varriale, 1 E. Bertoli, 3 S. D'Auria, 1 F. Tanfani**

1Dept. Biochem., UNIVPM, Ancona; 2Inst. Food Sci., CNR, Avellino; 3Lab. Mol. Sens., CNR, Naples; 4UMR FLAVIC Bourgogne

In order to reach their membrane receptors embedded in the membrane of olfactory neurons, airborne odorants have to be conveyed through the aqueous nasal mucus. The odorant-binding proteins (OBPs) which are secreted by the olfactory epithelium in the nasal mucus of vertebrates, are candidates for playing such a carrier role. OBPs belong to the lipocalin superfamily whose structure is characterized by a beta-barrel and a short alpha-helix. The beta-barrel defines a central apolar cavity, called calix, whose role is to bind and transport hydrophobic odorant molecules. The structural features of rat OBP were examined by using three different Fourier-transform infrared (FT-IR) spectroscopy methods based on the un-exchanged amide hydrogens of the protein sample. These are: 1) difference spectra analysis; 2) 2D-IR correlation spectroscopy; 3) phase diagram method. The data indicate that at high temperatures the OBP tertiary structure undergoes a relaxation process, suggesting the presence of a molten globule-like state. The above FT-IR spectroscopy analyses represent a valuable tool to study the stability of small and beta-sheet-rich proteins.

## THE SPECIFIC CO-CHAPERONE HSCB MODULATES THE ASSEMBLY OF IRON-SULFUR CLUSTERS ON THE SCAFFOLD PROTEIN ISCU

**F. Bonomi, A. Morleo, D. Ta, L. E. Vickery, F. Hanneman, S. Iametti**

DISMA, University of Milan, Italy; Physiology & Biophysics, UCI, Irvine, USA; Biochemie, UniSaar, Saarbrücken, Germany

IscU is a scaffold protein that functions in iron-sulfur cluster assembly and transfer, interacting with a chaperone protein HscA and a cochaperone protein HscB, that use ATP hydrolysis to “wrench” the geometry of a previously assembled cluster on holo-IscU, facilitating cluster transfer. No information is available on whether HscA or HscB affect the assembly of a cluster on apoIscU. HscB in a 1/1 molar ratio to apoIscU impairs formation of a cluster on apoIscU both when sulfide is added as such and when is generated by the cysteine desulfurase IscS. HscB does not affect rates and yields of 2Fe2S cluster transfer from preformed holo-IscU to various apoferredoxin (apoFds) when added together with apoFd. No holoFd forms upon addition of apoFd to IscU preincubated in the presence of HscB, iron and sulfide, but holoFd formation was accelerated when HscB was present in mixtures of apoIscU and apoFd, and cluster synthesis was started by adding iron and sulfide. HscB modulation of the biological activity of IscU, confirms that protein-protein interactions control the sequence and nature of events in FeS cluster assembly. This work was supported from PUR and from “Progetto Vigoni”.



## **Nucleic acids**

**Poster session:  
23/09/2009 (h. 14.00-15.00)**



## MRNA PROFILES IN FRESH OOCYTES (MET II) COMPARED TO CRYOPRESERVED OOCYTES AFTER SLOW FREEZING AND VITRIFICATION PROTOCOLS.

**S. Chamayou\*, A. Guglielmino\*, L. Alecci\*, G. Bonaventura, D. Tibullo\*, F. Diraimondo\*, M.L. Barcellona**

Dip. Chim.Biol. Chim.Med. Biol.Mol. Università di Catania. \*UMR Fondazione Hera. •Dip.Sc.Biom.Osp.Ferrarotto Catania.

The mammalian oocyte is responsible for a number of extraordinary biological processes. The human oocyte is susceptible to freeze-thaw damage and it has been suggested that cryoinjury is responsible for the relative lack of success in fertilization process. Nowadays two protocols are applied for oocytes cryopreservation: slow freezing and vitrification protocols. We assume that mRNA with its translational potentiality and structural uniqueness could be the targeted molecule damaged by the cryopreservation process. Our investigation is addressed to evaluate the modification of mRNA in content and quality in the MII oocytes after slow freezing and vitrification protocols in comparison with fresh oocytes. We analyzed the expression of different genes involved either in ultrastructural genes maintenance or in the regulation of transcriptional pathways such as: MAPK6, TOP1, DPPA, OCT-4, HSP family, SMC. Our data showed that fresh oocytes present an higher expression of these genes respect to the slow freezed, while there is a comparable expression with the vitrified ones and in particular for SMC and DPPA involved in the chromatid cohesion and developmental pluripotency respectively.

## A NEW REACTION OF NUCLEOTIDE METABOLISM

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Dipartimento di Medicina interna, Scienze Endocrino-Metaboliche e Biochimica, Università di Siena, Italia.

We have demonstrated that rat liver supernatant catalyses the new reaction 1) called AMP-AMP phosphotransferase: 1)  $AMP+AMP \rightarrow ADP + \text{Inosine} + NH_3$  resulting from reactions 2) and 3): 2)  $AMP+AMP \rightarrow ADP+Adenosine$  3)  $Adenosine + H_2O \rightarrow \text{Inosine} + NH_3$  Through purification, we demonstrated that the formation of ADP involves the cooperation of AdK (adenosine kinase, EC 2.7.1.20) and AK (adenylate kinase, EC 2.7.4.3). Reaction 1) needs also the cooperation of ADA (adenosine deaminase, EC 3.5.4.4). Physical contact between AdK and AK is required, while ADA fills the gap in the energy balance of the phosphoryl transfer and shifts the equilibrium towards ADP and inosine synthesis. The proposed mechanism involves the association of AdK and AK through non-covalent interactions, that induce slight conformational changes in the active site of AK, bringing two already bound molecules of AMP together for phosphoryl transfer to form ADP. Reaction 1) may play a physiological role, specially under conditions of energy drain (temporary, hypoxia, cancer tissues) when the involved enzymes cannot display their normal activity because of substrate deficiency.

## **EXPRESSION AND CHARACTERIZATION OF A CHIMERIC COSTRUCT CONTAINING RIBOSOME INACTIVATING PROTEIN (RIP) AND SERINE PROTEASE INHIBITOR (WSC1)**

**V. Capuzzi<sup>1</sup>, F. Tedeschi<sup>1</sup>, A.G. Ficca<sup>2</sup>, R. Tamburino<sup>3</sup>, E. Poerio<sup>1</sup>, and A. Di Maro<sup>3</sup>**

<sup>1</sup>Dip. ABAC & <sup>2</sup>Dip. SA, Università della Tuscia. <sup>3</sup>Dip. Scienze della Vita, Seconda Università di Napoli

A bifunctional chimeric protein, potentially able to act as an insecticidal agent, has been designed and expressed in *E. coli* cells. The first domain corresponds to the toxic/antiviral protein PD-L4 type 1 RIP, firstly isolated from *P. dioica* L. leaves (1). The second domain is made of the wheat protein inhibitor WSCI; this protein is able to interfere with subtilisin, pancreatic chymotrypsins and chymotrypsin-like activities isolated from the midgut of a number of phytophagous insect larvae (2, 3). The chimeric construct, pd-l4-cDNA~oligonucleotide linker~wsci-cDNA, was cloned in the expression vector pET22b and employed to transform *E. coli* (strain BL21-DE3). The recombinant chimera was recovered from the inclusion bodies with an appropriate refolding procedure. The expression product was characterized by electrophoretic (SDS-PAGE) and chromatographic (HPLC) analyses, N-terminal sequence determination and evaluation of anti-protease and polynucleotide adenosine glycosidase activities. 1. Di Maro, A. et al. (1999) *Planta* 208, 125-131. 2. Poerio, E. et al. (2003) *Biol. Chem.* 384, 295-304. 3. Di Gennaro, S. et al. (2005) *Biol. Chem.* 386, 383-389.

## **FUNCTIONAL INTERACTION BETWEEN THE ATP-DEPENDENT NUCLEOSOME REMODELING FACTOR ISWI AND THE HSR-OMEGA NON-CODING RNA**

**Maria Cristina Onorati, F.V. Davide Corona**

Dipartimento di Biologia Cellulare e dello Sviluppo ed Istituto Telethon Dulbecco c/o Università di Palermo

ISWI is chromatin remodeler playing essential roles in chromosome condensation, gene expression and DNA replication. Using an *in vivo* assay to identify factors that antagonize ISWI activity, we recovered a genetic interaction between ISWI and Hsr- $\omega$ . The Hsr- $\omega$  locus encodes for a non-coding RNAs (ncRNA) essential for the assembly and organization of omega speckles. These special nuclear compartments are localized in the nucleoplasm and are thought to play essential roles in the storage/sequestration of proteins playing important roles in RNA maturation. Remarkably, loss of Hsr- $\omega$  function results in a strong suppression of developmental and chromosome condensation defects caused by loss of ISWI activity. On the other hand, the organization of the omega speckles in ISWI mutant cells is profoundly altered when compared to wt cells. Interestingly, immunofluorescence analysis revealed a significant number of nuclear sites where ISWI overlaps with the Hsr- $\omega$  ncRNA. Remarkably, RNA-immunoprecipitation assays revealed a physical interaction between ISWI and Hsr- $\omega$  ncRNA. Our data strongly suggest that ISWI could act as a functional bridging factor between chromosomes and nuclear speckles.

## **HEPATIC MICRORNAS WITH POTENTIAL ANTIVIRAL ACTIVITY**

**Nicoletta Potenza, Umberto Papa, Francesca Zerbini, Nicola Mosca  
Valentina Nobile, and Aniello Russo**

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The analysis of the hepatitis B virus (HBV) genome by MiRanda led to the identification of 6 sites that are potential targets for human liver miRNAs. These sites are clustered in a 836 bp segment within the viral Polymerase ORF and are conserved among the most common HBV strains. The putative interactions between the identified miRNAs and HBV mRNAs were then verified by a primary validation test based on cultured cells, luciferase reporter genes, and synthetic miRNAs. The rationale for using this assay is that the binding of a given miRNA to its specific target will repress translation thus reducing luciferase activity compared to a control. The HBV targets validated in this assay were then subjected to a secondary validation step based on endogenous hepatic miRNAs expressed in HepG2 cells. In this assay, two miRNAs were found to interact with the viral sequence and to suppress translation effectively. No inhibition of the reporter activity was detected with control sequences (inverted, scrambled or mutated). These data suggest that human liver cells make use of the two identified miRNAs to down-regulate the expression of HBV polymerase gene, thereby affecting viral replication.

## **IDENTIFICATION OF GENES INVOLVED IN DECREASED SUSCEPTIBILITY OF E. COLI CELLS TO THE HUMAN ANTIMICROBIAL PEPTIDE LL-37**

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Antimicrobial peptides (AMPs) participate in the immunity of both animals and plants. In mammals, the most important AMP families are defensins and cathelicidins. The only human cathelicidin, LL-37, is a 37-residue,  $\alpha$ -helical, cationic peptide with a direct, membranolytic antibacterial activity and an immunomodulatory capacity. The aim of our research is to identify mutated genes that modulate the susceptibility of *E. coli* cells to LL-37 and thus to help clarify the peptide's mode of action. A library of random *E. coli* mutants was treated at inhibiting LL-37 concentrations, and 20 clones with a resistant phenotype were selected, allowing to identify six different mutated genes conferring this phenotype. The gene *rfaY* (*waaY*), identified in 15 out of 20 analysed mutants, encodes one of the kinases that phosphorylate the core region of bacterial lipopolysaccharide, suggesting that the resulting reduction in negative charge of the outer membrane in these mutants lowers susceptibility to LL-37. We performed a series of experiments to confirm this resistant phenotype and characterise it. Genetic complementation and overexpression are planned to clarify the mechanism of the resistance.

## IDENTIFICATION OF TWO PROTEINS BINDING THE 3'UTR OF MHC CLASS II MRNA

**C. Corso\***, **G. Manco#**, **L. Pisapia\***, **A. Citro\***, **P. Barba\***, **A. Maffei\***, **L. Cigliano°**  
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AIM: Multiple mRNAs belonging to the same functional biological pathway are co-regulated by RNA-binding proteins that orchestrate their processing. Major Hystocompatibility Complex class II molecules (MHCII) are heterodimeric cell-surface glycoproteins playing a pivotal role in the immune response. We aimed to study whether mRNAs for MHCII are co-ordinately regulated as post-transcriptional RNA operons through a ribonucleoprotein-driven mechanism. RESULTS: The 3'UTRs of HLA-DRA and HLA DQA1 mRNAs are involved in the interaction with nuclear and cytoplasmic proteins. We report that affinity and ionic exchange chromatography allowed to isolate from cytosol of Raji cells (B lymphoma) at least two proteins interacting with HLA-DRA mRNA. These proteins turned out by mass spectrometry to be Ebp1 and DRBP76. Both of them are known to regulate stability and translation of different mRNAs. We knocked down their mRNAs by specific silencing and following q-RT-PCR we observed a decrease in the accumulation of HLA-DRA, DRB1 and DQA01 mRNAs. CONCLUSIONS: Our results demonstrate that the two identified proteins have a role in the control of MHCII stability by binding the 3'UTR of mRNAs .

## LARGE OR LONG LINEAR DNA: WORM LIKE CHAIN OR GLOBULE ?

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DLS provides diffusion coefficient values of DNA, associated to a coil model or to a spherical shape, depending on DNA chain length (<10 kbp or >100 kbp). Statistic light and x-ray scattering allow to derive the so called form factor, that gives a diffraction pattern associated to the shape of the molecules. The form factor measured for 4.000 DNA bp is similar to a coil and for 115.000, close to a sphere. Renewed attention has been addressed to the formation of compact regular structures or cylindrical deformation spontaneous or salt induced and to the requirement of the collapsed state for proper biological functions. Consequently the implications of deriving form factors by using VIS light whose scattering vectors allows observations in a scale smaller than the light wavelength and X ray scattering, instead, in a larger scale of the scattering vectors, from 0.1 nm to hundred nanometers, are evident. Analysis of the 115kbp DNA evidences a repetitive structure in the atomic scale for large scattering vectors, being in the 10 nm scale a filament structure, a coil structure in the 100 nm scale and a collapsed in the LS range, where a globular structure in the  $\mu\text{m}$  scale, appears.

## **PATIENTS' COLLECTION FOR ARRAY-CGH (ACGH) ANALYSIS**

**Maria G. Bruccheri 1,2, Anna Maria Roccazzello 1, Elio Insirello 1, Manuela Andolina1  
Adriana Tiralongo 1, Giovanni Tringali 1**

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Many chromosomal defects are the most commonly cause of developmental delay(DD) or mental retardation(MR), which affect 1–3% of children and are often seen in conjunction with growth retardation, dysmorphic features, and multiple congenital anomalies(MCA). A large part of patients with MCA/MR is not detectable by karyotype. Only 15–40% of individuals with DD and MR demonstrate imbalances by this method, and only 3–5% of patients carry subtelomeric rearrangements(FISH). So, screening of selected patients with MCA/MR and normal conventional cytogenetic analysis results to be needed. The goal of the study is to determine which MCA/MR patients are predicted to have chromosomal aberrations based on clinical presentation of symptoms detectable only by high-resolution aCGH. Patients included in the study, had to have a diagnosis of MCA/MR and a previously documented normal karyotype and excluding those subtelomeric imbalances that could have been detected by FISH or MLPA/MAPH analysis. This patients' collection will allow the determination of the genomic distribution of disease causing imbalances and may reveal the underlying mechanisms causing chromosomal imbalances.

## **REGULATORY PROPERTIES OF PROTEIN REPAIR METHYLTRANSFERASE ON APOPTOSIS: ROLE OF RNA INTERFERENCE**

**Irene Sambri, Rosanna Capasso, Patrizia Galletti, Diego Ingrosso.**

Department of Biochemistry & Biophysics "F. Cedrangolo", Second University of Naples

**BACKGROUND.** Asparaginyl deamidation is a spontaneous post-biosynthetic modification causing protein structural and functional impairment. Isoaspartyl protein carboxyl-O-methyltransferase (PCMT) repairs the abnormal isoaspartyl residues at deamidated sites, thus reverting the functional consequences of deamidation. Evidence supports the role of protein deamidation in the acquisition of susceptibility to apoptosis. **AIMS.** To establish the role of PCMT in apoptosis and shed a light on the relevant mechanisms. **METHODS.** Various cell lines were transfected with vectors overexpressing PCMT (wild type, mutants) or in which PCMT was suppressed (antisense, dsRNA, siRNA oligonucleotides). The role of miRNA-dependent regulation of PCMT expression was also investigated (pre-miR and antago-miR trasfection). **RESULTS.** PCMT overexpression confers cell resistance to apoptosis; Bcl-xL, Hsp70/90 are among the PCMT substrates involved; miR-15a/16 downregulate PCMT. **CONCLUSIONS.** PCMT is involved in apoptosis by preserving the structural stability and biological function of key anti-apoptotic proteins. PCMT expression is in turn downregulated by miRNA involved in apoptosis effector modulation.

## ROLE OF MICRORNAS IN CELLULAR SENESCENCE

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Cellular senescence is an irreversible growth arrest that can be induced by various stress signals. Cellular senescence contributes to aging not only by accumulation of senescent cells in tissues but also by limiting the tissue renewal as a consequence of stem cell senescence. We reported that young human embryo fibroblasts (IMR90) exposed for few days to low doses of the GSH-depleting agent diethylmaleate (DEM) acquire a senescent phenotype. MicroRNAs (miRNAs) are a class of small non-coding RNAs that act as post-transcriptional “regulators” of gene expression. In order to investigate the role of miRNAs in the induction of cellular senescence, we analyzed the expression profiles of 365 known miRNAs using TaqMan Array Human miRNA Panel, in young and senescent fibroblasts (either senescent by repeated in vitro passages or by mild oxidative stress). 200 miRNAs out of the 365 tested resulted expressed in IMR90 cells. The miRNA profile was altered in senescent cells, with 11% of them being up-regulated, and 23% being down-regulated. This suggests that specific miRNAs are involved in the senescence program and demands functional experiments to prove their involvement in such process.

## THE CRYSTAL STRUCTURE OF A JUNCTION BETWEEN TWO Z-DNA HELICES

**M. de Rosa<sup>1, 2</sup>, D. de Sanctis<sup>4</sup>, A.L. Rosario<sup>2</sup>, M. Archer<sup>2</sup>, A. Rich<sup>3</sup>  
A. Athanasiadis<sup>1</sup> and M.A. Carrondo<sup>2</sup>**

<sup>1</sup>IGC and <sup>2</sup>ITQB, Oeiras Portugal; <sup>3</sup>MIT, Cambridge, USA; <sup>4</sup>ESRF, Grenoble. France

The double helix of DNA or RNA when composed of purine-pyrimidine repeats, can adopt a left-handed helical structure called Z-DNA and Z-RNA respectively. Such dinucleotide repeats in genomic sequences have been associated with instability leading to cancer, for reasons not entirely understood. Adoption of the left handed conformation in just a region of a polynucleotide sequence results in the formation of conformational junctions: a B-Z junction is formed at the boundaries of the left handed helix, a Z-Z junction is commonly formed in sequences where the dinucleotide repeat is interrupted by single base insertions or deletions that bring neighbouring left-handed helices out of phase.

We report the first crystal structure of a Z-Z junction stabilized by  $\alpha$ , the Z-DNA binding domain of the RNA editing enzyme ADAR1. The junction structure consists of a single base-pair and leads to partial or full disruption of the helical stacking. The junction region allows intercalating agents to insert themselves in the otherwise resistant to intercalation left-handed helix. However, unlike a B-Z junction the bases do not become fully extruded and the stacking between the two left handed helices is not continuous.

## IDENTIFICATION AND EXPRESSION PATTERN OF A SECOND ZEBRAFISH RELAXIN-3 GENE

**Aldo Donizetti, Marcella Fiengo, Sergio Minucci, Francesco Aniello**

Structural and Functional Biology, University Federico II; Dep. Experimental Medicine, Second University, Napoli

Relaxin-3 is thought to function as neurotransmitter mainly produced in the mammalian nucleus incertus and involved in different neural processes among them the stress response. We recently examined the spatial expression of the relaxin-3 gene in the developing zebrafish brain, showing the first evidence of the nucleus incertus in fish. Here we report the expression pattern of the duplicated zebrafish *rln3b* gene and compare it to the *rln3a* expression pattern. Both genes are active and show relevant differences in their expression patterns. In fact, whereas *rln3a* gene is expressed in the periacqueductal gray from 40 hpf, the *rln3b* gene expression becomes restricted in that region only from 48 hpf. In addition, no expression was observed for *rln3b* in the nucleus incertus cells that express *rln3a* gene from 72 hpf. In the adult, both genes are expressed in brain, but only *rln3b* transcript is revealed in testis at the similar expression level. Both the putative mature protein sequences are highly conserved, this feature and their differential expression patterns might indicate a sub-functionalization during evolution with the consequent retention of the two paralogs genes.



## **Macromolecules**

**Poster session:  
23/09/2009 (h. 14.00-15.00)**



## **HYALURONAN OLIGOSACCHARIDES INDUCE INFLAMMATION BY ENGAGING BOTH TOLL-LIKE-4 AND CD44 RECEPTORS IN HUMAN CHONDROCYTES**

**Angela D'Ascola, Angela Avenoso, Salvatore Campo, Giancarlo Nastasi, Dario Samà  
Alberto Calatroni, Giuseppe M. Campo**

DBPNS, section of Medical Chemistry, School of Medicine, University of Messina

Hyaluronan (HA) oligosaccharides are endogenous ligands for both CD44 receptor and toll-like receptor 4 (TLR4). HA fragments were suggested to induce pro-inflammatory cytokine expression by interacting both with CD44 receptor and TLR4. The CD44 and TLR4 stimulation activates different inflammatory pathways that culminate with the activation of the NF- $\kappa$ B responsible of the expression of the inflammation mediators such as  $\alpha$  and  $\beta$ ), interleukin beta (IL- $\alpha$ tumor necrosis factor alpha (TNF- interleukin-6 (IL-6). Aim of this study was to investigate the inflammatory effects of very small HA oligosaccharides, at different concentrations, in normal human articular chondrocyte. Addition of 6mers HA fragments to chondrocyte cultures upregulated CD44, TLR-4 and pro-inflammatory cytokines expression, and activated NF- $\kappa$ B translocation. The addition of a specific CD44 blocking antibody partially reduced these effects, except TLR4 expression, while TLR4 blocking antibody decreased TLR4 and, partially, inflammatory cytokines expression. CD44 expression was unaffected. The addition of both CD44 and TLR4 blocking antibodies significantly reduced CD44, TLR4 and inflammatory cytokine expression

## **MODULATION OF MEGAKARYOCYTE DIFFERENTIATION BY EXTRACELLULAR MATRIX COMPONENTS.**

**C. Gruppi, A. Malara, S. Badalucco, L. Visai, R. Tenni, M. E. Tira, C. Balduini, A. Balduini**

Dipartimento di Biochimica "A.Castellani", Università di Pavia (Italia)

Megakaryocytes (Mks) and their progeny, circulating platelets, are specialized cells that participate in haemostatic and inflammatory functions. Megakaryocyte differentiation occurs in the bone marrow and the mechanisms of this process are still poorly understood. A number of evidence indicates that the nature of the microenvironment surrounding Mks may play an important role in the regulation of platelet production within the bone marrow. In this work we analyzed the role of different matrix proteins in megakaryocyte spreading and proplatelet formation (PPF). We hypothesized an involvement of fibronectin in this process: in fact, immunofluorescence staining of spreaded Mks on collagen I (the only non-permissive substrate for PPF), but not on other matrix proteins, showed an exposure of endogenous fibronectin to the cell membrane. We report that fibronectin may finely regulate PPF and Mk spreading depending on substrate. Further investigations are directed to understand a possible biochemical pathway leading to Mk spreading or platelets release. This study highlights how the interactions with collagens or matrix proteins influence megakaryocyte behaviour and platelet release.

## **STRUCTURAL AND FUNCTIONAL ASPECTS OF DYSTROGLYCAN: TOWARDS THE ELUCIDATION OF ITS PATHOPHYSIOLOGICAL ROLE.**

**S. Morlacchi<sup>1,3</sup>, M. Bozzi<sup>3</sup>, A. Galtieri<sup>1</sup>, B. Giardina<sup>3</sup>, F. Sciandra<sup>2</sup> and A. Brancaccio<sup>2,3</sup>**

<sup>1</sup>Università degli Studi di Messina; <sup>2</sup>ICRM (CNR) Roma; <sup>3</sup>Università Cattolica del Sacro Cuore, Roma.

Dystroglycan (DG) is composed of two subunits, alpha and beta, the first being a highly glycosylated extracellular protein that interacts noncovalently with the second, whose transmembrane domain interacts with dystrophin. In a subgroup of congenital muscular dystrophies (defined as dystroglycanopathies) DG can be largely altered (1). Dystroglycan also plays a role in a wide range of pathophysiological processes involving different tissues and organs, which include cancer progression, infections and stabilization of post-synaptic elements (2). Our work has helped to elucidate and dissect the domain organization and structure of the two dystroglycan subunits, with particular reference to the maturation of the DG precursor and the molecular details of the intersubunit interface (3). Our analysis of the structural and functional aspects of the two subunits is currently based on different strategies, ranging from multiple fluorescent tagging to the establishment and investigation of novel knocked in transgenic mice lines. 1. Sciandra et al. 2007 Trends Biotechnol. 25:262-68 2. Bozzi et al. 2009 Matrix Biol. 28:179-87 3. Sciandra et al. 2009 FEBS J. in press.

## **THE EFFECT OF CHONDROITIN SULPHATE DEGRADATION PRODUCTS ADDITION TO HUMAN CHONDROCYTE CELL CULTURE**

**Angela Avenoso, Angela D'Ascola, Giancarlo Nastasi, Salvatore Campo, Paola Traina  
Giuseppe M. Campo, Alberto Calatroni**

Department of Biochemical, Physiological and Nutritional Sciences, University of Messina, Messina, Italy

Biomolecules degradation products exhibit often properties different from their parent compound. In the glycosaminoglycan (GAG) field, low molecular weight (MW) hyaluronan (HA) was reported to possess high inflammatory activity, while high MW HA demonstrated a marked anti-inflammatory effect (1). The sulphated disaccharides obtained from GAG chondroitin-4-sulphate (C-4-S) promote axonal growth, while C-4-S proteoglycans show growth inhibitory effect on neurons (2). C-4-S derived oligosaccharides are especially produced in some GAG storage diseases with skeletal deformities (3). In the present work commercial C-4-S is degraded by testicular hyaluronidase, an endopolysaccharidase that exhibits also transglycosydase activity of unknown function. The resulting C-4-S oligosaccharides were purified and added to human chondrocyte cell cultures, and the effect on cytokines and receptors expression was evaluated, and compared with those obtained with undigested C-4-S, human plasma derived C-4-S chains and HA fragments. (1) Campo GM et al., *Innate Immun.*, in press, 2009. (2) Rolls A and Schwartz M, *Adv. Pharmacol.*, 53: 357-374, 2006. (3) Traina et al., *Conn Tissue Res.*, 48: 110-11, 2006.

## **AORTIC: SMOOTH MUSCLE CELLS AND VASCULAR PATHOLOGY: ROLE OF HYALURONAN**

**A. Passi, B. Bartolini, D. Vigetti, M. Viola, E. Karousou, S. De Leonibus, M. Clerici  
A. Genasetti, G. De Luca**

DSBSC, Insubria University, VARESE ITALY

The crucial event in atherosclerosis initiation is the retention of lipoproteins (LDL) within the arterial wall, due to a combination of proteoglycan binding and LDL aggregation. This triggers a cascade of events, such as LDL oxidation and fusion, ultimately leading to foam cell formation and lipid deposition. Smooth muscle cells (SMCs) are essential in neointima formation because of their ability to migrate and proliferate in response to different stimuli, like HA which they themselves secrete. The regulation of HA production by SMCs is expected to be one of the crucial steps for the atherosclerotic plaque formation and development. Nevertheless the exact mechanism by which LDL particles modulate HA synthesis is not yet known. Our data reveal that, when added to the culture medium, LDL are able to increase the production of HA, with the greatest effect obtained by the oxidized LDLs. This seems to be due to an up-regulation of the hyaluronan synthase genes 2 and 3 and can be visualized by particles exclusion assay. The fine definition of HA synthesis throws new insights on the knowledge of atherogenic progression. Migration of the SMC cells depends on the CD44 receptor activity.

## **SPHINGOSINE 1-PHOSPHATE INDUCES DIFFERENTIATION OF MESOANGIOBLASTS TOWARDS SMOOTH MUSCLE CELLS**

**F. Cencetti 1,2, C. Donati 1,2, G. Marseglia 3, A. Magi 3, C. Bernacchioni 1,2, M. Benelli 3  
F. Torricelli 3, P. Bruni 1,2**

1Dip. Scienze Biochimiche Firenze, 2Istituto Interuniversitario Miologia (IIM), 3UO Citogenetica e Genetica AOUC Firenze

Smooth muscle cells (SMCs) control fundamental functions such as arterial tone and airway resistance. Recent studies proved that circulating, SMC progenitor cells can contribute to tissue repair following vascular injury. Mesoangioblasts (Mbs) are a new type of stem cells, capable of differentiating into mesoderm cell types, such as muscle and bone. Sphingosine 1-phosphate (S1P) is a lipid mediator that regulates many biological processes as vascular development and SMC growth and migration. We demonstrated that S1P acts as potent mitogen and antiapoptotic agent in Mbs. We also showed that TGF beta exerts a marked antiapoptotic action in Mbs, involving the regulation of SK1. In order to exploit the therapeutic potential of these cells, we performed a microarray study to establish transcriptional profiles of human Mbs treated with S1P for 6 h and 24 h. Results were validated by Real time PCR, WB and IF analysis, demonstrating that S1P promotes differentiation of human Mbs towards SMC. Moreover, TGF beta-induced differentiation of Mbs into SMC relies on SK regulation. This study provides a prominent role for S1P in Mbs which can be used to favour vascular regeneration.

## **EVALUATION OF THE PRESENCE OF AGE<sub>s</sub> (ADVANCED GLYCATION END-PRODUCTS) IN THE PERIODONTAL LIGAMENT**

**Anna M. D'Alessandro<sup>1</sup>, Davide Pietropaoli<sup>1</sup>, Annarita Lizzi<sup>2</sup>, Carla Tatone<sup>1</sup>  
Arduino Oratore<sup>2</sup>, Annalisa Monaco<sup>1</sup>**

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AGE<sub>s</sub> (Advanced Glycation End-products) have a role in the genesis of numerous pathologies and aging-related diseases associated with oxidative stress. These compounds play a role in inflammation and vascular complications by irreversibly cross-linking collagen and elastin with subsequent loss of elasticity and destabilization of basement membranes. A relationship between serum AGE<sub>s</sub> and deterioration of the periodontal ligament (PL) in type 2 diabetes patients has been documented. Nevertheless, there are no evidence about the presence of glycated molecules in this tissue. In the present study, PLs were mechanically isolated from teeth of healthy male subjects and processed for Western blotting analysis with a monoclonal antibody against argpyrimidine, an adduct formed by reaction of a powerful glycating agent, 2-oxopropanal, with arginine residues. This approach has lead us to detect for the first time AGE<sub>s</sub> in PL by identifying argpyrimidine-positive proteins. This finding reveals that formation of AGE<sub>s</sub> physiologically occurs in this tissue and represents a starting point for defining the role of protein glycation in PL degeneration associated with aging and periodontal disease.

## **CERAMIDE 1-PHOSPHATE STIMULATES PROLIFERATION OF MOUSE MYOBLASTS.**

**C. Bernacchioni (1), P. Gangoiti (2), C. Donati (1), F. Cencetti (1), A. Gómez-Muñoz (2), P. Bruni (1)**

1) Dipartimento di Scienze Biochimiche, Università di Firenze. 2) Department of Biochemistry and Molecular Biology UPV/EHU

Bioactive sphingolipids including sphingosine, sphingosine 1-phosphate, ceramide, GM3 ganglioside have been recently emerged as important regulators of skeletal muscle cell biology being capable of regulating key cellular parameters including myoblast proliferation and myogenesis. However, no information is presently available on the possible biological effects of ceramide 1-phosphate (C1P) in these cells. Here we provide the first experimental evidence that C1P acts as mitogen in C2C12 myoblasts. C1P (15  $\mu$ M) was found to stimulate DNA synthesis measured by determining radioactive thymidine incorporation as well as by assaying MTT dye reduction. Moreover, analysis by FACS revealed that challenge of myoblasts with C1P accelerated cell cycle. Specific inhibitors of PI3K or ERK1/2 signaling pathways strongly attenuated the C1P action, whereas pertussis toxin treatment, or inhibition of p38 MAPK or JNK were ineffective. In agreement, C1P was responsible for a rapid phosphorylation of Akt and ERK1/2. Thus, in this study a novel important biological action of C1P in myoblasts has been highlighted, which possibly will be exploited in the future to enhance skeletal muscle regeneration.

## **SMALL MOLECULES AND MACROMOLECULES FOR THE INHIBITION OF B2 MICROGLOBULIN FIBRILLOGENESIS**

**Riccardo Porcari, Sofia Giorgetti, Sara Raimondi, Loredana Marchese, Jan Steyaert  
Monica Stoppini & Vittorio Bellotti**

Discovery of new molecules able to inhibit protein fibrillogenesis represents a challenging demand for treatment of amyloid disease. Recent advances in the elucidation of the mechanism of amyloidogenesis of B2 microglobulin (B2m) are offering the opportunity to discover new anti-amyloidogenic compounds. Small molecules like tetracyclines and high affinity antibodies anti B2m represent two prototypic classes of interactors for potential pharmaceutical exploitation. From a series of ten analogues of tetracyclines we have singled out the two best anti-amyloidogenic compounds, able to inhibit B2m fibrillogenesis and capable solubilising preformed fibrils. Both compounds abrogate the cytotoxicity of oligomeric B2m. Whereas the tetracyclines exert their activity through a low affinity interaction with the amyloidogenic conformers, the monovalent nanobodies display an anti-amyloidogenic capacity through a nanomolar affinity against B2m. The contact of the nanobody with specific regions of B2m can switch off its amyloidogenic propensity. Comparative analysis of these molecules in the inhibition or disorganization of fibrils are essential steps toward the identification of new therapies against DRA.



## **Polyamines**

**Poster session:  
23/09/2009 (h. 14.00-15.00)**



## **PUTRESCINE INHIBITS THE DNA AGGREGATION INDUCED BY SPERMIDINE**

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The natural polyamines, putrescine, spermidine and spermine are aliphatic molecules fully protonated at physiological pH. It is described that, unlike putrescine, spermidine and spermine induce the DNA aggregation in aqueous solution. We analyzed this aggregation in low ionic strength solution each containing two different polyamines: spermidine and spermine, and either spermine or spermidine with putrescine. Spermidine and spermine show a synergistic effect: the concomitant presence of both polyamines induce the aggregation at concentrations at which the single polyamine are ineffective, whereas putrescine inhibits the spermidine- but not the spermine-induced DNA aggregation. By ethidium bromide exclusion assay we observed that only small amount of putrescine binds DNA; this binding could displace spermidine and inhibit the aggregation. Since spermine binds more strongly DNA than spermidine, its binding is not affected by the presence of putrescine. We are currently analyzing the behavior of polyamines in the presence of 150 mM NaCl. Preliminary data show that in this condition putrescine inhibits the aggregation in solutions containing both spermidine and spermine.

## **HUMAN ASTROGLIAL TUMORS: TRANSGLUTAMINASE 2 AND RETINOBLASTOMA PROTEIN EXPRESSION**

**V. Macaione, M. Aguenouz, M.G. De Pasquale, R. M. Di Giorgio, A. Vanella\*, G. De Luca, A. Campisi A\***

Bioch. Phys. and Nutritional Sciences, Messina \*Biol. Chemistry, Med. Chemistry and Molecular Biology, Catania

Transglutaminase 2 (TG2) can both promote apoptosis and protect against cell death depending upon cell type, apoptotic stimulus, and subcellular localization of TG2. Retinoblastoma protein (Rb) is involved in regulating the expression of genes that favor cell cycle progression and suppressing the expression of genes involved in apoptosis, and its phosphorylation plays an important role in the regulation of its function. Rb is also a substrate for the recently identified serine/threonine kinase activity of TG2. TG2 phosphorylates Rb at the critically important Ser780 residue. Astrocytomas are the most common glioma, accounting for about half of all primary brain tumours. Astrocytoma is graded as pilocytic, diffuse, anaplastic, and glioblastoma multiforme. Our aim is to investigate TG2, Rb and Rb (Ser 780) expression in different grade of human astroglial brain tumours, in order to clarify the relationship between apoptosis and cell proliferation. Our data show an over expression of TG2, Rb and Rb (Ser 780) in all samples tumors with different methods, suggesting the involvement of these proteins in cell proliferation and tumor progression.

## **INTERACTIONS BETWEEN POLYAMINES, ACETYLPOLYAMINES AND PCAF DURING HISTONE ACETYLATION**

**Gennaro Taibi, Concetta M.A. Nicotra**

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Polyamines are involved in several gene regulatory functions, as the transcription process. In order to clarify their effect, in an initial "in vitro" biochemical study, we considered their influence on the nuclear PCAF acetyltransferase (PCAF-HAT). In a model system, using both commercial histones and laboratory histone preparations from mammary epithelial cells, we evidenced the inhibitory effect played by 40-180  $\mu$ M spermidine (Spd) versus PCAF, assayed on 250 nM histone and 40  $\mu$ M acetyl-CoA. At the same concentrations N8-acetylspermidine (N8-AcSpd), the acetylated form of spermidine located in the nucleus, exerts a significant activating effect on the PCAF histone acetyltransferase. In addition, N8-AcSpd seems to be a substrate donor of acetyl group for PCAF histone acetylation. A new vision is thus taking shape on non-shared effects of polyamines and acetylpolyamines observed on PCAF-HAT activity, although acetylpolyamines has been until now considered excretory or export forms of polyamines.

## **ROLE OF AGMATINE AS REGULATOR OF MITOCHONDRIAL FUNCTIONS**

**Valentina Battaglia<sup>1</sup>, Silvia Grancara<sup>1</sup>, Maria Angelica Grillo<sup>1</sup>, Enzo Agostinelli<sup>2</sup>, Antonio Toninello<sup>1</sup>**

<sup>1</sup>Dip. di Chimica Biologica, Università degli Studi di Padova <sup>2</sup>Dip. di Scienze Biochimiche, Università di Roma La Sapienza

Agmatine (AGM) is a dicationic amine at physiological pH, formed by the action of arginine decarboxylase (ADC). It acts on polyamine metabolism by inhibiting nitric oxide synthase and activating spermidine/spermine acetyltransferase as well as the antizyme of ornithine decarboxylase. Indeed, it is metabolized by agmatinase (AGMase) to form urea and putrescine. AGM and its metabolic enzymes ADC and AGMase have also been recognized in mitochondria, as well as imidazoline receptor. The aim of this work is to study transport and action of AGM in isolated rat brain mitochondria (RBM) for evaluating its physiological roles in these mitochondria. Results show the characterization of AGM uptake by RBM which is responsible for a protective effect against the MPT in RBM, whereas in liver mitochondria it shows double behavior, depending on the concentration. The possible explanation is the presence of a specific amino oxidase in liver mitochondria. In conclusions the results obtained with this study evidence AGM as a physiological regulator of mitochondrial activity. Indeed, the action of AGM in MPT of isolated mitochondria explains its effect on cell proliferation and apoptosis.

## **ROLE OF TISSUE TRANSGLUTAMINASE IN NEURAL PLASTICITY IN AN EXPERIMENTAL MODEL OF EPILEPSY: EFFECT OF CYSTEAMINE**

**A. Campisi 1, S. Mancuso 2\*, M. Caruso 2, M. Maritati 1, G. Tringali 2  
A. Vanella 1, M. F. Serapide 3**

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Tissue transglutaminase(TG2) involvement in various neuronal diseases is well known, except for epilepsy. Previous works on TG2 expression showed an up-regulation of the protein in mice cerebral cortex (CC) and hippocampus (HP) in early stages and its decrease in late stages of kindling, an experimental model of epilepsy. In particular, in the early stages of kindling it is involved in apoptotic pathway activation, while in the late stages it translocates into the nucleus, where it exerts TK activity. To understand TG2 role during kindling, we evaluated TG2 expression in mice CC and HP treated 15 days before kindling with cysteamine, an inhibitor of TG2 expression. We also assessed its role in cell cycle progression. Comparing to the controls, in CC and HP of untreated-kindled mice, G0/G1 phase was reduced. S phase appeared almost doubled than control. G2/M phase diminished after 1 days, while slightly increased after 3 days. In CC and HP of cysteamine-treated mice G0/G1 phase reduced; moreover, S phase increased without progression towards G2/M phase after 3 days. The inhibition of TG-2 expression by cysteamine demonstrates TG2 key role in neural plasticity during kindling.

## **INTERACTIONS BETWEEN TRANSGLUTAMINASE 2 AND HEAT SHOCK PROTEINS IN CELL RESPONSE TO EXCITOTOXIC STRESS**

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Transglutaminase 2 (TG2) catalyzes the calcium-dependent formation of insoluble protein aggregates, characterized by (γ-glutamyl)-epsilon-lysine (GGEL) crosslinks, of disease-specific proteins, such as tau and amyloid-beta in AD, and α-synuclein in PD. Heat shock proteins (HSPs) provide the first line of defense against misfolded, aggregation-prone proteins. Interestingly, Hsp27 and Hsp20 have been reported to be in vitro cross-linked by TG2. In this study, we elucidate the possible interaction between TG2 and HSPs in neurodegeneration using a model of excitotoxic stress. In SH-SY5Y neuroblastoma cells, differentiated toward neurons, by retinoic acid, the basal levels of Hsp70 and Hsp27, were modified in NMDA-treated cells in function of time of exposure. These effects were counteracted in the presence of a specific TG2 inhibitor, demonstrating that enzyme activity is involved in HSP response to excitotoxic stress. Immunoprecipitation experiments evidenced the interaction between Hsp27 and TG2 in NMDA-treated cells. Also, an interaction between Hsp27 and Hsp20, as hetero-oligomeric complexes that are slightly affected by TG activity inhibition, could not be excluded.

## EFFECTS OF AGMATINE ON ROTENONE-INDUCED DAMAGE IN SH-SY5Y NEUROBLASTOMA CELLS

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Evidence has accumulated from several data demonstrating that in the brain agmatine exerts protective function against neurotoxic and ischemic injury (1). However, the sequence of events leading to protective effects and mode of action of agmatine to prevent cell damage have not been fully elucidated. Our experiments were designed to characterize the agmatine protective effects against oxidative stress produced by exposure to rotenone. RA-differentiated SH-SY5Y cells were exposed to 500 nM rotenone, in presence or absence of agmatine in a range concentration (50-500  $\mu$ M). The data from the MTT cytotoxicity assay demonstrated that agmatine (100  $\mu$ M) protected SH-SY5Y cells against rotenone toxicity. Contemporaneously, a significant reduction of rotenone-induced increase in ROS production was observed. We also demonstrated that agmatine can be able to antagonize rotenone effects through the reduction of NF-kappaB nuclear translocation. Our cumulative results may be considered as suggestive of neuroprotective effects of agmatine in neuronal damage associated with oxidative stress. 1. Halaris A. & Plietz J. (2007) *CNS Drugs*; 21, 885-900.

## SILENCING OF TG2 REDUCES THE $\beta$ -AMYLOID-INDUCED ACTIVATION OF HUMAN MONOCYTE THP-1 CELLS

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Several findings have shown that the increase in the expression of TG2 is associated with inflammatory diseases (1). Multiple physiological roles for TG2 have been demonstrated in various cell types, however its role in the inflammatory process is not yet clear. In order to characterize the TG2 role in the neuroinflammatory mechanisms, in this study we employed human monocytic THP-1 cells that have been accepted as a good model of monocytes/microglia cells. The exposure for 24 h to both  $\beta$ -amyloid(1-42) (200 nM) and LPS (100 ng/ml) produced a significant increase in TG activity. Moreover, mature macrophages stimulated by  $\beta$ -amyloid(1-42) elicited the production of IL-6 and TNF $\alpha$ . These effects were abolished by inhibition of TG activity by R283 a site-specific inhibitor of TG2. In addition, the reduction of TG2 expression by siRNA produced a strong depletion in TG2 expression, which was associated to a significant reduction in cytokine production. These data confirm that TG2 is involved in the inflammatory response, and increases in enzyme expression may be strongly associated with the pathogenesis of many inflammatory diseases. Kim S.Y., *Front Biosci.* 2006 ;11:3026-35

## **Neurosciences**

**Poster session:  
24/09/2009 (h. 14.00-15.00)**



## **GROUP IIA PHOSPHOLIPASE A2 (GIIA) IS INVOLVED IN NEURITOGENESIS IN NGF-TREATED PC12 CELLS**

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R. Donato§ and G. Goracci\***

Depts. Int. Med-Neurochem. Lab.\* , Exp. Med. Bioch. Sci. -Anatomy §, Clin. Med.-Imagine Lab.¶, University of Perugia

Neural cells express various low molecular weight (13-18 kDa) phospholipases A2 (sPLA2) isoforms which participate to cell signalling including neurodegeneration. Indeed, we have shown that GIIA is present in rat brain cortex mitochondria and it has been proposed its involvement in cell death (Macchioni et al., 2004). Another intracellular function of sPLA2 isoforms seems to be their participation to NGF-induced neuritogenesis. Masuda et al. (2005) provided evidences for the involvement of GX sPLA2 in this phenomenon. Preliminary results in our laboratory indicated that GIIA might also participate to neuritogenesis. Availability of specific antibodies is critical for investigating functional and pathological roles of PLA2 enzymes. Thus, a specific antibody against rat GIIA was produced in our laboratory. By using this antibody, we found that endogenous GIIA but not GX localizes to dendritic cones during NGF-induced neurite outgrowth. This has been confirmed by experiments with PC12 cells over-expressing myc-GIIA indicating that the newly synthesized enzyme is in part delivered to the site of neuritogenesis. Supported by Fondazione Cassa di Risparmio di Perugia Code 2008.021.321

## **REGIONAL REDOX PROTEOMICS ANALYSIS ON SENESCENT RAT BRAIN: INVOLVEMENT OF PROTEIN OXIDATION IN THE AGING PROCESS”**

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A. Giorgi, M.E. Schininà ,V. Calabrese\*°**

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Free radical-mediated oxidative stress plays a critical role in the age-related decline of cerebral functions as a result of the oxidation of proteins, nucleic acids and lipids. Because proteins are major components of biological systems and play an important role in a variety of cellular functions, an age-related increase in oxidative damage to proteins is considered one of the principal events involved in cellular impairment during aging. In the present study we identified, by a redox proteomics approach, oxidatively modified proteins in four brain regions generally affected by age-related neurodegeneration, i.e. hippocampus, cortex, cerebellum and striatum. Senescent (28 months old) rats in comparison with adult (12 months old) rats exhibited higher levels of protein oxidation, particularly for bioenergetic proteins involved in energy transduction processes, such as pyruvate kinase, ATP synthase, aldolase, creatine kinase and  $\alpha$ -enolase. The oxidative modification of these enzymes likely leads to their inactivation. These results further indicate that oxidative damage and decreased energy production are characteristic hallmarks of neurodegenerative processes associated to aging.

## **MODEL FOR INVESTIGATING NSAIDS WITH A POTENTIAL APPLICATION IN ALZHEIMER DISEASE**

**Agata Campisi<sup>1</sup>, Livia Basile<sup>2</sup>, Giuseppina Raciti<sup>1</sup>, Rosaria Acquaviva<sup>1</sup>  
Rosario Pignatello<sup>2</sup>, Giovanni Puglisi<sup>2</sup>,**

<sup>1</sup>Dip. di Chimica Biologica, <sup>2</sup>Dip. di Scienze Farmaceutiche Università degli Studi di Catania, Catania, Italy

Non-steroidal anti-inflammatory drugs (NSAIDs) have received attention for their possible role in the therapy of Alzheimer disease (AD). It has been reported that the conjugation of drugs to lipoamino acids moieties (LAA) could represent a new possible strategy to change the pharmacokinetic profile of drugs and also to improve their penetration into the brain tissues. The aim of this research was to realize and validate a novel model for the in vitro evaluation and screening of drugs and prodrugs with a potential interest for AD treatment. In particular, we assessed the effect of two NSAIDs, Naproxen (NAP) and R-Flurbiprofen (R-FLU), on excitotoxicity induced by glutamate in primary rat astroglial cell cultures. Furthermore, we evaluated if was able to counteract the up-regulation of tissue transglutaminase (TG2) induced by the neurotransmitter. Our preliminary experimental data demonstrate that NAP an R-FLU counteract the excitotoxicity induced by glutamate, restoring also the levels of TG2. The overall aim of the study is then to use this model to assay prodrugs of these and other drugs with LAA, as well as other derivatives of potential pharmaceutical interest.

## **DNA FRAGMENTATION IN PRIMARY RAT ASTROGLIAL CELL CULTURES EXPOSED TO LOW INTENSITY 900 MHZ ELECTROMAGNETIC FIELDS**

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People exposure to electromagnetic fields (EMF) has increased in the last decades, mainly due to the rapid rise in the use of mobile communications, but also to electrical power lines and electric household appliances. At radiofrequency waves, the thermal effects of EMF exposure, due to temperature increase by direct energy transfer to the biological samples, are considered as the only effects that can induce health issues. Instead it is controversial the occurrence of non thermal effects, caused by low intensity EMF exposure, and the possible consequences on the human health. The head and brain are usually the most exposed targets of RF appliances, so we assessed in primary rat neocortical astroglial cell cultures the effect of low intensity exposure to modulated and not modulated 900 MHz EMF. We found that low intensity modulated EMF induces, by non thermal effect, excitotoxicity in primary rat astroglial cell cultures, leading to the DNA fragmentation. These results point out that the electromagnetic fields used for telecommunications play an important role in astrocytes activation, which is involved in several acute and chronic neurodegenerative diseases.

## TARGETING ABETA WITH LIPID NANOPARTICLES

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M. Gobbi and M. Masserini**

DIMS, University of Milano-Bicocca, Monza; Mario Negri Institute, Milano; Hopital Salpetriere, Paris

The aim of this research, within the FP7 project “NAD, Nanoparticles for therapy and diagnosis of Alzheimer Disease”, is to create nanoparticles (NPs) able to bind Abeta peptide, playing a central role in Alzheimer Disease. METHODS: Human Abeta 1-42 was from Sigma. Liposomes of 100nm diameter were prepared by extrusion and composed of cholesterol/sphingomyelin (Chol/Sm:1/1) and glycerophospholipids or glycosphingolipids. The interaction between liposomes and Abeta was assessed using ultracentrifugation on a density gradient, followed by ELISA assay and by Surface Plasmon Resonance (SPR); interaction between fluorescently labeled Liposomes and Abeta plaques of autaptic Alzheimer Disease brain by confocal microscopy. The effect on Abeta aggregation was evaluated by AFM. RESULTS: Our results provide evidence that LIP composed of Chol/Sm/ anionic phospholipids display an apparent high affinity for Abeta with Kd value in the nanomolar range. Moreover, LIP containing phosphatidic acid or cardiolipin seem to specifically label plaques in Alzheimer brain and to inhibit the formation of fibrils from Abeta monomers. The liposome formulations have been patented

## CATASTROPHIC NAD<sup>+</sup> DEPLETION THROUGH NAMPT INHIBITION IN ACTIVATED T LYMPHOCYTES REDUCES DEMYELINATION AND DISABILITY IN EAE

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E. Moran 4, A. Uccelli 2, and A. Nencioni 4**

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Nicotinamide phosphoribosyltransferase (Nampt) inhibitors such as FK866 are potent inhibitors of NAD<sup>+</sup> synthesis used for the treatment of different forms of cancer. Based on Nampt upregulation in activated T lymphocytes, we investigated whether FK866 interferes with T lymphocyte function and survival. We show that activated, but not resting, T lymphocytes undergo massive NAD<sup>+</sup> depletion upon FK866 mediated Nampt inhibition. As a consequence, impaired proliferation, reduced IFN-gamma and TNF-alpha production, and cell death result. We demonstrate that upregulation of the of the NAD<sup>+</sup>-degrading enzyme poly-(ADP-ribose)-polymerase by activated T cells enhances their susceptibility to NAD<sup>+</sup> depletion. We also relate defective IFN-gamma and TNF-alpha production in response to FK866 to impaired Sirt6 activity. Finally, we show that FK866 strikingly reduces the neurological damage and the clinical manifestations of experimental autoimmune encephalomyelitis (EAE), a model of T-cell mediated autoimmune disease. In conclusion, Nampt inhibitors could be used to modulate T cell-mediated immune responses and thereby be beneficial in immune-mediated disorders.

## NEUROPROTECTIVE EFFECT OF A NEW SIGMA RECEPTOR LIGANDS CIS-(+)AND CIS(-)-PPCC.

**O. Prezzavento \***, **A. Campisi §**, **C. Parenti #**, **A. Marrazzo \***, **E. Arena \***, **S. Ronsisvalle \***  
**G. Aricò #**, **A. Vanella §**, **G.M. Scoto #**, **G. Ronsisvalle \***

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Sigma receptor is a distinct class of proteins with a widespread distribution in the central nervous system (CNS) and peripheral tissues. In previous studies performed in primary rat astroglial cell cultures, we observed that cis-(±)-methyl (1R,2S/1S,2R)-2-[(4-hydroxy-4-phenylpiperidin-1-yl)methyl]-1-(4-methylphenyl) cyclopropanecarboxylate [(±)-PPCC], a sigma selective ligand, was able to modulate the calcium-dependent protein tissue transglutaminase (TG2). Here we characterized the pure enantiomers cis-(+) and cis(-)-PPCC, assessing, both in in vitro and in vivo studies, their neuroprotective effects and pharmacological activity. Our results demonstrated that the single enantiomers possess a protective effects on glutamate excitotoxicity in primary astroglial cell cultures: indeed the pre-treatment with (+)-pentazocine, a putative sigma-1 agonist, (+)-PPCC or (-)-PPCC restored GSH and ROS levels to control values. Furthermore they reduced TG-2 up-regulation induced by glutamate. Since an anti opioid effect was also observed on KOP-mediated analgesia, we suggested that the single enantiomers could act as sigma-1 agonists.

## SEX DIFFERENCES IN NEUROACTIVE STEROID LEVELS IN THE NERVOUS SYSTEM OF DIABETIC AND NON-DIABETIC RATS

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**R.C. Melcangi b**, **D. Caruso a**

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Neuropathy and encephalopathy represent important complications of diabetes. Recent observations have suggested that, in male rats, neuroactive steroids are protective agents and that their levels in peripheral and central (CNS) nervous system are strongly affected by the disease. It is interesting to highlight that incidence, progression and severity of neurodegeneration are different in the two sexes. To this aim, we have evaluated the levels of neuroactive steroids in different CNS regions and in the sciatic nerve of control and diabetic (i.e., induced by streptozotocin, STZ) male and female rats. Data obtained by liquid chromatography–tandem mass spectrometry indicate that the levels of neuroactive steroids show sex and regional differences in control animals. STZ-induced diabetes resulted in a strong general decrease in neuroactive steroid levels. In addition, the effects of diabetes on neuroactive steroid levels also show sex and regional differences. These findings may have strong implications in the comprehension of the mechanisms involved in neurodegenerative processes and for the development of sex-oriented therapies aimed at the treatment of diabetic neurodegeneration

## **DYSFUNCTIONS IN N-CAM AND UNEXPECTED ACCUMULATION OF PSA-NCAM IN BRAIN OF AUTOSOMAL DOMINANT ADULT ONSET LEUKODYSTROPHY (ADLD)**

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In brain white matter (BWM) of two subjects with orthochromatic ADLD we described defective L-MAG and patchy distribution of myelin restricted to the elder one. L-MAG and N-CAM (namely N-CAM 180, 140, and 120) are structurally related and concur to myelin/axon interaction. In early developmental stages, in neurons and glia N-CAM is converted into PSA-NCAM by two sialyltransferases STX and PST. PSA-NCAM disrupts N-CAM adhesive properties and of note it is nearly absent in the adult brain. Here, both subjects were evaluated in BWM extracts and myelin for N-CAM and PSA-NCAM expression, and in BWM for N-CAM, STX and PST gene copy number and gene expression as mRNA. Biochemically we disclosed an unexpected accumulation of PSA-NCAM along with almost unvaried N-CAM 140, increased N-CAM 180 and decreased N-CAM 120; no duplication of genes encoding N-CAM, STX and PST was observed, whereas PST mRNA was clearly enhanced. Immunohistochemically, in BWM of both subjects an unusually diffuse accumulation of PSA-NCAM without inflammation markers was unveiled. PSA-NCAM persistence, up-regulated PST mRNA and previously uncovered defective L-MAG, may be early pathogenetic events in this ADLD form.

## **THE EXPRESSION PATTERN OF TAR DNA-BINDING PROTEIN 43 (TDP-43) IN PERIPHERAL BLOOD MONONUCLEAR CELLS (PBMC) IN INDIVIDUALS AFFECTED BY AMYOTROPHIC LATERAL SCLEROSIS (ALS) AND THEIR HEALTHY RELATIVES.**

**G. De Marco<sup>1,2</sup>, M. Piccinini<sup>1</sup>, E. Lupino<sup>1</sup>, A. Lomartire<sup>1</sup>, B. Buccinnà<sup>1</sup>, C. Ramondetti<sup>1</sup>  
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TDP-43 is a ubiquitous prevalently nuclear 43 kDa protein interacting with RNA and DNA. In brain cortex (BC) of patients with familial ALS and TDP-43 gene mutations (fALS+) or sporadic ALS without gene mutations (sALS-) TDP-43 is sequestered in the cytoplasm within insoluble inclusions. Recently, by biochemical and immunohistochemical procedures, we showed that in sALS- TDP-43 accumulates in BC cytoplasm not only within insoluble inclusions but also as soluble inclusion-free species, including the 43kDa protein and low molecular mass fragments. Here we focused on TDP-43 distribution, evaluated by western immunoblotting, in the cytoplasmic and nuclear fractions (CF and NF) of PBMC from healthy (CTRL), sALS-, fALS+ and fALS+ relatives. In NF of all cases TDP-43 was quantitatively comparable and had the expected molecular mass; in CF it was represented by two isoforms of about 43 and 41kDa respectively, both less appreciable than the nuclear form, that were quantitatively comparable in CTRL and sALS-, whereas one of the two forms prominently accumulated in fALS+ and in some of their healthy relatives. This feature might be an easily detectable marker of ALS even not yet manifest.

## **SERINE BASE EXCHANGE ENZYME IN TRITON-X100 INSOLUBLE FLOATING FRACTIONS FROM RAT CEREBRAL CORTEX**

**S. Buratta, G. Ferrara and R. Mozzi**

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Phosphatidylserine (PS), strictly required for PKC activity, is synthesized in mammals by serine base exchange enzyme (SBEE). The coexistence of SBEE and PKC in Triton insoluble floating fractions (TIFFs) from rat cerebellum led us to suggest that SBEE could play a role in signal transduction, modulating PS level in the binding area for PKC(1). Results of this study support this hypothesis. Indeed, a similar colocalization has been found in TIFFs from cerebral cortex of 60 day old rats and in those prepared from cerebrocortical plasma membranes. The assay of SBEE in the presence or absence of unlabelled ethanolamine or choline demonstrated that TIFF-PS synthesis was mainly due to an enzyme able to utilize serine and ethanolamine (SE-SBEE) but not choline as free exchanging base. This isoform (rev 2) could convert PS into phosphatidylethanolamine and vice versa. No SBEE activity was found in TIFFs from cerebral cortex of 30 days old rats. From preliminary results, the age 33-40 days is crucial for the appearance of SBEE activity, and in particular of the SE-SBEE, in TIFFs from cerebral cortex. 1) Buratta et al (2007) J Neurochem 103, 942 2) Mozzi et al (2003) Neurochem Res 28, 195

## **FACTORS AFFECTING PLASMA SEROTONIN AND TRYPTOPHAN IN DICENTRARCHUS LABRAX**

**A.M. Ferlazzo, G. Bruschetta, S. Campo\*\*, P. Di Pietro, E. Fazio\*, F. Marino\*\*\*, P. Medica\***

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Hypoxia and temperature stress frequently occurred in aquatic environments and intensive rearing density may affect fish metabolism and welfare. Serotonin (5-HT) that integrate nervous, behavioural and neuroendocrine stress-response also in fish has not been detected in all fish trombocytes (1). In this study plasma 5-HT with tryptophan (Try) were measured by HPLC (electrochemical detector) in *Dicentrarchus labrax*, in fishes caught from off-shore cages subjected to different handling stressors. Fish (N.30, mean weight 230 g) were transferred to a tank of water (150 l). Sampling was done without anaesthetic at pH 7,5, T 18°C (Group I), with MS 222 (50 mg/l): at pH 6,2, T 22°C (Group II), at pH 6,2, T 30°C (Group III). 5-HT plasma levels measured in fish of different size (N.30, 70-230 g) without anaesthetic is hardly detectable. Time exposure to MS-222 (2-10 min) seems to have any effect on 5-HT levels. Both plasma 5-HT and Try levels are strongly increasing in Group III vs. Group I, II (P<0,001) probably suggesting the presence of an acute stress-response to variation of hypoxia, temperature, pH, confinement. 1. Maurer-Spurej E. (2005) Cell. Mol. Life Sci. 62, 1881-1889

## **ROLE OF PARP-1 AND PARP-2 IN CELL DEATH AND MODULATION OF THEIR EXPRESSION BY NEUROPROTECTIVE AGENTS IN PRIMARY RAT ASTROCYTES AND OLIGODENDROCYTES**

**V. Spina Purrello, S. Giliberto, V. Barresi, V. G. Nicoletti, E. Rizzarelli, and A. M. Giuffrida Stella.**

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PARPs play an important role in cell cycle regulation, genome stability, apoptotic cell death. Seventeen PARP family members have been identified, with PARP-1 and PARP-2 sharing the highest homology (69%). We examined the effects of L-carnosine and trehalose on PARP-1 and PARP-2 expression through PCR and Western analysis, in primary rat astroglial and oligodendrocyte cells, treated with lipopolysaccharide (LPS) and interferon gamma (INF $\gamma$ ) to induce stress conditions with or without carnosine or trehalose. After 24 h of LPS and INF- $\gamma$  treatment, we observed an increase of nitrite production and LDH release and a decrease of cell viability (MTT). Carnosine or trehalose treatment decreased nitrite and LDH release and increased cell viability. Due to the NO binding activity of carnosine, such effect is attributable to a down-regulation of PARP-1 and PARP-2 expression by carnosine and trehalose treatment under stress conditions. These data suggest that besides their antioxidant role, carnosine and trehalose may also modify the pathways regulated by PARPs. To further verify our hypothesis we are investigating the role of these molecules in neurodegenerative disorders.

## **EFFECT OF $\alpha$ LIPIC ACID ON GFAP, VIMENTIN, NESTIN, CYCLIN D1 AND MAP-KINASE EXPRESSION IN GLUTAMATE-PRETREATED ASTROCYTE CULTURES**

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We studied the effect of  $\alpha$ -lipoic acid (R+)enantiomer or raceme on the expression, by Western blot and ELISA analysis, of GFAP, vimentin, nestin, cyclin D1 and MAP-kinase in 15 DIV chronic or acute 100  $\mu$ M  $\alpha$ -lipoic acid-treated astrocyte cultures pretreated with 5 mM glutamate for 24h. GFAP expression significantly increased after (R+)enantiomer acute-treatment and also in glutamate-pretreated ones. R(+)enantiomer acute-treatment increased vimentin expression, but it decreased after raceme acute-treatment. Nestin expression drastically increased after acute raceme-treatment in glutamate-pretreated or unpretreated cultures, but significantly decreased after (R+)enantiomer acute and chronic-treatments. Cyclin D1 expression much increased in raceme acute-treated astrocyte cultures pretreated with glutamate. MAP-kinase expression slightly increased after (R+) enantiomer acute treatment with in glutamate-pretreated or unpretreated ones. Immunostaining analysis are well correlated with Western blot and ELISA data. Finally, our findings may represent a "tool" to better clarify the antioxidant and metabolic role played by lipoic acid in proliferating-differentiating astrocyte cultures

## **ENERGETIC METABOLISM OF MYELINATED AXONS: A NEW CORRELATION AMONG AXONAL DEGENERATION, DEMYELINATION, AND ENERGY SUPPLY**

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During demyelinating diseases, the loss of myelin does not simply cause a lowering of speed of conduction but also an axonal necrosis, as in Multiple Sclerosis (MS) or Charcot Marie Tooth 1a disease (CMT1a), but the mechanism is poorly understood. Some Authors hypothesize that axonal degeneration depends on energy depletion. We have demonstrated that myelin sheath is a site of aerobic respiration, producing ATP. This suggests that myelin sheath not only surrounds the axon, but supplies it, through energy production. We have observed that the level and the functionality of redox chain are less in myelin extracted from MS plaque and CMT1A sciatic nerve, than in healthy myelin and that the loss is directly proportional to the degree of demyelination. These data may correlate the neuronal MS symptoms to an ATP depletion of the axon, helping to shed light on the etiopathogenesis of demyelinating diseases. So we hypothesize that the demyelination implies a dramatic ATP depletion and consequent loss of functionality, axon survival and cytoarchitecture, which may be the biochemical mechanism leading to axonal degeneration, suggesting a new neuro-trophic role for the sheath.

## **COMPUTER-AIDED DRUG-DESIGN OF COMPETITIVE INHIBITORS OF L-DOPA-DECARBOXYLASE A TARGET FOR THE TREATMENT OF PARKINSON'S DISEASE**

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Parkinson's Disease (PD) is a neurodegenerative disease, which involves the loss of dopaminergic neurons of the substantia nigra. Modern treatment of PD is based on L-DOPA, which is transformed by the Pyridoxal-5'-phosphate (PLP) dependent enzyme L-DOPA-decarboxylase (DDC) into dopamine in dopaminergic neurons. L-DOPA is administered together with a DDC-inhibitor (e.g. Carbidopa) unable to pass the blood-brain barrier, in order to increase central concentration of L-DOPA and prevent the side effects caused by its peripheral metabolism. However, Carbidopa reacts irreversibly both with free PLP and other PLP-dependent enzymes, causing diverse side effects. The aim of this work is to develop new, more selective and competitive inhibitors of DDC by means of computer-assisted techniques. Pharmacophore modeling and molecular docking were used in a structure and ligand based approach to filter a ~8 million compounds database and select potential inhibitors of DDC; the identified compounds will be then experimentally tested.

## EXPRESSION OF GAD ISOFORMS AND NEUROACTIVE AMINO ACID LEVELS IN MOUSE BRAIN AREAS: EFFECTS OF PENTYLENETETRAZOLE AND MINOCYCLINE

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Pro-inflammatory and anti-inflammatory molecules are synthesized in glial cells during epileptic activity in those brain areas, where seizures initiate and spread. Minocycline (MIN), a semi-synthetic, second-generation tetracycline analogue, in addition to its own antibacterial properties, exerts neuroprotective effects in various experimental models. The neuroprotective role of MIN has not been investigated in animal models of epilepsy. In this study, we investigated whether MIN is neuroprotective against pentylenetetrazole (PTZ)-induced seizure in mice and measured the levels of some neuroactive amino acids by HPLC and the expression of GAD65 and GAD67 isoforms by Western blotting. MIN was able to antagonize PTZ-induced seizure with an ED<sub>50</sub> of 2.31 (1.25-4.27) mg/kg. Administration of PTZ led to an increase of GABA and glutamate in the cortex and a reduction in the hippocampus. Instead, the administration of MIN alone increased GABA and glutamate in both areas. Both GAD isoforms were increased by MIN and unmodified by PTZ in most brain areas studied. In conclusion, MIN shows good anticonvulsant properties in this animal model and the increase in GAD65 might underlie this effect.

## IN-VITRO EVALUATION OF NEW BENZOMORPHAN-BASED LIGANDS

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°G. Maria Scoto, #Z. Georgoussi \*G. Ronsisvalle**

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Unrelieved cancer pain significantly decreases the quality of life of patients. Cancer pain is a complex symptom associated with a range of diseases and is particularly difficult to treat effectively. In an first screening on benzomorphan-based compounds, LP1 was found to have affinity for  $\mu$  and  $\delta$  receptor in nanomolar range ( $K_{i\mu} = 0.83$  nM and  $K_{i\delta} = 29,1$  nM, respectively). Moreover, in tail flick test LP1 showed an analgesic effect comparable to morphine. In chronic subcutaneous administration, LP1 maintained its analgesic profile until the eighth day while chronic morphine administration determined a significant loss of analgesic effect already at the third day of treatment. These results indicate that LP1 could be a new long-acting opioid compound with lower tolerance development. To evaluate its functional activity profile, LP1 was tested in the mouse vas deferens (MVD) isolated tissue assays and in [<sup>35</sup>S]GTP $\gamma$ S binding assay.



## **Mitochondria**

**Poster session:  
24/09/2009 (h. 14.00-15.00)**



## **HISTONE DEACETYLASES PARTICIPATE IN THE REGULATION OF MITOCHONDRIAL BIOGENESIS IN C2C12 CELLS**

**Emma De Fabiani 1, A. Galmozzi1, N. Mitro1,3, F. Gilardi1, D. Rotili2, S. Valente2, A. Mai2, M. Crestani1**

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The coactivator of gene transcription PGC-1 $\alpha$  is one of the key regulators of mitochondrial biogenesis and of energy metabolism. Since PGC-1 $\alpha$  activity can be modulated by Histone Deacetylases (HDACs), the aim of this work was to study the mechanisms underlying the effects of known HDAC pan-inhibitors (HDACi) in a model of muscle fibers, which play a crucial role in energy metabolism. Treatment of C2C12 cells with HDACi induces the expression of PGC-1 and its target genes. Consistently with this, we observed increased mitochondrial density, activity and DNA content, most likely linked to  $\alpha$ -dependent activation of gene transcription. We also found PGC-1 increased active form of AMPK and upregulation of GLUT4 mRNA level, which is associated with improved insulin-stimulated glucose uptake. Results obtained with new class-specific inhibitors suggest that both class I and II HDAC are involved in these processes, although selective inhibition differently affects gene expression and mitochondrial biogenesis, through mechanisms that still need to be investigated and that can be exploited for the treatment of metabolic diseases. (Funded by EC grant LSHM-CT-2006-037498 SOUTH)

## **CYTOCHROME C (CYT C) REDOX STATE AND RELEASE FROM BRAIN MITOCHONDRIA: A H<sub>2</sub>O<sub>2</sub>-CARDIOLIPIN INDEPENDENT MECHANISM**

**Magdalena Makedonschi, Lara Macchioni, Ermelinda Francescangeli and Lanfranco Corazzi**

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Cyt c promotes the early stages of apoptosis if released outside mitochondria. Its anchorage to IMM requires a cardiolipin (CL) acyl chain inserting into the protein. In cyt c-CL liposomes, phosphate (Pi) and fatty acids (FA) breach lipid-protein interactions. Pi caused a higher release of the reduced protein, compared to oxidized, suggesting a stronger anchorage of cyt c in its Fe<sup>+3</sup> form. FA caused transition from low to high spin Fe<sup>+2</sup> heme spectrum. The propensity to form FA-protein complexes was higher for reduced cyt c, with palmitate

## MULTIFACTORIAL IMPAIRMENT OF MITOCHONDRIAL OXIDATIVE PHOSPHORYLATION IN HUMAN SKIN FIBROBLASTS WITH CHROMOSOME 21 TRISOMY

**Daniela Valenti, Riccardo S. Merafina, A. Gabriella Manente\*, Ersilia Marra and Rosa Anna Vacca**

Istituto di Biomebrane e Bioenergetica, CNR, Bari, Italy; \*DISCAFF Università del Piemonte orientale A. Avogadro, Novara

A central role for mitochondrial dysfunctions has been proposed in the pathogenesis of Down syndrome (DS), a multifactorial disorder caused by trisomy of chromosome 21. Mitochondrial respiratory function and ATP synthesis through oxidative phosphorylation in fibroblasts from subjects with DS (DS-HSF) has been thoroughly investigated. We found in DS-HSF a multifactorial impairment of oxidative phosphorylation machinery which involves respiratory-chain complex I, ATPase, the ADP/ATP translocator and adenylate kinase. In spite of complex I, ADP/ATP translocator and adenylate kinase reduced activities, their gene expression was unchanged and protein content increased whereas, F1ATPase amount was found significantly decreased. The observed increase in the amount of mitochondrial proteins was due to an increase in the number of mitochondria in DS-HSF, perhaps to compensate the deficit of mitochondrial functions. Measurements of reactive oxygen species (ROS) revealed accumulation in mitochondria of DS subjects of both H<sub>2</sub>O<sub>2</sub> e O<sup>•-</sup> produced by the defective complex I. ROS act as signaling molecules mediating the increase of mitochondrial mass.

## BIOGENESIS OF MITOCHONDRIAL CARRIER PROTEINS: ROLE OF THE CHAPERONES HSC70 E HSC90

**Alessandra Ferramosca\*, Vincenzo Zara\*, Philippe Robitaille-Foucher°  
Ferdinando Palmieri\*\* and Jason C. Young°**

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Metabolite carrier proteins carry targeting signals to mitochondria in their transmembrane domains. In addition, some carrier proteins possess cleavable presequences which are dispensable for mitochondrial targeting, but have some other function before import. The cytosolic chaperones Hsc70 (heat-shock cognate 70) and Hsp90 (heat-shock protein 90) bind to carrier precursors and interact specifically with the Tom70 import receptor to promote import. In this study, we examined the interactions of the mature and precursor forms of PiC (phosphate carrier) and CIC (citrate carrier), and for comparison, of OGC (oxoglutarate carrier), which naturally lacks a presequence, with the cytosolic chaperones. The binding of each protein to Hsc70 and Hsp90 was tested in relation to solubility before import. The function of chaperones in import was analysed by inhibition of Hsc70 and by competition of Tom70 targeting using an Hsp90 fragment. We found that the presequences of PiC and CIC improve import competence by different mechanisms, as PiC provides a binding site for a particular chaperone, Hsc70, and CIC reduces the aggregation of the polypeptide independent of any external chaperone activity.

## **N-3 AND N-6 POLYUNSATURATED FATTY ACIDS DIFFERENTLY AFFECT CITRATE CARRIER PROMOTER ACTIVITY**

**Fabrizio Damiano, Simone Alemanno, Eleonora Stanca, Luisa Siculella and Gabriele Vincenzo Gnoni**

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Citrate carrier (CiC), a mitochondrial membrane protein, plays an important metabolic role by transporting, in the form of citrate, acetyl-CoA from mitochondria into the cytosol for fatty acid and cholesterol synthesis. A PUFA (polyunsaturated fatty acids) response region, composed of a NF-Y site, an E-box like site, a SRE1 like site and four Sp1 sites, has been identified within the CiC promoter. Transcription factor SRE-Binding Protein-1 (SREBP-1c) is target for PUFA down-regulation of CiC transcription. Transfection and gel mobility shift assays indicated that a functional E-box like confers responsiveness to SREBP-1c. In H4IIE cells overexpression of SREBP-1c overrides arachidonic acid suppression but does not prevent the repression by docosahexaenoic acid. ChIP assay showed that docosahexaenoic acid affects the binding of NF-Y, Sp1 and SREBP-1 to PUFA response region whereas arachidonic acid alters only the binding of SREBP-1. PUFA inhibition of CiC gene transcription is mediated not only by the SREBP-1c but might also involve a reduction in Sp1 and NF-Y DNA binding, suggesting differential mechanisms in the CiC gene regulation by different PUFA.

## **KINETIC CHARACTERIZATION OF A NOVEL COPPER AMINE OXIDASE ACTIVITY FROM RAT LIVER MITOCHONDRIA MATRIX**

**Sara Cardillo, A. De Iuliis, V. Battaglia, G. Sinigaglia, M. A. Grillo, M. Magro, A. Toninello  
R. Stevanato and F. Vianello**

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The present study reports preliminary results on the presence of a novel Copper containing amine oxidases (Cu-AO, EC 1.4.3.6.) in rat liver mitochondria lysates. Such enzymatic activity was found in the soluble mitochondrial fraction, obtained by simple osmotic shock. The enzyme was isolated by a new procedure based on the binding on iron oxide nanoparticles chemically modified with an enzyme substrate. SDS-PAGE showed a single band at about 60 kDa. The crude enzyme activity was tested by spectrophotometric measurements, determining hydrogen peroxide production following oxidative deamination of different substrates, such as polyamines (spermine, spermidine, putrescine and cadaverine) and monoamines (dopamine and benzylamine). The highest activity was found with 1,2-diamino-ethane and the highest affinity with 1,5-diamino-pentane. The enzyme was preliminary kinetically characterized, using spermine, spermidine and putrescine as substrates, as a function of ionic strength.

## **SEROTONIN INTERACTIONS WITH RAT LIVER MITOCHONDRIA AND ITS EFFECTS ON BIOENERGETIC FUNCTIONS**

**Silvia Grancara, Valentina Battaglia, Mario Mancon, Maria Angelica Grillo, Renzo Deana and Antonio Toninello**

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Serotonin is a biogenic amine known as a neurotransmitter, but also involved in various processes of peripheral tissues, including cell proliferation and differentiation, and liver regeneration. Catabolism of serotonin is mediated by the mitochondrial monoamine oxidase, with the generation of hydroxyindolic acid and H<sub>2</sub>O<sub>2</sub>, which plays a central role in inducing mitochondrial and cellular damages like cardiomyocyte death and steatohepatitis. Considering the effects in liver and that serotonin is degraded in this organ, the aim of this study is to clarify the involved mechanisms. Results show that serotonin does not affect the  $\Delta\Psi$  of RLM or their capacity to synthesize ATP. Indeed, it is able to amplify the mitochondrial permeability transition (MPT), induced by high Ca<sup>2+</sup> levels. The mechanism of this effect is doubtful but it seems linked to an oxidative stress, as suggested by the observed thiol oxidation. H<sub>2</sub>O<sub>2</sub> by interacting with the pore forming structures, already predisposed by the action of Ca<sup>2+</sup>, should be responsible for the increased extent of MPT. The observed effects suggest a significant role of serotonin in the amplification. Human sialidase NEU4 long and short are extrinsic proteins bound to outer mitochondrial membrane and the endoplasmic reticulum, respectively

## **HUMAN SIALIDASE NEU4 LONG AND SHORT ARE EXTRINSIC PROTEINS BOUND TO OUTER MITOCHONDRIAL MEMBRANE AND THE ENDOPLASMIC RETICULUM, RESPECTIVELY**

**A. Bigi, L. Morosi, C. Pozzi, M. Forcella, G. Tettamanti, B. Venerando, E. Monti and P. Fusi**

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Sialidases are widely distributed glycohydrolytic enzymes removing sialic acid from glycoconjugates. In mammals, several sialidases with different subcellular localizations and biochemical features have been described. NEU4, the most recently identified member of the human sialidase family, is found in two forms, NEU4 long and NEU4 short, differing in the presence of a 12 aminoacid sequence at the N-terminus. Contradicting data are present in the literature about the subcellular distribution of this enzyme, their membrane anchoring mechanism being still unclear. In this work we investigate human NEU4 long and NEU4 short membrane anchoring mechanism and their subcellular localization. Protein extraction with Triton X-114 and Na carbonate and cross-linking experiments demonstrate that both forms of NEU4 are extrinsic membrane proteins, anchored via protein-protein interactions. Moreover, through immunofluorescence and subcellular fractionation, we show that the long form localizes in mitochondria, while the short form is associated with the endoplasmic reticulum. Finally, mitochondria subfractionation experiments suggest that NEU4 long is bound to the outer mitochondrial membrane.

## **BACULOVIRUS EXPRESSION SYSTEM AS VERSATILE TOOL FOR MITOCHONDRIAL CARRIER EXPRESSION**

**1 M. Madeo, 2 C. Carrisi, 1 D. Iacopetta, 2 L. Capobianco, 1 A.R. Cappello, 1 R. Curcio  
3 F. Palmieri, and 1 V. Dolce**

1Dep. of Pharmaco-Biology, Calabria University; 2DiSTeBA, Salento University; 3Dep. Pharmaco-Biology, Bari University

Heterologous expression of recombinant proteins is an essential technology for protein characterization. A major obstacle to investigating the structure and the biochemical properties of membrane proteins is the difficulty in obtaining sufficient amount of functional protein. Here we report the successful expression and characterization of the tricarboxylate (or citrate) carrier (CIC) from eel (*A. anguilla*) liver mitochondria, by baculovirus expression system. CIC is one member of the mitochondrial carrier family proteins, that transport different metabolites across the inner mitochondrial membrane. Our results show firstly, a correct subcellular localization of recombinant CIC, so demonstrating that this expression system is able to target the expressed protein to the appropriate cellular compartment. Secondly, we establish that the characteristic functional properties of the purified recombinant CIC are the same of those determined for eel liver mitochondrial CIC. Therefore, it is likely that this procedure can be applied successfully to other mitochondrial transport proteins, thus providing enough protein quantity for functional characterization and structural studies.

## **PGC-1ALPHA-DEPENDENT PATHWAY FOR MITOCHONDRIAL BIOGENESIS ACTIVATION IS UPREGULATED IN TYPE I ENDOMETRIAL CANCER.**

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An increase of mitochondrial DNA (mtDNA) and mtDNA mutations have been found in type I endometrial carcinoma but up to now the role of mitochondrial biogenesis in this cancer has never been investigated. The aim of this work was to determine if there is a change of mitochondrial biogenesis in this type of cancer. Therefore, we measured in samples of endometrial carcinoma and in proliferative endometrium used as control: 1) the mtDNA/nDNA content, 2) the citrate synthase activity as a measure of mitochondrial mass, 3) the expression of PGC-1 $\alpha$ , of NRF-1 and of TFAM proteins. A 2-fold increase of mtDNA content and mitochondrial mass were found in endometrial carcinoma compared to normal endometrium. In cancer endometrial tissue the doubling of mitochondrial mass and mtDNA content were associated to the doubling of TFAM expression level as well as to a 1.5-fold increase of PGC-1 $\alpha$  and NRF-1 expression. This suggests that the mitochondrial biogenesis increased in type I endometrial carcinoma through the upregulation of PGC-1 $\alpha$ -dependent pathway. Acknowledgements This project is co-funded by Fondazione Cassa di Risparmio di Puglia.

## **“IN SILICO” STUDIES ON TOM40: STRUCTURAL RELATIONSHIPS AMONG MITOCHONDRIAL OUTER MEMBRANE BETA-BARRELS.**

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The biological process of the protein translocation through the mitochondrial outer membrane is fascinating for its highly coordinated biomolecular mechanisms. In particular, the main component of this process is the protein Tom40 which allows the transport of all the precursors of the mitochondrial proteins encoded in the nucleus. At the moment, structural informations about the TOM complex are missing and this hampers the explanation of the available physiological data at a mechanistic level. On the other hand, in a recent paper (Bayhuber et al. 2008) a structural similarity was proposed between Tom40 and VDAC (Voltage Dependent Anion Channel), one of the most abundant protein of the mitochondrial outer membrane. Consequently, by means of homology modeling and using the X-ray structure of VDAC1 (PDB:3EMN) as a template, we created 6 Tom40 models of the most representative organisms for the three kingdoms Animalia, Plantae and Fungi. Finally, considering the structure of the aldolase presequence (PDB:2V1T) and through protein-protein docking studies, we have also sketched a model of the Tom40-presequence complex in order to indicate a likely protein translocation mechanism.

## **IDENTIFICATION OF PUTATIVE TRANSCRIPTION PROMOTERS IN SEA URCHIN MITOCHONDRIAL DNA**

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Dipartimento di Biochimica e Biologia Molecolare “E. Quagliariello”, Università di Bari, Italia

The mitochondrial transcription factor TFAM is central to the assembly of the mitochondrial transcription complex. It binds DNA at the promoters and recruits the proteins TFB1/2M and RNA polymerase. We cloned and expressed TFAM cDNA from sea urchin mitochondria. The protein is 237 residues long, shares a significant homology with the human counterpart and displays two tandem HMG box domains. Interestingly, the C-terminal tail, which in vertebrate is essential for specific DNA recognition and transcription activation, is very short, suggesting unexpected features in transcription initiation of sea urchin mitochondria. By DNaseI footprinting assays on sea urchin mtDNA and by measuring the stability of the protein-DNA complexes, we identified a TFAM specific binding site at the boundary of ATPase6/COIII genes; furthermore we found binding sites inside the genes for tRNASer(UCN) and tRNAPro, close to short conserved AT-rich sequences that were previously suggested to act as promoters. We intend to employ the recombinant TFAM, RNA polymerase and TFB2M to reconstitute in vitro the basal transcription machinery and to assess open complex formation at the candidate promoter sequences.

## OXYDATIVE PHOSPHORYLATION IN RETINAL ROD OUTER SEGMENT DISKS

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Visual transduction in vertebrate retinal rod Outer Segments (OS) is an energy demanding process. Up to now ATP supply for phototransduction in OS is believed to come from glycolysis or diffusion from the mitochondria of the Inner Segments, however location and timing do not seem adequate. Our previous proteomic analysis of purified bovine rod disks identified proteins involved in vision and mitochondria-specific proteins. By confocal laser scanning microscopy imaging we report that respiratory chain complexes and F1Fo-ATP synthase are present on disk and display an activity sensitive to mitochondrial oxidative phosphorylation common inhibitors. The presence of a proton gradient across disks is also demonstrated by fluorescence quenching experiments of Rhodamine 123 (RH 123). Confocal microscopy of bovine retinas ex vivo show that RH 123 stains OS; rhodopsin and MitoTracker fluorescence co-localize on rod OS. Data are suggestive of the presence of an aerobic metabolism in rod disks, that can provide sufficient energy for rod visual transduction. Our findings shed light on many retinal pathologies related to oxidative stress and energy supply in rod OS.

## THE MITOCHONDRIAL CITRATE CARRIER IS REQUIRED FOR CHROMOSOME INTEGRITY IN DROSOPHILA AND HUMAN CELLS

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G. Cenci<sup>°</sup> and L. Capobianco<sup>\*</sup>**

<sup>\*</sup>University of Salento; <sup>°</sup>University of L'Aquila; <sup>^</sup>Sapienza University; <sup>\*\*</sup>University of Palermo; <sup>°°</sup>ITB Pisa

Chromosomal aberrations are key events in the initiation and progression of cancer. We isolated a P-element induced mutation in *Drosophila* that causes chromosome fragmentation. We named sea the gene specified by this mutation. The P-element is inserted in CG6782 which encodes a protein orthologous to the mammalian citrate carrier SLC25A1. It exports citrate from mitochondria supplying the cytosol with acetyl units. Sea shows biochemical properties similar to those of SLC25A1 indicating a functional conservation of this carrier. Sea is reduced in mitochondria of sea mutants which also exhibit a reduced citrate transport activity and low levels of citrate in cytosolic extracts as revealed by LC/MS. Western blot of nuclear protein extracts with anti-acetylated histone antibodies revealed a reduction of Ach3 and Ach4 in mutants. Therefore, the phenotype observed in mutants seems to be the consequence of reduced levels of histone acetylation. Notably, SLC25A1 siRNA-treated human fibroblasts exhibited a phenotype similar to *Drosophila* mutants. These results suggest an unexpected role for Sea/SLC25A1 in the chromosome integrity providing a link between cellular metabolism and epigenetics.

## THE HUMAN SLC25A42 GENE PRODUCT TRANSPORTS COENZYME A AND ADENOSINE 3',5'-DIPHOSPHATE IN MITOCHONDRIA

**G. Fiermonte, E. Paradies, S. Todisco, C. M. T. Marobbio, G. Parisi and F. Palmieri**

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Mitochondrial functions require highly specific transport of metabolites, nucleotides and cofactors between the cytosol and the mitochondrial matrix. To a large extent, this is mediated by a family of mitochondrial carriers. Coenzyme A (CoA) is an essential cytosolic-synthesized cofactor required in many intra-mitochondrial metabolic pathways. In this work, we have identified SLC25A42 as the human gene responsible for mitochondrial CoA transport. The gene product is localized in the inner mitochondrial membrane and is ubiquitously expressed although at different levels. The recombinant protein expressed in *Escherichia coli* has been functionally characterized upon its purification and reconstitution into phospholipid vesicles. In the reconstituted system the protein exhibited a high transport affinity for CoA, dephospho-CoA, ADP and adenosine 3',5'-diphosphate (PAP). The main physiological role of SLC25A42 is to import CoA into mitochondria in exchange for intra-mitochondrial adenine nucleotides and PAP. This is the first time that a mitochondrial carrier for CoA and PAP has been identified and biochemically characterized.

## STRUCTURE/FUNCTION RELATIONSHIPS OF THE MITOCHONDRIAL CARNITINE/ACYLCARNITINE CARRIER

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The carnitine/acylcarnitine carrier (CAC), which is responsible for the transfer of acylcarnitines into the mitochondrial matrix, belongs to the mitochondrial carrier family, the members of which contain three repeated segments of about 100 amino acids with the conserved motif P-X-[DE]-X-X-[RK]. So far, only the tertiary structure of the ADP/ATP carrier is available. To define the structure/function relationships of CAC, mutants of rat and human CAC have been over-expressed in *E. coli*, reconstituted in liposomes and assayed for the transport function as 3H-carnitine(ex)/carnitine(in) antiport. The multialignment of the CAC subfamily members revealed that several amino acid residues are conserved in the CAC, like the specific motif RX2PANAAXF and an H (H29) residue. These residues have been mutated with different amino acids and the variations in the transport activity and kinetics have been evaluated in comparison with the WT protein. The experimental results together with bioinformatic data suggest that R275, N280 and F284 of the RX2PANAAXF motif and H29 are important for the function. One hypothesis under investigation is that they are involved in substrate binding.

## IDENTIFICATION AND CHARACTERIZATION OF NAD<sup>+</sup> TRANSPORTERS IN ARABIDOPSIS THALIANA

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The *A. thaliana* genome encodes 58 putative members of the mitochondrial carrier family (MCF). Two MCF members of *A. thaliana* (accession Nos. FM211595 and FM211594) were overexpressed in *E. coli*, and the purified proteins were reconstituted into liposomes. In the reconstituted system both proteins, named AtNDT1 and AtNDT2, transported NAD<sup>+</sup> in exchange for internal NAD<sup>+</sup>, ADP, AMP and other nucleotides. In contrast, negligible activity was detected with internal  $\alpha$ -NAD<sup>+</sup>, NADH and cAMP. The transport affinities ( $K_m$ ) of AtNDT1 and AtNDT2 for NAD<sup>+</sup> were somewhat lower than the  $K_m$  of the *S. cerevisiae* NDT1 (Todisco et al, J Biol Chem 2006, 281:1524-31), and their specific activities ( $V_{max}$ ) were similar to those exhibited by most mitochondrial carriers characterized so far. However, AtNDT2 was more active and displayed a lower affinity for NAD<sup>+</sup> than AtNDT1. Furthermore, both AtNDT1 and AtNDT2 complemented the growth defect on nonfermentable substrates of the *S. cerevisiae* cells devoid of their NAD<sup>+</sup> mitochondrial carriers ( $\Delta ndt1\Delta nt2$ ). Finally, the expression of AtNDT1 or AtNDT2-short (without the 47 C-terminal amino acids) increased the mitochondrial NAD<sup>+</sup> content of the  $\Delta ndt1\Delta nt2$  strain.

## THE MITOCHONDRIAL OXALOACETATE CARRIER TRANSPORTS $\alpha$ -ISOPROPYLMALATE NEEDED FOR LEUCINE BIOSYNTHESIS IN YEAST

**Carlo M.T. Marobbio, Giulia Giannuzzi, Eleonora Paradies, Ciro L. Pierri  
Franchino Mariateresa, and Ferdinando Palmieri**

Department of Pharmaco-Biology, Laboratory of Biochemistry and Molecular Biology, University of Bari

In *S. cerevisiae*,  $\alpha$ -isopropylmalate ( $\alpha$ -IPM), which is produced in mitochondria, must be exported to the cytosol where it is required for leucine biosynthesis. Recombinant and reconstituted mitochondrial oxaloacetate carrier (Oac1p) efficiently transported  $\alpha$ -IPM. Though not transported,  $\alpha$ -ketoisocaproate, the immediate precursor of leucine in the biosynthetic pathway, inhibited Oac1p activity competitively. Leucine,  $\alpha$ -ketoisovalerate, valine and isoleucine neither inhibited nor were transported by Oac1p. Moreover,  $\Delta OAC1$  cells required leucine for optimal growth on fermentable carbon sources. Single deletions of other mitochondrial carrier genes or of LEU4, which is the only other enzyme that can provide the cytosol with  $\alpha$ -IPM in addition to Oac1p, exhibited no growth defect, whereas the double mutant  $\Delta OAC1\Delta LEU4$  did not grow at all on fermentable substrates in the absence of leucine. This defect was restored by adding  $\alpha$ -ketoisocaproate and  $\alpha$ -IPM to these cells as well as by complementing them with one of the two unknown human mitochondrial carriers. Oac1p is important for leucine biosynthesis on fermentable carbon sources because it catalyzes the export of  $\alpha$ -IPM.

## FUNCTIONAL CHARACTERIZATION OF THE HUMAN MITOCHONDRIAL CITRATE CARRIER GENE PROMOTER

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We have functionally characterized the gene promoter of the human mitochondrial citrate carrier (CIC) that exports citrate from mitochondria to the cytosol, where it is needed for fatty acid and sterol biosynthesis. Insulin upregulated and polyunsaturated fatty acids downregulated CIC gene transcription through the SRE/SREBP-1 system; demethylation of the proximal promoter and histone acetylation activated CIC gene expression by promoting binding of both Sp1 and acetylated histone H3 to the CIC proximal promoter; FOXA acted as a strong enhancer of CIC gene expression by binding to a FOXA site; and luciferase (LUC) gene reporter and EMSA experiments led to the identification of an inhibitory domain (from -742 to -499 bp) within which a CIC silencer activity extended over 26 bp, from -595 to -569. The transcription factor binding to the silencer region was purified from HepG2 cell nuclear extracts by DNA affinity and identified as ZNF224. Overexpression of ZNF224 reduced LUC transgene expression activity as well as CIC transcript and protein levels, whereas ZNF224 silencing enhanced LUC reporter activity and CIC gene expression.

## MT-ND5 GENE NUCLEOTIDE VARIANTS DECREASE MITOCHONDRIAL ENERGY COUPLING

**Dell'Aglio<sup>1</sup>, Zaccagnino<sup>1</sup> Artuso<sup>1</sup>, Trentadue<sup>1</sup>, D'Oria<sup>1</sup>, Carrozzo<sup>2</sup>, Guerriero<sup>3</sup>, Papa<sup>1,4</sup>, Lorusso<sup>1,4</sup>, Petruzzella<sup>1,4</sup>**

<sup>1</sup>DIBIFIM., Univ. Bari <sup>2</sup>UMM, Bamb. Gesù Hosp., Rome <sup>3</sup>Dep. Ophth. and Otolaryng., Univ. Bari <sup>4</sup>CNR, IBBE, Bari

Optic atrophy is a quite common sign in the context of mitochondrial disorder mostly associated to mutations in mt-ND genes encoding for complex I subunits, but the association with renal involvement is rather unusual. Herein, we present a case with optic subatrophy and renal failure in which the sequencing of the entire mtDNA excluded primary LHON mutations but revealed two nucleotide changes in ND5 gene, A13528G and C13565T, both present in apparently homoplasmy. The same association of ND5 mutations was recently reported in a MELAS patient (1). Although the viability of patient's fibroblasts and NAD-dependent oxygen consumption were comparable to normal control cells, further biochemical analysis revealed reduction of isolated complex I activity and a significant decrease in the rate and extent of the glutammate/malate-driven membrane potential, suggestive of a possible decoupling of proton pumping in complex I. Our results imply that in cultured fibroblasts the variation of membrane potential might be not crucial for the energetic requirements but it could be relevant for optic nerve and renal cells metabolism. REFERENCES 1. McKenzie M. et al., JBC 2007; 282(51):36845

## **COMPARATIVE STUDY OF RESPIRATORY CHAINS IN FOUR BACILLUS CLAUSII STRAINS**

**A. Abbrescia<sup>1</sup>, A. Gaballo<sup>1</sup>, S. Maiorano<sup>2</sup>, P. L. Martino<sup>2</sup>, L. L. Palese<sup>2</sup>, D. Panelli<sup>1</sup>, S. Papa<sup>1,2</sup>, G. Sgaramella<sup>1</sup>, A.M. Sardanelli<sup>2</sup>**

<sup>1</sup>Ist. of Bioenerg. and Biomembr., CNR, Bari, Italy, <sup>2</sup>Dept. of Med. Biochemistry, Biology and Physics, Univ. of Bari, Italy

Alkaliphilic *Bacillus* species have been shown to exert a probiotic activity useful in preventing and treating gastrointestinal disorders. They are resistant to antibiotics and have been found to have an H<sup>+</sup> translocating respiratory chain (r.c.). The focus of this work is the analysis of the different bioenergetics responses adopted by 4 *Bacillus clausii* strains. The analysis of r.c. revealed striking differences among strains. Despite belonging to a unique genospecies, they differ in thermodynamic efficiency. The CN<sup>-</sup> resistance threshold effect indicates that two of these strains have a functional redundancy of the terminal part of the r. c.. These data correlate with the expression level of the mRNA of heme-copper oxidases. In order to gain further insight in the oxygen biochemistry in 4 strains, studies on the ROS scavenging enzymes catalase have been carried out. Our data indicate that different bioenergetics strategies can be adopted by similar strains. It is conceivable that these strategies may be related to the antibiotic-resistance mutations in RNA polymerase and S12 protein which characterize two of the 4 strains here analysed. Acknowledgement: Grant from FAR-MIUR; DM 23154

## **BINDING OF EXOGENOUS Zn<sup>2+</sup> AT INTERNAL SITE(S) OF CYTOCHROME C OXIDASE CAUSES DECOUPLING OF PROTON PUMPING**

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Cytochrome c oxidase (COX) is the terminal oxidase of mitochondria. The effect of Zn<sup>2+</sup> binding at the N-side of bovine COX reconstituted in phospholipid vesicles (COVs) on proton pumping in the various phases of the catalytic cycle is presented. In the aerobic oxidation of reduced COV, the H<sup>+</sup>/COX decreased for proton release from 2.7 to 1.8 with increasing pH; anaerobic ferricyanide oxidation resulted in H<sup>+</sup>/COX of 1.7-1.9 in the pH range 6.2-8.5. In both cases Zn<sup>2+</sup> decreased the H<sup>+</sup>/COX to 1.2-1.5 regardless of the pH. In the reduction of oxidized COV, the H<sup>+</sup>/COX increased from 0.3 to 1.6 with increasing pH. In the presence of Zn<sup>2+</sup>, the H<sup>+</sup>/COX was lowered to 0-0.4 in the pH range 6.5-8.5. The H<sup>+</sup>/COX ratio at level flow exhibited a pH dependence reaching a maximum of 0.8 at pH 7.2. Zn lowered the H<sup>+</sup>/COX to 0.3. Zn<sup>2+</sup> had no effect on H<sup>+</sup>/COX in the anaerobic oxidation of the CO and CN<sup>-</sup> inhibited oxidase. Zn<sup>2+</sup> binding at internal site(s) of COX uncouples proton transfer steps associated with oxidoreduction of heme a<sub>3</sub> and the steps of the reduction of oxygen to water. Acknowledgements: FIRB 'Italian Human Proteome Net' No. RBRN07BMCT\_014

## **cAMP RESPONSE ELEMENT BINDING PROTEIN (CREB) IS IMPORTED IN MITOCHONDRIA AND PROMOTES PROTEIN SYNTHESIS**

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The cAMP response element binding protein (CREB) is an ubiquitous transcription factor, that binds a DNA consensus sequence in gene promoters. Phosphorylation of CREB by PKA, as well as other protein kinases, in response to cellular signals, promotes transcription of CRE-regulated genes. We have studied the mitochondrial import of CREB and its effect on the expression of mtDNA encoded proteins of the respiratory chain. [35S] Methionine labelled human CREB, synthesized in vitro in the RRL system, was imported in rat liver mitochondria by a membrane potential and TOM complex-dependent process. The imported CREB exerted cAMP-dependent promotion of the synthesis of mitochondrially encoded subunits of oxidative phosphorylation enzyme complexes that was inhibited by the PKA inhibitor, H89. Treatment of fibroblast cell cultures with 8Br-cAMP resulted in an enhancement of the synthesis of mitochondrially encoded subunits. This effect could be due to the activation of mitochondrial CREB. Thus, CREB moves from the cytosol to mitochondria, in addition to the nucleus, and, when phosphorylated by PKA, promotes the expression of mitochondrial genes.

## **MITOCHONDRIAL BIOGENESIS OCCURS DURING DENDRITIC CELL DIFFERENTIATION.**

**M. Saltarella #, P. Zaccagnino #, A. Abbrescia°, A. Gaballo°, G. Santoro\*, A. Del Prete#, M. Lorusso° #**

#DIBIFIM and \*DIMO, University of Bari; °Institute of Biomembrane and Bioenergetics (IBBE), CNR, Bari, Italy

Dendritic cells (DC) are sentinels of the immune system deriving from circulating precursors recruited to sites of inflammation. After antigen sampling, DC undergo a maturation process that culminates in their migration to draining lymph nodes to induce an adaptive immune response. We have reported that mitochondrial ROS play a crucial role in DC differentiation. These results prompted us to investigate dynamic changes in energy metabolism accompanying the differentiation. TEM analysis revealed a significant increment in the number of mitochondria in DC compared to monocytes. Moreover western blot analysis showed an increase of NRF-1 and Tfam, together with NDUF3 protein (a subunit of complex I) and COX IV. These findings are suggestive of an active mitochondrial biogenesis accompanying DC differentiation. Accordingly, DC showed an increase of i) the respiratory activity and ATP content; ii) the activity of the enzyme citrate synthase; (iii) the amount of mtDNA; (iv) the activity and the expression of complex I and IV of the respiratory chain. These changes are likely required by DC to support energy-dependent processes, such as their endocytic activity and antigen presentation.

## **ALL-TRANS RETINOIC ACID (ATRA) INDUCES IN HUMAN KERATINOCYTES ACCUMULATION OF INACTIVE COMPLEX I OF THE RESPIRATORY CHAIN**

**Annarita Nicastro 1, Salvatore Scacco 1, Francesco Papa 2, Raffaella Trentadue1 and Sergio Papa 3**

1Dept. of Biochem., 2Dept. of Odont. and Surg., University of Bari-Italy; 3IBBE-CNR-Bari

Treatment of Keratinocytes cultures with ATRA results in marked inhibition of the activity of complex I accompanied by enhancement of Grim19 and other subunits of the complex. The inactivation of complex I is reversed by the addition of dibutyryl-cAMP or okadaic acid. Simultaneous addition of ATRA and okadaic acid during 72 hours of Keratinocytes cultivations presents inhibition of the activity of the complex and accumulation of complex I subunits. The effect of ATRA on complex I is apparently mediates by dephosphorylation of complex I subunits and/or other factors which regulate the turnover of complex I subunits and its catalytic activity. Reference: 1. Soprano K.J. et al. *Oncogene*. 2006 Aug 28;25(38):5315-25; 2. Palmisano G. et al. *Proteomics* 2007, 7, 1575-1583; 3. Scacco S. et al. *Jbc* 2000, Vol. 275, 23:17578-17582. Supporter by FIRB project, Rete Nazionale per lo studio della proteomica umana (Italian human ProteomeNet). Progetto RBRN07BMCT\_014



## **Analytical methods**

**Poster session:  
24/09/2009 (h. 14.00-15.00)**



## **EPSTEIN BARR VIRUS (EBV) DNA RESEARCH BY PCR HAS A HIGHER DIAGNOSTIC EFFICACY THAN THE ANTIBODY RESEARCH**

**Giovanni Tringali, Anna Maria Roccazzello, Rosa Veca, Venerando Torrisi, Elio Insirello**

Istituto Ricerca Medica ed Ambientale (I.R.M.A.)

Epstein Barr Virus(EBV) belong to Herpesviridae family and it causes persistent infection, mononucleosis and slight forms of hepatitis. After an EBV infection it is possible to have relapses with poorly antibodies movements. EBV genome is made up of a 172 Kb double strand DNA with different repeated sequences. During replication phase the active viral genes(VCA and Gp350) activate the EBNA genes. It is possible to sub-diagnose the EBV infections by research of specific antibody to viral antigens. In order to check this hypothesis we selected 108 subjects that showed presence of EBV DNA in peripheral blood by molecular biology method(PCR detection) and we investigated for the presence of anti-EBNA IgG/IgM. Results obtained showed that only the 48,1% of infected subjects presented anti-EBNA IgM of which 14,8% in presence of anti-EBNA IgG. In 27,7% of subjects there was present only anti-EBNA IgG(re-infection/re-activation). Finally 24% of subjects were negatives for anti-EBNA antibodies. Despite diagnosis of EBV infection often is performed by IgM detection, we proved that in 51,7% of the cases (56 subjects on 108) we could not diagnose the EBV infection properly only by this method.

## **EVALUTATION OF AMINO-PENICILLINS SENSITIZATION ON SUBJECTS ALLERGIC TO PENICILLIN G USING BASOPHILS ACTIVATION TESTS (BATS)**

**Massimo Caruso, Stefania Mancuso, Pietro L.M. Di Giuseppe, Adriana Pennisi  
Maria A. Cosentino, Rosa Attaguile, Giovanni Tringali**

I.R.M.A. (Institute for Medical and Environmental Research) – Acireale (CT)

The aim of the study is to evidence the incidence of Aminopenicillins sensitization on subjects affected by hypersensitivity to Penicillin G (PENG). Flow cytometry was employed in order to detect two basophil markers of allergic reactions. BATs are reliable tools on adverse drug reaction (ADR) prevention, as receptors and cytokines basophils profile is similar to mast cells. Tests employed are Flow2-CAST (Bühlmann Labs) and Allergenicity Kit (Beckman Coulter). Flow2-CAST reveals the degranulation marker gp53 (CD63), externalized in consequence of degranulation process, while Allergenicity Kit detects the expression of an activation marker typical of allergen stimulated basophil (CD203c). From 93 subjects hypersensitive to PENG, peripheral whole blood samples were collected and stimulated by PENG, Ampicillin (AMP) and Amoxicillin (AMOX). Obtained data evidenced that 24 subjects developed hypersensitivity to AMOX (25.8%) and 19 to AMP (20,43%). Of these subjects (n=35), 22,85% showed hypersensitivity to both AMOX and AMP (n=8). It is possible to hypothesize that these subjects are hypersensitive to Penicillin Determinants (MDM, PPL). This query needs further carefully evaluations.

## **INTERPRETATING DATA FROM HAIR TISSUE MINERAL ANALYSIS (HTMA) BY INDUCTIVELY-COUPLED PLASMA – MASS SPECTROMETRY FOR A TARGETED NUTRITIONAL SUPPLEMENTATION**

**Adriana Tiralongo, Stefania Mancuso, Deborah Martino, Massimo Caruso, Giovanni Tringali**

I.R.M.A. (Istituto Ricerca Medica e Ambientale) – Acireale(CT)

HTMA is used in preventive medicine to assessing mineral imbalances and toxicities. The sampled hair, obtained by cutting the first 3 centimetres of growth closest to the scalp at the nape of the neck, is prepared through chemical and physic digestive procedures. The samples provide indication of mineral status and toxic metal accumulation, revealing intracellular activity and providing a blueprint of the biochemistry during the period of hair growth. The interpretation of mineralogram results enables to describe how the ANS manages stress and determines metabolism (sympathicotonia or parasympathicotonia conditions related to Ca/P ratio), and identify the metabolic-oxidative type. Through the Ca/K and Na/Mg ratios the energy production by the thyroid gland can be analyzed, as well the adrenal gland response to stress agents. Ca/Mg ratio influences the immune system, the parathyroid activity, and the level of glucose intolerance. Fe/Cu balance is crucial for the immune system, and altered Cu/Zn reflects female hormones unbalance. The mineralogram has become an original and reliable tool for analysing the nutritional conditions of the body and estimate the speed of oxidative processes.

## **DNR FROM PSEUDOMONAS AERUGINOSA: STRUCTURAL AND SPECTROSCOPIC PROPERTIES OF A BACTERIAL NO-SENSOR**

**G. Giardina, S. Rinaldo, N. Castiglione, M. Caruso, A. Arcovito\*, S. della Longa+, P. D'Angelo°  
M. Brunori, F. Cutruzzolà**

Dip. Scienze Biochimiche and ° Dip. Chimica, Sapienza Univ. Roma; \* Univ. Catt. Sacro Cuore, Roma; + Univ. L' Aquila

*Pseudomonas aeruginosa* keeps the steady-state concentration of nitric oxide (NO) below cytotoxic levels by controlling the expression of denitrification gene clusters via REDOX signalling using specific transcriptional regulators, such as the NO-sensitive master regulator DNR. We have studied the structural and functional properties of the DNR regulator. We have shown that DNR binds heme in vitro, suggesting a possible mechanism for the NO-dependent activity. We have determined the structure of the NO-sensing domain and of the entire protein. Comparison with other structures among the same class of regulators (CRP-FNR superfamily) reveals that DNR may undergo an unexpected and very large conformational rearrangement on activation. In solution, we have investigated the heme local structure of the ferric and ferrous derivatives of DNR by XAS: the Fe K-edge XANES spectrum of the ferric adduct displays typical features of bis-histidine coordination. In parallel, analysis of DNR site-directed mutants is ongoing in vitro in order to assign residues involved in heme binding.

## **DIAGNOSIS OF FANCONI ANAEMIA (FA) BY MULTIPLEX LIGATION-DEPENDENT PROBE AMPLIFICATION (MLPA – MRC HOLLAND).**

**A.M. Roccazzello, A. Tiralongo, E. Insirello, L. Russo, V. Scavo, M. Andolina, G. Tringali**

I.R.M.A. Biology Molecular Division Acireale (Catania), Italy

Fanconi anaemia (FA) is an autosomal recessive disease characterised by congenital abnormalities, defective haemopoiesis, and a high risk of developing acute myeloid leukaemia and certain solid tumours. There are at least 13 genes of which mutations are known to cause FA: FANCA, FANCB, FANCC, FANCD1, FANCD2, FANCE, FANCF, FANCG, FANCI, FANCL, FANCM and FANCN. Cells derived from FA patients demonstrate chromosomal instability and heightened sensitivity to DNA cross-linking agents, such as mitomycin C (MMC), diepoxybutane (DEB), and cisplatin (CDDP), a feature that is used to make the diagnosis. The recently developed multiplex ligation-dependent probe amplification (MLPA – MRC Holland) technique has been accepted as a simple and reliable method for multiplex detection of copy number changes of genomic DNA sequences using DNA samples derived from blood, amniotic fluid or tumors. Here, we describe a rapid and easy method to apply MLPA based method, for the detection of mutations in FANC genes.

## **ASSESSMENT OF CEPHALOSPORIN CROSS-REACTIVITY ON ALLERGIC SUBJECTS BY BASOPHILS ACTIVATIONS TESTS (BATS)**

**Manuela Andolina, Giovanni Tringali, Maria Angela Cosentino, Stefania Mancuso  
Giuseppe Belluomo, Massimo Caruso**

Institute for Medical and Environmental Research (I.R.M.A.)

In recent years flow cytometric BATs have been drawing attention, particularly for Adverse Drug Reactions (ADR). BATs comprise the assessment of both CD63 and CD203c basophil expression. Aim of our work was to investigate the possible cross-reactivity among different cephalosporins on subjects sensitized to one of them: cephalosporin C (CC), cefuroxime (CX), cefamandole (CM) and cefazolin (CZ). The major differences between these molecules stand on side chains (R1 and R3). On 89 sensitized subjects confirmed by BATs, 40 were sensitized to CC (44,94%), 32 to CX (35,65%), 31 to CZ (34,83%) and 15 to CM (16,85%). The main incidence of cross-reactivity (n=5) was observed for CZ/CX (5,62%); 3 cases (3,37%) for CM/CZ, CC/CX/CZ, CZ/CM/CX, CM/CX, 2 cases (2,89%) for CC/CZ and 1 case (1,12%) for CC/CM. Cross-reaction among CC and others was expected because of the basic structure, while for CZ and CM the similarity of R3 was determinant. To understand the interactions that cephalosporins undertake with blood proteins, molecular models would be of great help. It could be important to follow temporal evolution of incidence of cross-reactions to see if the trend is likely to grow.

## CHARACTERIZATION OF HUMAN HMGR CATALYTIC SUBUNIT EXPRESSED IN ESCHERICHIA COLI BY MALDI MS AND MS/MS.

**1 D. Aiello, 2 AR. Cappello, 1 L. Di Donna, 2 D. Iacopetta, 2 V. Dolce, 1 F. Mazzotti, 2 A. Santoro  
1 A. Napoli, and 1 G. Sindona**

<sup>1</sup>Department of Chemistry, University of Calabria; <sup>2</sup>Department of Pharmaco-Biology, University of Calabria.

Mevalonic acid (MVA) is the precursor of isoprenoids, a class of compounds involved in several cellular functions such as sterol synthesis and growth control. Products of the MVA pathway include cholesterol and heme. Within cells, the concentration of mevalonate is tightly controlled through the activity of 3-hydroxy-3-methylglutaryl CoA reductase (HMGR), the tetrameric enzyme that catalyzes the NADP-dependent reduction of HMG-CoA to MVA. HMGR is among the most highly regulated enzymes yet known. Three regions can be identified within HMGR: a membrane anchor N-terminal domain; a C-terminal catalytic domain; a linker region between these two domains. The human HMGR catalytic portion was expressed in Escherichia Coli. Two different protein products were obtained, a full-length and a shortened form. The two HMGR forms were characterized by MALDI MS and MS/MS. Protein identification was performed by searching a comprehensive protein database using Mascot programs ([www.matrixscience.com](http://www.matrixscience.com)). Sequence specific peptides were automatically identified by database searching of the MS/MS spectra against the NCBI. All spectra were manually checked to verify the validity of the MASCOT results.

## REAL-TIME PCR ANALYSIS OF APP ISOFORMS EXPRESSION IN PLATELETS FROM ALZHEIMER'S DISEASE PATIENTS

**Davide Sartini\*, Monica Emanuelli\*, Leandro Provinciali#, Laura Mazzanti\*, Arianna Vignini\***

\*Dipartimento Biochimica, Biologia e Genetica e #Dipartimento Neuroscienze, Università Politecnica Marche, Ancona

In the past few years, it has been tried to identify peripheral markers of Alzheimer's Disease (AD), mostly focusing on the amyloid precursor protein (APP) which exists in different isoforms generated by alternative splicing. Platelets represent an important peripheral source of APP since it has been demonstrated that the three major APP isoforms are inserted into the membrane of resting platelets. Several studies independently described alterations in APP metabolism in platelets of AD patients. The present study aims to investigate the expression level of different APP platelet isoforms using Real-time PCR in patients affected by AD (n=20) and in age-matched controls (n= 10). Differential gene expression measurements (AD versus control) revealed a significant up-regulation of all APP platelet isoforms (APP 1.5-fold; APP-KPI 1.6-fold; APP 770 1.4-fold; APP 751 1.4-fold; APP 695 1.3-fold). Our results show that high levels of platelet-derived APP isoforms are present in patients with AD and suggest that APP may be considered a potential peripheral marker for the diagnosis of AD.

## **SIMULTANEOUS LIQUID CHROMATOGRAPHY DETERMINATION OF HYPOXANTHINE, XANTHINE, AND URIC ACID IN BLOOD WITH PHOTODIODE ARRAY DETECTOR**

**1 Maria Concetta Gueli, 2 Vincenza Cusimano, 2 Maria Antonietta Mazzola, 2 Giuseppe Salemi**

1Dipartimento di Scienze Biochimiche, 2Dipartimento di Neuroscienze Cliniche Università di Palermo

Uric acid (UA), a polyedric functional compound, is a strong natural antioxidant in humans where it is the end product of purine metabolism. The rational process for this study was the clinical interest of the metabolites as markers for energy disturbance in ischemia/hypoxia, antioxidant capacity, and disease activity in vivo. Unfortunately, reports about changes in UA levels in the neurodegenerative disorders have been conflicting. We propose a fast one-step HPLC method with photodiode array detector (PDA) for the simultaneous determination of hypoxanthine, UA, and xanthine (PD) in human plasma from the blood donors. Waters-HPLC-PDA system consisted of an Empower TM2 DS; AtlantisT3 column. The m.f. was a 40 mmol/L phosphate buffer, pH 2.2. The spectral range of the PDA was 200-400nm, and the optimal wavelength was 254nm. We have obtained an excellent base-line separation of PD and the RT were 5.5; 6.6; 8.7 min, respectively. The reported reference ranges were (1.2-17.9, 151-442, and 0.2-5.8  $\mu$ M), for PD respectively. Peaks were identified by spiking the samples with authentic standards. This HPLC-PDA system is very useful tool both in research and in clinical laboratories.

## **LIGAND TUNNELING STUDIES IN PROTOGLOBIN THROUGH X-RAY CRYSTALLOGRAPHY**

**Marco Nardini<sup>1</sup>, Alessandra Pesce<sup>2</sup>, Sylvia Dewilde<sup>3</sup>, Paolo Ascenzi<sup>4</sup>, Massimo Coletta<sup>5</sup>,  
Luc Moens<sup>3</sup> & Martino Bolognesi<sup>1</sup>**

<sup>1</sup> Univ. of Milano <sup>2</sup> Univ. of Genova <sup>3</sup> Univ. of Antwerp, Belgium <sup>4</sup> Univ. of Roma "Tre" <sup>5</sup> Univ. of Roma "Tor Vergata"

The structural adaptability of the classical 'globin fold' has been highlighted by the recent discovery of 2-on-2 Hbs, of neuroglobin and cytoglobin. Protoglobin from *Methanosarcina acetivorans* C2A (MaPgb), a strictly anaerobic methanogenic Archaea, is the latest entry adding new variability and functional complexity to the Hb superfamily. We have recently reported the 1.3 Å crystal structure of MaPgb, together with first insight into its ligand binding properties. MaPgb was shown to host specific loops and a N-terminal extension that completely bury the heme within the protein matrix. Access of O<sub>2</sub>, CO, and NO to the heme is based on two Pgb-specific apolar tunnels that originate at the B/G and B/E helix interfaces. Here we present the crystal structure of wt MaPgb in complex with Xe atoms and the 3D structures of several mutants (including a truncated form devoid of 20 N-terminal amino acids) which substantially alter the heme distal site accessibility through the tunnels. In all cases large conformational changes affect residues TrpB9, TyrB10, and PheE11 that build the main part of the heme distal site hosting the ligand. Nardini, M. et al. EMBO Rep. (2008) 9, 157-163

## **BIOLOGICAL MOLECULES IMMOBILIZATION ON SI-BASED SURFACES FOR MINIATURIZED BIOSENSOR APPLICATIONS**

**V. Aiello\***, **S. Libertino\*\***, **A. Scandurra#**, **P. Fiorenza\*\***, **F. Sinatra°**, **S. Lombardo\*\***, **M. Renis\***

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The use of biomolecules in microelectronic devices could produce great progresses in biomedical applications (biosensors). The growing interest in biosensors (a tool to monitor both environment and health) is a strong stimulus to find innovative devices that could allow mass production and accurate analysis. An ideal substrate to accomplish such breakthrough is Si. The goal of our study was to verify the feasibility of a multi-sensor chip, anchoring various biomolecules: glucose oxidase and horseradish peroxidase enzymes, the methallotioneine proteins and DNA oligonucleotides. All the molecules were anchored on SiO<sub>2</sub> surfaces using a four step protocol optimized by us and consisting of the following steps: 1) oxide activation; 2) silanization; 3) Glutaraldehyde deposition; 4) bonding of amino terminated biomolecules. To fully characterize the biological layer, X-Ray Photoelectron Microscopy, Atomic Force Microscopy and contact angle measurements were used. Electrical measurements were performed to verify their use as biosensors. The results show that sensing biomolecules can be performed utilizing a simple structure that can be easily integrated in a more complex design.

## **QUANTIFICATION OF BONE TURNOVER MARKERS IN CONTROL HUMAN URINE BY HPLC-FLUORESCENCE USING A NEW PROPOSED INTERNAL STANDARD**

**E. Monticelli 1**, **C.S. Aman 1**, **L. Costa 2**, **P. Rota 2**, **P. Allevi 2**, **G. Cighetti 1**, **M. Anastasia 2**

1 Dip. Scienze Precliniche, 2 Dip. Chimica, Biochimica e Biotecnologie per la Medicina, Università degli Studi di Milan

The aim of this study is to validate an HPLC-fluorescence method for the quantification of free pyridinoline (Pyd), free deoxypyridinoline (D-Pyd), indices of bone degradation, together with galactosyl (Gal-Pyd) and glucosyl-galactosyl (Glu-Gal-Pyd) pyridinolines in non-hydrolyzed healthy women urine (n=7, age=34±7). For the first time, a synthesized D-Pyd superior homologue is proposed as internal standard. For all these compounds, the method is linear in the range 0-170 pmol injected and no matrix effect is found. The coefficients of variation for intra- and inter-day repeatability are between 3.1 and 10.4%. The limit of detection is around 2.3 pmol injected. Free D-Pyd, free Pyd and Glu-Gal-Pyd evaluated in urine account for 40.5±4.7, 215.1±25.0 and 96.4±20.1 pmol/mL (mean±SD), respectively. Gal-Pyd is absent in control urines or under our detection limit. The addition of the new proposed internal standard to urine before any pre-analytical step, allows the unequivocal quantification of the selected bone resorption indices. Moreover, the correct HPLC identification of all selected analytes is guaranteed by the availability of pure corresponding synthesized standards.

## **A TWO-DIMENSIONAL ELECTROPHORESIS AND MASS SPECTROMETRY PROTEIN ANALYSIS OF FOUR BACILLUS CLAUSII STRAINS**

**A. Abbrescia<sup>1</sup>, A. Gaballo<sup>1</sup>, A. Gnoni<sup>2</sup>, R. Lippolis<sup>1</sup>, L. L. Palese<sup>2</sup>, D. Panelli<sup>1</sup>, M. S. Paternoster<sup>2</sup>  
S. Papa<sup>1,2</sup>, A. M. Sardanelli<sup>2</sup>**

<sup>1</sup> Ist. of Bioenerg. and Biomembr., CNR, Bari, Italy; <sup>2</sup> Dept. of Med. Biochemistry, Biology and Physics, Univ. of Bari, Italy

The alkaliphilic *Bacillus* species constitute a large, heterogeneous group of microorganisms which have relevant applications and commercial interest. Probiotic species have been proved to contribute in preventing and treating various gastrointestinal disorders by improving the host's intestinal microbial balance. The focus of this work are four probiotics *B. clausii* strains which display a low level of intraspecific genome diversity. Aims of this study are, the characterization of the protein expression profile and the identification of proteins differentially expressed in the four *B. clausii* strains during the stationary phase of growth. Few protein spots show statistically significant expression changes, and, among this group of proteins, alcohol dehydrogenase (ADH) and aldehyde dehydrogenase (ALDH) show a significant decrease in the stationary phase only two out of four strains. The comparative proteomic data correlate with the enzyme activity and the expression level of the mRNA of ADH and ALDH. Acknowledgement: Grant from FAR-MIUR; DM 23154.

## **BETA-AGONISTS IN LIVER - VALIDATION METHOD**

**FRANCESCA DI GAUDIO**

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Administration of beta-agonists through feeds in meat-producing livestock has been illegally introduced in EU in order to improve muscle tissue to fat tissue ratios. Only few methods have been validate for Clenbuterol like anabolic drugs determination in bovine liver using stringent criteria imposed by Decision 657/2002/EC. The aim of this work was to validate a method following the EU guidelines, using LCMS QqQ after cleanup procedure. 5,00 g of tissue were homogenized, added with IR, extracted and digested with  $\beta$ -glucuronidase/arylsulfatase and cleaned up. The validation procedure was carried out on a LCMS QqQ. Several MS/MS spectra, at increasing collision energy, were recorded. Some of the fragments in the MS/MS spectra can be explained by a common fragmentation pathway that imply competitive losses of water and alkene. Identification and quantification was performed using Clenbuterol D9 as IS, specific SRM transition and a calibration curve in certified matrix. The validation following the Decision is challenging for retrieval of certified matrices and isotopically Labeled Standards and a large number of samples necessary in order to optimize parameters.



## **Natural compounds**

**Poster session:  
25/09/2009 (h. 14.00-15.00)**



## **A RED ORANGE EXTRACT COUNTERACTS THE PRO-INFLAMMATORY EFFECTS OF IFN- AND HISTAMINE ON NORMAL HUMAN KERATINOCYTES NCTC 2544**

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Paolo Rapisarda<sup>3</sup>, Francesco Bonina<sup>2</sup>**

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The purpose of this study was to evaluate the anti-inflammatory activity of a red orange (*Citrus sinensis* varieties: Moro, Tarocco, Sanguinello) complex (ROC), characterized by high levels of anthocyanins, flavanones, hydroxycinnamic acids, and acid ascorbic, on human and histamine.) keratinocyte line NCTC 2544 exposed to interferon-gamma (IFN- The expression of immuno-modulatory membrane molecules such as intercellular adhesion molecule-1 (ICAM-1) by Western blot analysis, and release of chemokines like monocyte chemoattractant protein-1 (MCP-1) and interleukin-8 (IL-8) through ELISA kits, were determined. ICAM-1 modulates the permanence and activation of T lymphocyte in the epidermis. MCP-1 is a specific chemoattractant for monocytes and dendritic cells. IL-8 is important for the recruitment of both neutrophils and T lymphocytes. Addition of ROC together and histamine induced a dose-dependent inhibition of ICAM-1 with IFN- expression and MCP-1 and IL-8 release. ROC shows interesting anti-inflammatory properties in human keratinocyte cells NCTC 2544. This natural complex could have topic employment and mitigate the consequences of some skin pathologies.

## **SYNTHESIS, STRUCTURAL CHARACTERIZATION AND ACTIVITY OF ACID ABSCISIC ANALOGS**

**E. Millo, A. Salis, E. Poskovic, A. Grozio, G. Damonte, A. Armirotti, S. Bruzzone,  
E. Zocchi, U. Benatti, A. De Flora**

Department of Experimental Medicine Biochemistry section and CEBR, University of Genoa, Italy

The plant hormone abscisic acid (ABA) is a sesquiterpene involved in the regulation of many physiological processes. Recently it has been identified as a new endogenous pro-inflammatory hormone in human granulocytes. In these cells, ABA stimulates functional activities through a signalling pathway leading to an increase of the intracellular Ca<sup>2+</sup> concentration. As monocytes play a key role in both inflammation and immunity, the research of ABA antagonists can lead to the development of new anti-inflammatory drugs. Our work involved synthesis and characterization of a series of ABA analogs useful as potential ABA antagonists. All the synthesized compounds were assayed in the ability of inhibiting increase of cAMP, fundamental step in the ABA signalling pathway, on the human granulocytes. This screening allowed to obtain a compound with an ABA inhibitory action ranging from 70% to 100% at concentrations 0.1 to 1 microM. Among the produced compounds, some could be useful to clarify the structural requirements for antagonist action. These informations will assist in the development of more effective inhibitors as potential anti-inflammatory drugs.

## **ABILITY OF RHODIOLA ROSEA EXTRACT TO PROTECT HUMAN CULTURED KERATINOCYTES AGAINST OXIDATIVE STRESS**

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Keratinocytes are strongly exposed to oxidative stress and exogenous antioxidants could play an useful role in minimizing the adverse skin responses associated with oxidant species. For this reason it was paid attention to the extract of *Rhodiola rosea* L. roots by using the phytocomplex as a whole. We have measured the protection afforded by the extract to GSH levels, GAPDH activity and TBARS levels in cultured human keratinocytes (NCTC 2544) exposed to different oxidative insults: Fe(II)/ascorbate, Fe(II)/H<sub>2</sub>O<sub>2</sub> and tert-butyl-hydroperoxide. We also have investigated the influence of the *R. rosea* extract on the production of intracellular reactive oxygen species (ROS) and on the activity of several antioxidant enzymes. Furthermore, we have evaluated the *R. rosea* extract ability to increase, in a time and dose-dependent manner, the activity of the trans plasma membrane oxidoreductase activity. In conclusion, NCTC 2544 are able to better counteract to several oxidative insults if incubated with *R. rosea* extract demonstrating a very good antioxidant activity of this phytocomplex.

## **GASTRIC INFLAMMATION: INHIBITION OF ELASTASE AND METALLOPROTEASE-9 BY CHAMOMILE INFUSIONS**

**O.Maschi\*, M. Bulgari\*, M. Dell'Agli##, E. Bosisio## and D. Caruso##**

#Research Centre 'Giovanni Galli' and \*Dept. of Pharm. Sci., Università degli Studi di Milano, Italy

The mechanisms involved in the protective effect of chamomile infusions on gastric complaints are not well established. Matrix metalloproteases (MMPs) and neutrophils elastase (NE) are proteases involved in gastric inflammation. Therefore the aim of this work was the evaluation of the effect of chamomile infusions prepared from capitula (CFI) and sifted (SFI) flowers on MMP-9 and NE, and the identification of the compounds responsible for this effect. Although, LC-ESI-MS/MS analysis showed different compositions in infusions made with CFI and SFI, they are able to inhibit MMP-9 catalytic domain. Api7glu and lut7glu, two major component of SFI and CFI, showed an inhibitory activity, demonstrating their contribute to the effect of the infusions. The inhibitory effect of CFI and SFI was also confirmed on MMP-9 released by AGS cells. Both the infusions were able to inhibit MMP-9 secretion from AGS cells. CFI, SFI and the individual compounds api7glu, lut7glu, pat7glu and chlorogenic acid were also able to inhibit NE. In conclusion, the present study supports the use of chamomile in the treatment of gastrointestinal inflammation.

## BEYOND THE GUT. EFFECTS OF PLANT STEROLS IN CARDIAC CELLS

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1Food Science University Campus; 2Dept. of Biochemistry; 3Interdept. Research Centre for Cancer - University of Bologna

Plant sterols (PSs) are considered safe cholesterol lowering agents [1]. Unlike cholesterol, PSs are poorly absorbed and actively re-excreted in bile. Despite their low plasma concentration PSs can incorporate into cell membrane. The consequent alteration of fluidity, enzyme activity, and signal transduction could be the mechanism by which low PS concentrations inhibit proliferation and induce apoptosis in cancer cells [2]. Very few data on the effects of PSs in normal cells are reported in the literature. In this study, we evaluated the effect of the supplementation of beta-sitosterol (SS), the most common PS in foods, in primary cultures of neonatal rat cardiomyocytes. SS was supplemented at concentrations similar to the physiological human blood range. GC/MS analysis showed an increase of SS and a decrease of cholesterol content in cardiomyocytes. No differences were detected in cell growth curve and apoptosis, evaluated by flow cytometry, but at confluence cells viability was decreased by SS. This opens new questions on the effect of PSs in normal cells. 1. Rozner and Garti. *Colloids Surf A Physicochem Eng Asp* 2006, 282-283:435-456. 2. Awad and Fink. *J Nutr* 2000, 130:2127-2130.

## PHENOLIC ACIDS, GOOD ANTIOXIDANTS IN LIPOSOMES AND IN CULTURED CELLS, EXHIBIT PRO-APOPTOTIC EFFECTS IN LEUKEMIC CELLS

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In this study we investigated the potential antioxidant/co-antioxidant and pro-apoptotic activities of some simple dietary phenolic acids, i. e. caffeic (CAF), syringic (SYR) and protocatechuic (PRO) acids, small molecules deriving from metabolic degradation of large molecular weight polyphenols and present in considerable amount in seeds, fruit and vegetables. In membrane models, CAF behaves as a very efficient chain-breaking antioxidant, better than Trolox, the water-soluble analogue of vitamin E; SYR and PRO are only able to retard the peroxidation, but not to completely stop it. It is noteworthy that all the phenolic acids, when used simultaneously with vitamin E, are able to spare or even recycle alpha-tocopherol in liposomes and micelles. When pre-incubated in the concentration range 5-20 microM, these compounds act as good antioxidants in the cells under investigation subjected to an oxidative stress caused by 50 microM hydrogen peroxide. Moreover, phenolic acids decrease basal intracellular ROS levels in cells characterized by a very high ROS content, such as leukemic cells, where their antioxidant activity can be correlated with a mild pro-apoptotic effect.

## **OLEIC ACID AND OLIVE OIL ANTIOXIDANTS SYNERGICALLY INHIBIT CHOLESTEROL AND FATTY ACID SYNTHESIS IN C6 GLIOMA CELLS**

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Bio-active molecules in olive oil are oleic acid (OA) and some minor components showing high antioxidant activity, as hydroxytyrosol (OH-Tyr) and quercetin (Que). In C6 glioma cells, we found that OA inhibited fatty acid biosynthesis and cholesterologenesis by reducing activity and expression of key enzymes of these pathways: acetyl-CoA carboxylase (ACC) and 3-hydroxy-3-methyl-glutaryl-CoA reductase (HMGCR), respectively. Fatty acid synthase (FAS) activity was not affected by OA. Besides OA, OH-Tyr and Que down-regulated the aforementioned biosynthetic pathways. A simultaneous incubation of OA with OH-Tyr or Que showed inhibition of ACC and HMGCR activities stronger than that observed when they were singularly utilised, whereas these antioxidants had no effect on FAS. A noticeable reduction of palmitic, stearic and oleic acid synthesis was also observed. ACC and HMGCR inhibition was mediated by AMP-activated protein kinase (AMPK) activation. These findings suggest a synergic action of olive oil components on lipogenesis in C6 glioma cells. This might play a role in the reduced incidence in the Mediterranean area of diseases related to alteration of lipid metabolism.

## **SAGE (SALVIA OFFICINALIS L.) LEAF: A PROTEOMIC APPROACH**

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L. Pistelli 2, A. Lucacchini 1**

Department of: 1Psychiatry, Neurobiology, Pharmacology, Biotechnology 2Pharmaceutical Sciences, University of Pisa

Many studies have been focused on various biological activities such as antibacterial and anti-inflammatory of the secondary metabolites in *Salvia officinalis* L. but at this time are not performed any work about evaluation of presence and role of proteins and peptides in the extracts. We used a proteomic approach to study the leaf protein pattern of *S. officinalis* L.. To obtain a significant representation of leaf proteome, different protocols were tested and protein extracts in each samples were separated by 2D electrophoresis. Spots of interest were cut and analysed by MALDI-ToF-ToF. By using TCA-acetone based protocol a major number of spots (n = 623) was obtained, moreover the addition of PVPP during leaf grinding resulted in higher protein resolution. A total of 13 spots were identified, 9 of them with a probability over 95%. One spot is a protein belong to *Salvia* genome (*S. dentata*) while the others were identified through the homology with other plants genome (mainly *Oryza sativa* and *Pisum sativum*). This work is the first step to study medicinal plants which metabolites have traditional therapeutic and nutraceutic properties that are also used as food.

## **PROTEOMIC ANALYSIS OF MUCUNA PRURIENS SEEDS AND LEAVES TO LOCALIZE THE PROTEASE INHIBITOR (GPMUC) AND STUDY ITS PHYSIOLOGICAL AND FUNCTIONAL ROLE**

**N.S. Hope-Onyekwere, A.Cortelazzo, C. Muzzi, H. Cerutti, R. Pagani, R. Guerranti**

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*Mucuna pruriens* is a leguminosae known for its pharmacological importance. Previously, we studied the in vivo protective effect of its seeds extract (MPE) against the toxicity of *Echis carinatus* venom. The immunogen is a multiform glycoprotein (gpMuc). Its N-Terminal sequencing showed similarity with soybean Kunitz-type trypsin inhibitor. We separated the major proteins of MPE, than subjected to SDS-PAGE and 2-DE performed to study changes in proteins expressions and to localize gpMuc. Protein evidenced by silver stain were detected and analysed using TotalLab and ImageMaster 2D Platinum software. 2-DE maps show: 34 albumin spots in range of 20-30 KDa and 55-66 KDa, which contains gpMuc; 10 globulin spots; 8 glutelin spots. Western blot and reverse zymogram confirm which albumin fraction contains gpMuc, trypsin and chymotrypsin inhibitor. Changes in proteins are shown: after 1 week of germination in which 13 new spots appeared like enzymes or their subunits; after 1 month and in leaves in which all spots almost completely disappeared like reserve proteins. Protease inhibitors are localized in albumin like gpMuc which may be enzymic in nature as it degrades during germination.

## **THE ARACHIDONIC EFFECT ON PLATELET NITRIC OXIDE BIOAVAILABILITY**

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The aim of the present study was to investigate the molecular mechanisms involved in the arachidonic acid effect on platelet NO level. Thus NO, cGMP and superoxide anion level, the phosphorylation status of nitric oxide synthase, the protein kinase C (PKC) and NADPH oxidase activation were measured. It was shown that arachidonic acid dose-dependently reduced NO and cGMP level. This effect was abolished by the inhibitor of the thromboxane A2 receptor SQ29548 and partially reversed by the PKC inhibitor GF109203X or by the phospholipase C pathway inhibitor U73122. Moreover arachidonic acid activated PKC and reduced nitric oxide synthase (eNOS) activities. The phosphorylation of the inhibiting eNOSThr495 residue mediated by PKC was increased by arachidonic acid, while no changes at the activating ser1177 residue was shown. Finally arachidonic acid induced NADPH oxidase activation and superoxide anion formation. These effects were reduced by GF109203X, U73122 and apocynin. Likely arachidonic acid through these mechanisms that reduce NO bioavailability could potentiates its platelet aggregating power.

## **POSSIBLE ENDOPHYTISM OF THE PATHOGENIC FUNGUS *T. WIESNERI* IN CHERRY TREE (*PRUNUS AVIUM*)**

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In our study of the aetiology of the cherry rusty spot disease (CCRS) to which are associated viral and viroid RNAs, we investigated on fungi that could possibly be hosts of those agents. In previous communications we presented data showing that the pathogenic fungus *Taphrina wiesneri* was invariably found in leaves and bud structures of both healthy and symptomatic cherry trees. We report here results of PCR experiments on the innermost structures of healthy cherry trees, determining the relative abundance of the fungus with respect to the plant genome, and in situ hybridization and fungus specific stain studies localizing the fungus in bud sections. Data show that *Taphrina* genome is as abundant as the plant genome, as determined by PCR amplification of the relative 18S rDNA. Hybridization of bud sections with a 18S rDNA probe, shows the presence of layers of structures atypical for fungi. The same structures are reactive with the fungal specific stains Trypan blue and Lactophenol blue. These results add evidence of *T. wiesneri* in healthy cherry trees, in high number and atypical structure, as might be for an endophyte condition that would be a first example for woody plants.

## **PROTEIN SEQUENTIAL FRACTIONATION BASED ON SOLUBILITY CRITERIA OF PROTEIN BODIES ISOLATED FROM *MUCUNA PRURIENS* L. DC. SEEDS**

**Stefania Giglioni\*, Nicolina Martino\*\*, Lorenza M. Bellani\*\*, Roberto Guerranti\*, Simonetta Muccifora\*\***

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*Mucuna pruriens* L. DC., belongs to the Fabaceae family. Its seed have reserve proteins endowed into protein bodies different in number and in size. In the present study protein bodies were isolated from cotyledons of dry seeds and their proteins sequentially extracted and purified according to their solubility into appropriate extraction solutions. Albumins, globulins, glutelins, prolamins were evidenced by SDS-PAGE under reducing and non-reducing conditions. The polypeptide band profiles indicated that, under reducing conditions, albumin and globulin show a complex pattern of MW between 120 and 5 KDa and no varietal differences. However, differences were observed in number and intensity of bands. Glutelin showed a fewer number of prominent bands than albumin and globulin. Prolamins had only prominent bands with MW lower than 34 KDa. Under non-reducing conditions the polypeptide banding pattern observed evidenced mainly in albumins the presence of proteins that contain disulfide linkages, while for globulins, glutelins and prolamins no visible differences with the band profile of reducing conditions were evident.

## **CHARACTERIZATION OF THE C-TERMINAL DOMAIN IN TWO SWEET ORANGE TAU-TYPE GLUTATHIONE S-TRANSFERASES BY SITE-DIRECTED MUTAGENESIS**

**Angela Roberta Lo Piero, Ivana Puglisi, Valeria Mercurio, Goffredo Petrone**

University of Catania, Agrarian Faculty, Department of Agronomic, Agrochemistry and Animal Production Sciences

Glutathione transferases catalyse the conjugation of glutathione to a broad range of both endobiotic and xenobiotic compounds. This is considered a crucial step in the reclamation process of toxic chemicals because the S-glutathionylated metabolites are tagged for vacuolar sequestration. All soluble GSTs have a dimeric structure with each subunit divided into two distinct domains: the N-terminal domain which forms the highly conserved glutathione binding site, and, the C-terminal domain providing the more divergent hydrophobic substrate binding site. In a previous study we isolated from sweet orange leaves two tau GST genes, namely GSTU1 and GSTU2. The encoded proteins differ only for three amino acids all of them included in the H-site of the enzymes (R89P, E117K, I172V respectively). In order to understand the significance of the single mismatched residues between U1 and U2 site-directed mutagenesis experiments were undertaken to generate several mutate enzymes. Overall, the analysis of kinetic parameters highlights that the mismatched amino acid residues are located in fundamental positions of the H-site and all of them contribute to the enzyme biochemical properties.

## **SATURNIA SPA: BIOLOGICAL ACTIVITY AND CHEMICAL CHARACTERIZATION OF THERMAL MUD AND BIOGLEATM**

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The many therapeutic and cosmetic treatments offered in Spas are focused on mud-therapy, which possesses the capability of moisturizing the skin and preventing skin ageing and rheumatic diseases. Our studies were carried out on samples of thermal mud and Bioglea<sup>TM</sup> collected at Terme di Saturnia (Grosseto, Italy), which are characterized by several sulphureous pools. The aim of this work was to identify Bioglea<sup>TM</sup> species composition and determine organic substances which contribute to mud functionality. The chemical analysis was performed by extractive methods and chromatographic evaluation (TLC, HPLC, GC-MS). The research showed that Bioglea<sup>TM</sup> is mainly composed of cyanobacteria, particularly from the Oscillatoriales subsection, and also it showed that in the mud maturation phase organic substances, are provided by the development of Bioglea<sup>TM</sup>, which could contribute to mud activity. Mud and Bioglea<sup>TM</sup> didn't show any cytotoxic activity in *Artemia salinas* assay. No antibacterial activity and a very weak antifungal activity were detected for Bioglea<sup>TM</sup> and mud samples. Also DPPH and ORAC tests were performed on mud and Bioglea<sup>TM</sup> to evaluate radical scavenging activity.

## **METALS CONCENTRATIONS IN EDIBLE MUSHROOMS FROM THE MOUNTAINS OF THE PROVINCE OF LUCCA IN TUSCANY, ITALY**

**L. Betti, G. Giannaccini, L. Palego, G. Mascia, F. Fusi, A. Mela, L. Fabbrini, L. Schmid  
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Fungi are important living organisms in nature, present almost everywhere. Within this reign, there are numerous species of edible mushrooms. Since ancient times, mushrooms have been consumed by humans not only as a part of the normal diet but also as a delicacy, because they have a highly pleasant taste and aroma. This study was initiated to evaluate metal contents in some species of mushrooms edible and soil samples collected in province of Lucca. The fruiting bodies of 3 mushrooms species, namely *Boletus edulis*, *Boletus rufus* and *Macrolepiota procera*, were collected in 6 different areas. Surface soil samples were always collected at appropriate sampling places. All samples were analysed for metal contents by inductively coupled argon plasma atomic emission spectrometry (ICP-AES). Results showed a comparable soil compositions in all samples together species dependent differences in the uptake of metals. Heavy metals (As,Cr,Pb,Cd,Hg) resulted accumulated in amounts never harmful to humans. Moreover, all mushrooms analysed, especially *B. edulis*, were rich of Se, an element which plays an important role in human nutrition and metabolism. We thank the Administrations of Toscana

## **BIOGUIDED FRACTIONATION AND ISOLATION OF ANTIOXIDANT COMPONENTS FROM MEDICINAL PLANT TEUCRIUM POLIUM L**

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Free radicals provoke cellular oxidative stress, a condition linked to many health problems. In this frame, there is a growing interest in antioxidants from medicinal and dietary plants. The present work deals with the investigation on the antioxidant activity of components isolated from *Teucrium polium* L., an edible herb widely distributed in Molise. Aerial parts were extracted with methanol and hence partitioned in n-hexane, chloroform, n-butanol and water. The n-butanol extract resulted the most effective as radical-scavenger (IC<sub>50</sub> 8.6 µg/mL), as well as the most effective in xantine oxidase inhibition (XOI). Hence this extract was fractionated by Droplet counter-current chromatography (n-ButOH/Me<sub>2</sub>CO/H<sub>2</sub>O, 3:1:5). Among the fractions collected the most active ones as radical-scavengers contained a phenylpropanoid triglycoside as major component. On the other hand, the highest XOI was found in different fractions, essentially composed of flavonoid glycosides. The structures of bioactive compounds will be determined by NMR and mass-spectral data. To our knowledge, this is the first report dealing with the antioxidant properties of compounds purified from this plant.

## HUMAN PLATELETS EXPRESS TWO ECTO-NOX PROTEINS WITH DIFFERENT SENSITIVITY TO CAPSAICIN

**Luciana Avigliano, Rosaria Arnone, Antonello Rossi, M. Valeria Catani  
Domenico Del Principe and Isabella Savini**

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By modulating the cellular redox state, the plasma membrane electron transport (PMET) is important in platelet biology; indeed, the oxidant/antioxidant balance plays a central role during activation of the coagulation pathway. Nonetheless, in human platelets the PMET system has not yet been fully characterized and the molecular identities of most components are unknown. Here, for the first time, the presence of two members of the plasma membrane hydroquinone(NADH)oxidase family (namely Ecto-NOX1 and Ecto-NOX2) in human platelets has been described. Although similar, Ecto-NOX1 and Ecto-NOX2 showed distinct sensitivity to the quinone analogue capsaicin. Indeed, Ecto-NOX2 was directly inhibited by capsaicin, as it had a drug-responsive domain, whereas Ecto-NOX1 was up-modulated through a mechanism requiring capsaicin binding to its receptor, namely the transient receptor potential vanilloid subtype 1 (TRPV1). Ligand-receptor interaction triggered a signalling cascade leading to ROS production, which in turn enhanced the expression and activity of Ecto-NOX1. Redox regulation of Ecto-NOX1 may be important to platelet recruitment and activation during inflammatory diseases.

## CHEMOTHERAPEUTIC EFFECTS OF RESVERATROL AND ITS ANALOGUE 3,5,4' TRANS-TRIMETHOXYSTILBENE ON DU145

**G. Malfa\*, B. Tomasello\*, C. Spatafora<sup>o</sup>, V. Cardile<sup>^</sup>, C. Scifo\*, C. Tringali <sup>o</sup>, M. Renis\***

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Resveratrol (RV, 3,5,4'-trans-trihydroxystilbene) is a phytoalexin produced by a variety of plants in response to stress. In vivo and in vitro studies have evidenced its anti-inflammatory, anti-oxidant, anti-aging, chemopreventive and chemotherapeutic properties. Different RV analogues have been synthesized to improve the efficacy of the lead compound. We tested, on human prostate cancer cell lines (DU-145), different concentrations of RV and its analogue 3,5,4'-trans-trimethoxystilbene (TRV), examining growth inhibition, cell cycle progression, Sirtuin activity and DNA fragmentation. TRV, employed at half concentration than RV, is more efficient than lead compound in eliciting the chemotherapeutic effects, by inhibiting DU145 cell growth, inducing DNA fragmentation, decreasing both the percentage of cells in G1 phase and sirtuin activity. We hypothesize that the methoxy substitution in 3,5,4' to the stilbene rings, ameliorates lipophilicity of TRV so raising the cytotoxic effect on DU145. Our data encourage both to investigate on new synthetic compounds and to use stilbenoids, in particular TRV, as chemotherapeutic agents.

## LICHEN COMPOUNDS-CAUSED APOPTOSIS OF HUMAN PROSTATE CARCINOMA LNCAP CELLS IS MEDIATED VIA MODULATION OF TUMOR NECROSIS FACTOR-RELATED APOPTOSIS-INDUCING LIGAND (TRAIL)

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Pre-clinic studies have permitted to hypothesize the possible use of lichen compounds as anticancer agents. Our previous data showed a growth inhibition of prostate cancer cells by pannarin and vicanicin. A critical factor in prostate cancer progression is the altered expression of apoptotic regulatory proteins which renders cells resistant to both hormone- and chemo-therapies. Tumor necrosis factor related apoptosis inducing ligand (TRAIL) is a naturally occurring anticancer agent that preferentially induces apoptosis in cancer cells and is not toxic toward normal cells. However, emergence of drug resistance limits its potential use. The androgen-sensitive human prostate cancer cell line LNCaP is only slightly susceptible to TRAIL. In the current study, the ability of lichen compounds pre-treatment to sensitize androgen-sensitive (LNCaP) human prostate cancer cells to apoptosis and the mechanisms involved were investigated. Pannarin and M for 48 h induced TRAIL surface expression in vicanicin at 12 and 25 the cell line examined, resulting in a rapid development of apoptosis, as demonstrated by a significant increase of caspase-3 enzyme activity and a high DNA fragmentation.

## ISOLATION OF A POLYGALACTURONASE-INHIBITING PROTEIN (PGIP) FROM LATHYRUS SATIVUS L.

**Rachele Tamburino, Antimo Di Maro and Augusto Parente**

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Polygalacturonase inhibiting proteins (PGIPs) isolated from plants are defensive proteins being able to stop pathogens' polygalacturonase activity. PGIPs have been purified from a variety of dicotyledonous plants (tomato, apple) and from some monocotyledons (onion, wheat). Their size varies between 37 kDa (bean) and 54 kDa (inhibitor purified from citrus [1]). In general, the primary structure of mature PGIPs is characterized by the presence of repeats derived from a 24-amino acid leucine-rich peptide (LRRs; [2]). PGIPs may have interesting applications, being possible to produce transgenic plants resistant to phytopathogens. In this communication we report the isolation of PGIP from *Lathyrus sativus* L. seeds. The N-terminal sequence revealed an high sequence identity percentage with the PGIP purified from *Phaseolus vulgaris* L.. This novel inhibitor is able to inhibit commercial endopolygalacturonase extracts from *Aspergillus* spp and *Rhizopus* spp. 1. Barmore and Nguyen, 1985. *Phytopathology* 75: 446-449. 2. De Lorenzo et al., 1994. *Biochem. Soc. Trans.* 22: 396-399.

## UPTAKE OF BETANIN AND INDICAXANTHIN BY CACO-2 CELL MONOLAYERS

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Betanin (Bet) and Indicaxanthin (Ind) are bioavailable and bioactive dietary pigments. This study used Caco-2 human intestinal cell monolayers to assess uptake of Bet and Ind as purified compounds, and from betalainic foods after simulated gastrointestinal digestion (bioaccessible fraction). Experiments carried out under initial velocity conditions, showed that the rate of uptake of both purified betalains was linear in the 25-200 µM range, indicating a trans-epithelial transport system not saturable. Permeability of Ind appeared 1.5 fold higher than Bet, as indicated from the slope of the linear response. Kinetic studies of uptake with either pigment indicated a much more rapid uptake of Ind, although the calculated efficacy of absorption was identical for both betalains ( $0.9 \pm 0.08\%$ ,  $p < 0.01$ ,  $n = 5$ ) after 20 min incubation. Uptake of betalains from bioaccessible fraction of betalainic foods was measured. With respect to purified compounds, the absorption efficacy of Bet, but not of Ind, is strongly decreased ( $p < 0.001$ ) by the food matrix, suggesting interactions of Bet with matrix components. Present data may provide a rationale for the bioavailability of the two betalains observed in humans.

## PARA-APOPTOTIC EFFECT OF RED WINE EXTRACT ON U2Os OSTEOSARCOMA CELL LINE

**I. TEDESCO 1, A. NAPPO 1, G. IACOMINO 1, M. RUSSO 1, P. PETRILLO 1, G. IANNACONE 1  
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Programmed cell death (PCD) can be classified in apoptosis, a caspase-dependent process, apoptosis-like (para-apoptosis), autophagic and necrosis-like PCD, which are predominantly caspase-independent processes. Triggers for these death pathways are largely unknown (Houwerzijl et al., Leukemia. 2006;20:1937). Here, we described the activation of a caspase-independent apoptotic by a red wine lyophilized extract (RWL) in U2Os cells derived from a human osteosarcoma. Exposure of U2Os to RWL determined a 50% reduction of cell viability. Absence of increased LDH activity excluded necrotic cell death. Accordingly with the hypothesized para-apoptotic phenotype, RWL did not induce neither activation of caspase-3, nor PARP degradation. Para-apoptosis process is also characterized by an active protein synthesis and phosphatidylserine exposure. The block of the protein synthesis induced by cycloheximide (CHX) in RWL treated U2Os cells determined a recovery of cellular vitality. Finally, Annexin V test showed that RWL induced increased exposure of phosphatidylserine residues of about 18%.

## EFFECT OF CELTIS AETNENSIS STROBL TWIG EXTRACT ON COLON CANCER CELL LINE

**R. Acquaviva, R. Santangelo, C. Di Giacomo, V. Sorrenti, V. Cardile\*, S. Caggia\*, C. Genovese°  
S. Puglisi°, L. Iauk°**

Dpt. Bioch. Med. Chem., Mol. Biol., \*Dpt. of Physiol. Sci., °Dpt. Microbiol. Ginecol. Sci., University of Catania

*Celtis aetnensis* Strobl is a bushy shrub present on Mount Etna (Sicily). The genus *Celtis* (Ulmaceae) includes about 70 species of shrubs or trees, primarily distributed in temperate and tropical regions. In previous reports on species of this genus, the presence of some antitumor triterpenes was shown, in particular in twigs. Since there is an increasing interest in the *in vivo* protective effects of natural compounds contained in plants against oxidative damage involved in several human diseases such as cancer, in this study we investigated the effects of *Celtis aetnensis* twig extract on the viability of Caco2 cell line. In order to elucidate mechanisms of action of this extract, LDH release, GSH content and ROS levels were also evaluated. Our results evidenced the ability of this extract to significantly reduce cell viability of Caco2. Data obtained, regarding LDH release, ROS and GSH levels suggested the involvement of oxidative stress in the reported effect. Then, our study support the growing body of data suggesting the bioactivities of *Celtis aetnensis* and its potential impact on cancer therapy and on human health.

## **Oxidative stress**

**Poster session:  
25/09/2009 (h. 14.00-15.00)**



## **THE TRANSCRIPTION FACTOR DNR FROM PSEUDOMONAS AERUGINOSA SPECIFICALLY REQUIRES NITRIC OXIDE AND HEME FOR THE ACTIVATION OF A TARGET PROMOTER IN ESCHERICHIA COLI**

**Nicoletta Castiglione, Serena Rinaldo, Giorgio Giardina, Manuela Caruso  
Maurizio Brunori and Francesca Cutruzzolà**

Department of Biochemical Sciences, University of Rome La Sapienza, 00185 Rome, Italy

*Pseudomonas aeruginosa* is a common pathogen in chronic respiratory diseases (cystic fibrosis); its pathogenicity is related to the ability to grow under oxygen-limited conditions using the anaerobic metabolism of denitrification, in which nitrate is reduced to N<sub>2</sub> via nitric oxide (NO). Denitrification is activated by a cascade of redox-sensitive transcription factors, including the DNR regulator, sensitive to Nitrogen-oxides. To study the mechanism of NO-sensing by DNR, we have developed an *E. coli*-based reporter system. Our results demonstrate that in *E. coli* DNR responds to NO and is able to transactivate the *P. aeruginosa* norCB promoter. The direct binding of DNR to the target DNA is required: mutations in the DNR HTH domain and substitutions in the norCB promoter abolish the transcriptional activity. Since previous evidences suggest that DNR binds heme in vitro, we have also confirmed, using an *E. coli* strain deficient in heme biosynthesis, that heme is required also in vivo. Moreover, DNR responds specifically to NO, but is not activated by CO. In order to assign residues putatively involved in heme binding, we have also studied several DNR site-directed mutants.

## **PROTEOMIC PROFILE OF ENDOTHELIAL PROGENITOR CELLS (EPC) FOLLOWING HIGH-GLUCOSE TREATMENT**

**Lara Milone, Alfonso Giovane, Luigi Servillo, Maria Luisa Balestrieri**

Dipartimento di Biochimica e Biofisica "F. Cedrangolo", Seconda Università degli Studi di Napoli

Circulating endothelial progenitor cells (EPC) play an important role in neoangiogenesis and re-endothelization of injured blood vessels. Improvement of EPC levels and functional activity is of considerable clinical interest. We recently showed that high dose of glucose downregulates EPC number via SIRT1/FoxO pathway. Here, we aimed to study the signal transduction related to SIRT1/FoxO pathway by analyzing the EPC proteomic profile following high glucose treatment. Results of 2D-DIGE and 2D-SDSPAGE showed changes in the expression levels of proteins involved in oxidative metabolism. In particular, an increase in the expression levels of the superoxide-dismutase, galectine, and cathepsin was observed. Moreover, the 2D-dige analysis showed an increased expression of nicotinamide phosphoribosyltransferase (Nampt), which controls the levels of nicotinamide, an inhibitor of SIRT1 activity. In conclusion, the results suggest that EPC are able to counteract to the oxidative stress and highlight possible signaling pathways influencing Nampt levels which are likely to impact on SIRT1 activity.

## **THE PECULIAR PROPERTIES OF THE REDOX COUPLE FERREDOXIN-NADP+ REDUCTASE/ FERREDOXIN OF PLASMODIUM FALCIPARUM**

**Danila Crobu and Alessandro Aliverti**

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The apicoplast of *P. falciparum* contains a plant-type electron transfer system formed by ferredoxin-NADP+ reductase (PfFNR) and its substrate ferredoxin (PffD). This protein couple has been shown in vitro to provide reducing power to LytB(1), the last enzyme of the biosynthetic pathway that produces isoprenoid precursors. This pathway is a known site of action of antiplasmodial drugs and thus PfFNR could represent a new target for the development of antimalarial compounds. PfFNR displays a *k<sub>cat</sub>* value 5-fold lower than that of homologous FNRs. Rapid kinetic studies of the reductive half-reaction pointed out that the hydride transfer from NADPH to PfFNR-bound FAD was significantly slower than in other FNRs. Furthermore, redox studies on the enzyme-bound FAD showed that the apoprotein provide no stabilization of the FAD semiquinone. These two peculiar features of PfFNR, i.e. low rate of hydride transfer and poor stabilization of the FAD semiquinone, are possibly responsible for its low catalytic efficiency in comparison to homologous enzymes(2). 1. Röhrich R.C. et al. (2005) FEBS Lett. 579, 6433-6438 2. Balconi E. et al. (2009) FEBS J. 276, 4249-4260

## **BASAL NITRIC OXIDE RELEASE ATTENUATES CELL MIGRATION OF HELA AND ENDOTHELIAL CELLS**

**S. Bulotta<sup>1</sup>, M.V. Ierardi<sup>1</sup>, J. Maiuolo<sup>1</sup>, M.G. Cattaneo<sup>2</sup>, L.M. Vicentini<sup>2</sup>, N. Borgese<sup>1,3</sup>**

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Nitric oxide (NO) generated by endothelial NO synthase (eNOS) is a key regulator of endothelial cell migration and vascular homeostasis. Whereas the effects of acute NO generation are generally stimulatory, the role of chronic basal NO release has not been explored so far. We addressed this question both in HeLa and in human endothelial cells. In stably transfected HeLa cells, inducibly expressing eNOS, expression of the enzyme per se blunted the phosphorylation of Akt/PKB in response to serum and strongly inhibited chemotaxis, an effect partially blocked by eNOS- and soluble guanylyl cyclase (sGC) inhibitors. Likewise, long-term pre-treatment of non-transfected HeLa cells with nanomolar concentrations of an NO donor inhibited subsequent migration, an effect blocked by sGC inhibition and mimicked by a cGMP analogue. Finally, EC migration was stimulated by chronic pretreatment with an eNOS inhibitor. Thus, in addition to its well-known stimulatory role, eNOS also attenuates migration through basal long-term NO release. These results have implications for studies aimed at modulating cellular migration by enhancing or inhibiting NO-mediated signalling pathways.

## MERCURY STRESS INDUCED GENES IN TRICHODERMA HARZIANUM

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Goffredo Petrone\*, Santa Olga Cacciola\*\***

\*DACPA, University of Catania \*\*Dip. Chim. Biol., Chim. Med. e Biol. Mol., University of Catania

The particular characteristics of *Trichoderma* and at the same time its high tolerance to toxic metals may be useful in metals recovery systems. The specific response to different heavy metal stresses may be the key to the understanding of metal tolerance strategies. In particular *T. harzianum* IMI 393899 increased the dry weight in liquid culture supplemented with mercury. A molecular approach to investigate the mercury resistance mechanisms and the tolerance strategies exhibited by the strain IMI 393899 of *T. harzianum* was carried out by the application of the suppression subtractive hybridization technique. Since the cadmium tolerance mechanisms have been extensively investigated, we applied a particular strategy: a cDNAs from *T. harzianum* IMI 393899 grown in the presence of cadmium was used as driver, in order to reset the differential expression pattern due to the general heavy metals stress, knocking out the genes expressed both in the presence of mercury and cadmium. The differential expression level of the eight specific mercury induced genes was also confirmed by quantitative real-time PCR.

## INCREASED LEVELS OF OXIDATIVE STRESS IN AMNIOTIC FLUID FROM DOWN SYNDROME FETUSES

**A. Fiorini\*, E. Bucai\*, A. Cocciolo\*, C. Blarzino\*, C. Foppoli°, C. Cini\*°, R. Coccia\* and M. Perluigi\***

\*Biochemical Sciences Dpt. -"Sapienza" University of Rome °CNR Mol. Biol. & Pathol. Inst.-Rome, Italy

Growing interest is currently given to investigate the role of oxidative stress (OS) in Down syndrome (DS). In order to test if OS occurs early in DS pregnancies, we measured some OS markers in amniotic fluid. Samples from normal and DS fetuses were analyzed for protein and lipid oxidation (protein carbonyls, protein-bound HNE), heat shock response (Hsp70, Grp78, HO-1) and antioxidant systems efficiency (GPx, glutathione, thioredoxin). We observed significantly higher levels of carbonyls and HNE in the Down group with respect to control group. In addition, the expression levels of Hsp70, Grp78 and HO-1 in the DS group were significantly higher than in the normal one. In DS samples we also found an impairment of the major thiol-disulfide reductive systems (thioredoxin, glutathione) while GPx activity remained unchanged. These results clearly indicate that OS conditions occur early in DS pregnancy and therefore it can be reasonably hypothesized the possibility of a prenatal antioxidant therapy that may prevent or delay the onset of OS diseases in the DS population. The assays of OS markers in amniotic fluid could be used as additional parameters for prenatal screening of trisomy 21.

## SYLIBIN INDUCED HUMAN HEPATOMA HEPG2 CELL DIFFERENTIATION BY NITRIC OXIDE PRODUCTION

**P. Stiuso, I. Scognamiglio, D. Vanacore, M. Murolo, A. Federico, C. Tuccillo, M.P. Sommella  
M. Carteni, C. Loguercio**

Dip. Bioc. e Biof.; Dip.Inter. Clin. e Sper.; Dip.Med. Sper. SUN

Silybin, extracted from *Silybum marianum*, exerted hepato-protection, anticancer properties, and antioxidant functions. The aim of this study is to investigate the proliferation-inhibiting and differentiation-inducing actions of silybin on human hepatoma Hep G2 cells after H<sub>2</sub>O<sub>2</sub> treatment. The HepG2 was maintained in RPMI 1640 supplemented with 10% fetal bovine serum. After incubation for 4 h the cells were treated with 50 μM H<sub>2</sub>O<sub>2</sub>. H-HepG2 cells were incubated with different concentration of Silybin and the cell proliferation was measured by MTT-assay. Superoxide anion and nitrite was assessed as oxidative damage, while MnSOD and alkaline phosphatase activity was utilized with markers of antioxidant scavenger and differentiation respectively. The results indicated that the IC<sub>50</sub> value was 78 μM. The treatment of silybin induced an decrease of superoxide anion value, increased nitric oxide production, MnSOD, and ALP activity. The silybin shift the undifferentiated H-HepG2 cells in differentiated hepatocytes, then undergo a process of programmed cell death strongly suggests that this compound should be further investigated for its potentiality in cancer combination chemotherapy.

## QUANTITATIVE CHANGES IN THE THIOL PROTEOME UPON HEAVY METALS STRESS IN TRICHODERMA HARZIANUM

**Roberto Faedda (1), Goffredo Petrone (1), Santa Olga Cacciola (2), Vito Rappa (2), David Sheehan (3)**

(1) D.A.C.P.A., Univ. CT (2) Dip. Chim. Biol., Chim. Med. Biol. Mol., Univ. CT (3) Dpt. Biochemistry, UCC, Ireland

*Trichoderma harzianum* is ubiquitous in soil and tolerant towards several heavy metals (HMs). Toxicity of HMs is due to interaction with protein -SH groups, formation of ROS and displacement of cations from protein binding sites. Protein thiols are both highly-susceptible to oxidation and implicated in cell signaling. In this study we have used activated thiol sepharose as a means to enrich for -SH-containing protein of *T. harzianum*. 2-D electrophoresis of thiol proteins was applied to investigate the effect of CdCl<sub>2</sub> and HgCl<sub>2</sub> on the fungus thiol proteome. Thiol proteins of *T. harzianum* account for 1.4% of total protein and the exposure to HMs did not alter this value. The 2-D electrophoresis profiles of thiol proteins showed an average of 596 spots per gel and revealed 19 differentially expressed polypeptides. Cd-treated culture showed 13 up- and 5 down-expressed thiol proteins, whereas Hg-treated culture revealed 8 up- and 9 down-expressed thiol proteins with respect to control. These indicate that each metal affects protein profiles uniquely. Also, the differential spots observed support the hypothesis that a thiol-buffer is a putative defence response to oxidative damage.

## **ROLE OF SEMAPHORIN 4A IN THE INFARCTED MYOCARDIUM AFTER ISCHEMIA/REPERFUSION**

**Claudia Meda, Fabiola Molla, Roberto Latini, Federica Maione, Federico Bussolino, Enrico Giraud**

Dept. of Oncological Sciences, University of Torino, Torino, IRCC, Candiolo, Mario Negri, Milano

Tissue remodeling after myocardial infarction involves various processes including angiogenesis. Semaphorin4A (Sema4A) is involved in the regulation of angiogenesis and immune system. Heart ischemia/reperfusion (I/R) was induced in C57/Bl6 mice. Real Time RT-PCR of RNA purified from the hearts and confocal analysis revealed significant increase in Sema4A expression after 24h I/R associated with the precursors of monocytes/macrophages and mature macrophages (M0). Elicited peritoneum exudates collected after treatment with thioglycollate showed a strong increase of Sema4A in peritoneal M0. Human M0 cells treated with TNF $\alpha$  and LPS showed enhanced Sema4A gene levels, compared to controls. In contrast, among the Sema4A receptors, the expression of PlexinD1 and PlexinB2 was not affected by both I/R and generic inflammation, while PlexinB1 was detected only in ischemic tissues. Recombinant Sema4A induced a dose-dependent decrease in the chemotactic activity of endothelial cells (ECs) and human M0. In conclusion, here we show that Sema4A is involved in both angiogenic and inflammatory processes after I/R by increasing its expression in resident M0 and inhibiting ECs and M0 migration.

## **EFFECT OF PHYSIOLOGICAL CONCENTRATION OF PHLORIDZIN ON NORMAL AND DIABETIC HUMAN ERYTHROCYTES UNDER OXIDATIVE STRESS.**

**L. Massaccesi, C.J. Baquero, G. Goi**

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Type 2 diabetes mellitus (T2DM) is the most prevalent metabolic disease. Oxidative stress (OS) plays a crucial role in T2DM (1), antioxidant properties of dietary polyphenols (but in very high and far to in vivo concentrations) on human erythrocytes (RBCs) have been reported. We evaluate the potential antioxidant effect of phloridzin (PRZ) (2), at in vivo-like concentration (1 $\mu$ M), on RBCs membrane fluidity (MF) and on some membrane and cytosolic glycohydrolases activities indicated as sensitive markers of RBCs OS (3). We analysed RBCs (from healthy and T2DM subjects) for: a) membrane fluorescence anisotropy; b) cytosolic O-GlcNAcase and Hexosaminidase (Hex) activities; c)  $\beta$ -D-glucuronidase (GCR) and Hex activities in plasma membrane. We found: a) decrease of MF and an increment of membrane GCR activity in T2DM patients. b) no effect of PRZ on enzyme activities nor in membrane fluidity. We don't find, at [1 $\mu$ M], the protective effect of PRZ against OS reported in previously studies in-vitro (2). Possible synergistic in vitro effects with other polyphenols and/or their derivative metabolites at physiological levels will be studied. 1 Kaneto H. 2005 2 Boyer J. 2004 3 Goi G. 2005.

## **MOLECULAR MECHANISMS OF HYDROGEN PEROXIDE PRECONDITIONING IN CULTURED CARDIOMYOCYTE**

**Cristina Angeloni, Emanuela Leoncini, Marco Malaguti, Elisa Motori, Silvana Hrelia**

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In cardiac cells a short-timed stress, also called preconditioning (PR), may exert a protective role against a subsequent prolonged stress. In this study, we explored whether H<sub>2</sub>O<sub>2</sub>-PR protects against H<sub>2</sub>O<sub>2</sub>-induced oxidative stress in cardiac cells and the roles of phase II enzymes and MAPK signaling pathways in this adaptive protection. Primary cultures of cardiomyocytes were grown at confluence. PR was simulated with 1-100  $\mu$ M H<sub>2</sub>O<sub>2</sub> for 10 min, while oxidative stress was induced by 100  $\mu$ M H<sub>2</sub>O<sub>2</sub> for 30 min. Cell viability was evaluated by MTT and cytofluorimetric assays. Enzyme activities were determined by spectrophotometric methods. Phosphorylation of p38, Akt and ERK1/2 was analyzed by immunoblotting. MTT and cytofluorimetric analysis indicate a significant protective effect of PR against oxidative stress. PR was able to modulate the activities of different phase II enzymes and to increase the phosphorylation of ERK1/2, p38 and Akt protein kinases. Specific kinase inhibitors demonstrated that only p38 and Akt are involved in the protection elicited by PR. Supported by Fondazione del Monte di Bologna e Ravenna.

## **INTRACELLULAR OXIDATIVE STATUS AND OSTEOGENIC ACTIVITY OF SAOS-2 OSTEOSARCOMA CELLS**

**C. Romagnoli<sup>1</sup>, S. Catarzi<sup>1</sup>, F. Favilli<sup>1</sup>, S. Sorace<sup>2</sup>, I. Tognarini<sup>2</sup>, R. Zonefrati<sup>2</sup>  
T. Iantomasi<sup>1</sup> and M.T. Vincenzini<sup>1</sup>**

<sup>1</sup>Dipartimento di Scienze Biochimiche, Università di Firenze <sup>2</sup>Dipartimento di Medicina Interna, Università di Firenze

Intracellular oxidative status has been related to bone metabolism and osteoporosis. Some data relate osteogenic activity to the GSH, the main intracellular antioxidant. This study investigates in SaOS-2 osteoblast-like cells the relationship between the processes of proliferation and differentiation and the ratio GSH/oxidized GSH (GSSG), used to measure cellular redox status. A significant increase of GSH/GSSG ratio during the early times of cell differentiation in comparison with levels measured in proliferating cells was determined. Variations of oxidative status, obtained by the modulation of the GSH/GSSG ratio, showed that the proliferation was not affected by an increase of oxidative status, whereas, when this decreased the cell growth was significantly reduced. Differently, the differentiation process was activated in GSH treated cells; this effect seems to be prevalently related to the early phase of the differentiation. A possible involvement of P38 MAPKinase activity was observed. These data can clarify metabolic processes of bone regeneration and bone diseases related to intracellular oxidative stress. Grants: Cassa Risparmio di Pistoia e Pescia, MIUR.

## **SELDI-PROTEOMIC PROFILE AND OXIDATIVE STRESS EVALUATION IN HUMAN BLADDER CANCER: A PILOT STUDY**

**S. Grasso (1), M. Falsaperla (2), M. Puglisi (2), B. Tomasello (1), M. Renis (1), A. Vanella(1)**

1 Dept. Biol.Chem,Med.Chem and Molec.Biol; University of Catania 2 Dept. of Urol.- Vittorio E.Hospital-ASL3,Catania

Proteomics research useful to discovering new biomarkers for early diagnosis and therapeutic response is constrained by numerous analytical limitations in the sensitivity and throughput of mass spectrometry-based methods. SELDI ProteinChip accomplishes the separation of a subsets of proteins on a chromatographic chip surface analyzing the discrepancies in protein profiling of hundred samples. Our pilot study on bladder cancer patients focus on both pre and after surgery measurement of haematic redox status and the differences in protein expression examined in healthy and normal biptic samples of each patient. CM10 and Q10 proteinchip arrays were selected as best surfaces capable of grouping the healthy/disease samples and maximize the number of differentially expressed protein. Wizard software and high sensibility of SELDI technology, let us to compare healthy and cancerous tissue of the same patient and to evidence possible biomarkers. The initial high oxidative status observed (dROM, BAPtest, oxidative DNA damage) in all the enrolled patients was partially modified after surgical and therapeutic intervention, emphasising the importance of redox status management in cancer.

## **ROLE OF HEME OXYGENASE-1 DURING OSTEOLASTIC DIFFERENTIATION FROM HUMAN MESENCHYMAL STEM CELLS CULTURED IN HIGH GLUCOSE MEDIUM**

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\*\*D. Asprinio, \*\*N. G. Abraham**

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Bone-mineral density and other biochemical markers of bone turnover are very much affected in people with diabetes. We hypothesized that the induction of heme oxygenase (HO-1) and increased HO activity, during differentiation of Mesenchymal stem cells (MSCs) in osteoblasts, following high glucose exposure, would ameliorate the osteogenic process. Glucose concentrations used during osteoblastic differentiation corresponds to healthy individuals (5.5 mM) and to level frequently recorded in patients with hyperglycemia (30 mM). We found a negative effects of high glucose on the osteoblastic differentiation revealed by osteoblastic markers expression (OCN, BMP-2, RUNX-2) and by increase of ROS formation. Using CoPP, an inducer of HO activity, we reported that induction of HO-1 during high glucose differentiation is associated with reduction of ROS formation and was able to restore osteoblastic markers to normal glucose differentiation values. In conclusion HO-1 overexpression could be useful as a protective agent against high glucose toxicity during osteoblastic differentiation and could provide a possible therapeutic strategy in metabolic diseases such as diabetes.

## **A CARBON MONOXIDE RELEASING MOLECULE (CORM-3) PROTECTS AGAINST HEPATIC ISCHEMIA/REPERFUSION DAMAGE**

**R. Acquaviva \*, C. Di Giacomo \*, V. Sorrenti \*, R. Santangelo \*, L. Vanella \*, R. Motterlini\*\***

\*Dpt. Biol. Chem, Med. Chem & Mol. Biol., University of Catania. \*\*Italian Institute of Technology, Genova.

Ischemia/reperfusion liver injury occurs during resection or cold preservation before transplant surgery. A narrow balance among vasoconstrictors and vasodilators normally ensures microcirculation integrity. Carbon monoxide (CO) plays an important role in hepatic microcirculation as this endogenous gas produced by heme oxygenase enzymes is involved in the regulation of systemic vascular tone by exerting smooth muscle cell relaxation through activation of cGMP. CORM-3, a CO-releasing agent, has been reported to promote vasodilatation, regulate arterial pressure and prolong graft survival after transplantation. We report here that treatment with CORM-3 improved several parameters of hepatic function in an experimental model of rat liver ischemia/reperfusion injury. A direct role for CO was confirmed by the lack of effects in animals treated with an inactive compound (iCORM-3) that does not liberate CO. These results confirm the feasibility of using CO as therapeutic agent in hepatic surgery and indicate that CO-releasing molecules could be utilized to limit hepatic injury subsequent to cold storage prior to transplantation.

## **SULFORAPHANE AS AN ANTIAPOPTOTIC NUTRACEUTICAL IN CARDIAC CELLS**

**Emanuela Leoncini, Cristina Angeloni, Marco Malaguti, Elisa Motori, Silvana Hrelia**

Department of Biochemistry "G. Moruzzi", University of Bologna, via Imerio 48, 40126 Bologna, Italy

Myocyte loss through apoptosis has been reported in a variety of cardiovascular diseases. Being a highly regulated cell death program its inhibition is cardioprotective. We previously demonstrated that sulforaphane (SF), present in many Cruciferous vegetables, boosts cell defence system acting as an indirect antioxidant<sup>1</sup>. The aim of this study was to evaluate SF ability to modulate pro and anti-apoptotic proteins resulting in the inhibition of H<sub>2</sub>O<sub>2</sub>-induced apoptosis. SF treatment was able to protect cells against oxidative damage increasing cell viability, and decreasing apoptosis markers like DNA fragmentation and PS exposure. Accordingly, SF reduced Bax translocation to mitochondria, cytochrome c release and caspase 3 activation in comparison to untreated cells. Specific protein kinases inhibitors revealed that SF protection is mainly mediated by Akt activation. Taken together these results show that a reduction in apoptotic cell death contributes to cardioprotection, suggesting SF as a promising nutraceutical in the prevention of oxidative stress related cardiac diseases. Supported by Fondazione del Monte di BO e RA 1 Angeloni C et al, J Agric Food Chem. 2009; 57:5615-22

## NEU3 SIALIDASE OVEREXPRESSION PROTECTS SKELETAL MYOBLASTS FROM HYPOXIA

**R. Scaringi<sup>1,2</sup>, N. Papini<sup>1,2</sup>, A. Garatti<sup>1</sup>, L. Menicanti<sup>1</sup>, P. Allevi<sup>2</sup>, B. Venerando<sup>1,2</sup>,  
G. Tettamanti<sup>1</sup> and L. Anastasia<sup>1,2\*</sup>**

<sup>1</sup> IRCCS Policlinico San Donato; <sup>2</sup> Dep. of Med. Chem., Biochem. and Biotech., U Milan.

Sialidase NEU3 has been implicated to participate in critical regulatory function including cell differentiation, cell growth and apoptosis. Moreover, NEU3 increase during skeletal muscle differentiation has been shown to protect myoblasts from apoptosis and drive the differentiation process [1]. Along this line, it has been shown that NEU3 overexpression is a common feature of several tumours, and that the enzyme plays a crucial role in cancer cell increased proliferation and resistance to the apoptotic stimuli. On these premises, we envisioned a possible function of the enzyme in protecting skeletal muscle cells from apoptotic stimuli, including cell hypoxia. Indeed C2C12 myoblasts overexpressing NEU3 were found to be remarkably more resistant to hypoxia than C2C12 cells. Moreover, when induced to differentiate under 1% oxygen, they survived without any significant cell loss. Moreover, metabolic labelling with [3-<sup>3</sup>H]-Sphingosine showed a significant increase of ceramide content in wild-type C2C12 cells after 24–48 hrs of hypoxia, but no significant variation could be detected in NEU3-overexpressing cells. [1] Anastasia L. et al. J.Biol.Chem. 2008, 283 (52): 36265-36271.



## **Cancer**

**Poster session:  
26/09/2009 (h. 12.00-13.00)**



## **EFFECTS OF A NEW CLASS HDAC SELECTIVE INHIBITOR IN U2OS OSTEOSARCOMA CELLS**

**N. Calonghi, C. Cappadone, C. Parolin, C. Mangano, L. Masotti**

Department of Biochemistry "G. Moruzzi", Alma Mater Studiorum University of Bologna, Bologna, Italy

Aberrant levels of HDACs have been proved to be correlated with the onset of tumors. We have previously demonstrated that MC1855, an HDAC inhibitor, causes an antiproliferative effect in HT29. The treatment with this compound induces a G2/M arrest correlated with the upregulation of p21WAF1 gene expression. Furthermore, MC1855 administration leads to an induction of the expression of specific proteins implicated in the cell cycle regulation, like p21 and BAX. We have also observed an increase of caspase 9 activity, demonstrating the activation of a p53-independent apoptotic pathway. In order to study the effect in p53 w.t. cells, MC1855 has been tested in U2OS. 24 h of treatment with MC1855 induces a strong G2/M arrest, which is correlated with the increase in p21, p53, and BAX proteins, as revealed by both confocal microscopy and spectral analysis. 24h administration of MC1855 leads U2OS to apoptosis, as demonstrated by the Annexin V assay and by caspase 9 activation. Our studies are now focused to understand the mechanisms of the apoptotic pathway activation using in parallel p53 independent (HT29) and p53 w.t. cells (U2OS).

## **SOMATIC COPY NUMBER ABNORMALITIES AND COPY NEUTRAL-LOSS OF HETEROZYGOSITY IN CYTOGENETICALLY NORMAL ACUTE MYELOID LEUKEMIA (CN-AML)**

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40-50 % of AML patients are CN-AMLs, do not have clonal chromosomal aberrations detectable by conventional cytogenetics or targeted molecular techniques and are categorized in the intermediate-risk group. Recent advances in genome-wide analysis of submicroscopic DNA copy number variations (CNVs) may allow the identification of novel molecular tumor-associated abnormalities (somatic copy number abnormalities: CNAs). Here we report a study aimed to test the ability of the last generation of Affymetrix single nucleotide polymorphism (SNP)/CNV platform (SNP Array 6.0) to distinguish tumor-associated CNAs and LOHs from germ-line CNVs and LOHs. 20 de novo CN-AML samples (diagnosis with blasts >90% versus remission phase) have been analyzed. Analysis confirmed the presence of a large number of tumor-associated somatic CNAs in CN-AMLs (median value 32 per patient, median sizes of losses, 19 kb, and gains, 28 kb). An analysis of recurrent CNAs revealed the presence of some recurrent segments in 15-20% of the examined population. Somatic interstitial CN-LOH (average size 1 Mb) were detected in 92 % of the patients. The LOH of chromosome 21 was associated to point mutations of AML1 gene.

## **FIBROBLAST GROWTH FACTOR RECEPTOR 2 (FGFR2) IS A NOVEL MOLECULAR PARTNER OF THE MLL-AF4 LEUKEMIC ONCOPROTEIN**

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The mixed-lineage leukemia (MLL) gene is involved in chromosomal aberrations associated with human leukemia. In ALLs, a balanced translocation fuses in-frame the N-term portion of MLL with the C-term of AF4. The resulting fusion gene encodes a MLL-AF4 chimeric oncoprotein. Human MLL-rearranged ALLs could be distinguished from other ALLs by the H3K79 methylation profile that is important for maintenance of MLL-AF4-driven gene expression. There are evidences that MLL-AF4 cellular oncogenic pathways involve some receptor tyrosine kinases (RTKs). To identify molecular partners that the chimera shares with AF4, we expressed the recombinant MLL-AF4 in HEK293 cells. We demonstrated for the first time that FGFR2, a RTK, is a new molecular partner of AF4 and MLL-AF4. Moreover, in this system, we found, by real time PCR, that the GRB2 and FGFR2 genes were over-expressed. GRB2 links FGFR2 phosphotyrosines to the Ras-Erk pathway. In conclusion, our data suggest a key role of FGFR2 in cellular pathways activated by MLL-AF4 chimeras. (Grants: Regione Campania-Conv. CEINGE G.R.27/12/07 N 2495;L.R.5/2002,Es. 2005; MIUR-Rome PS35-126/IND, PRIN 2007).

## **NOTCH RECEPTORS EXPRESSION IN GLIOMA CELL LINES UNDER DIFFERENT CULTURE CONDITIONS.**

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Some of the signalling pathways involved in the differentiation and proliferation of glial progenitors are altered in gliomas. Notch signalling, essential for the maintenance of neural stem cells (NSCs), is one of the most important pathway in the development of the CNS. Notch signalling constitutive activation in glioma cells promotes growth and increases the formation of neurosphere-like colonies in presence of growth factors. A differential expression of Notch receptors and altered Notch signalling have been observed in human gliomas. We analyzed Notch receptor expression in glioma cell lines under different culture conditions. Glioma cells were grown in the presence of serum or growth factors such as bFGF and PDGF-AA. Under these different growth conditions glioma cell lines showed morphological modifications and a differential Notch receptor expression, suggesting that growth factors might influence gene expression and differentiation of glioma cell lines. Glioma cell lines could be a model for the determination of Notch-dependent gene expression in different conditions that might mimic similar conditions in in vivo specific niches both during development or tumorigenesis.

## TELOMERE LENGTH MODULATION IN ASTROGLIAL BRAIN TUMORS

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Telomere alteration in tumorigenesis is well known. Bidirectional telomere dysfunction is characteristically heterogenous in almost all solid tumors. This study was designed to clarify the pathways that modulate telomere maintenance in astroglial brain tumors. A cohort of 38 surgical specimens, obtained in adult patients who underwent craniotomy for microsurgical tumor resection, histologically grade 2-4 astrocytomas, was used for the study. The Authors studied terminal restriction fragment (TRF) length, and the expression levels of a panel of genes controlling the length and structure of telomeres. The correlation among the levels of gene expression, telomere length, and histological grading, were also studied. Up-regulation of TRF1 and shorter telomere resulted in the low grade gliomas, while down-regulation of TRF1 and up-regulation of both telomerase and PARP1 resulted mainly in high grade gliomas. Moreover, a statistically inverse correlation between TRF1 binding proteins and telomere length was found. These results support the hypothesis that in human astroglial brain tumors typical biomolecular features dealing with biological behavior of malignancy may exist.

## HEPCIDIN MRNA EXPRESSION IN THE ASTROCYTOMA CELL LINE U373MG

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Hepcidin is an hormone peptide that carries out a key role in iron homeostasis inside the organism. Its expression is regulated by several factors such as inflammation, hypoxia and iron overload (Nemeth E et al, 2006, Ganz T, 2006). The peptide is formed of 25 aminoacids, is produced predominantly from hepatocytes and is secreted in the circulating system (Park CH et al 2001) where it regulates the concentration of iron through the interaction with ferroportin. This mechanism is cause of the degradation of the protein and accumulation of iron inside the cells, principally in enterocytes and macrophages (Nemeth E et al, 2004). Mutations that influence its expression can cause a state of anemia or haemochromatosis (Nicolas G et al, 2002; Roetto A et al, 2003). In the last years, was discovered the presence of hepcidin mRNA and protein in neurons (Zechel S et al, 2006; Wang Q et al, 2008), but its role was not investigated at all. In our preliminary studies, we have found that two astrocytoma cell lines produce hepcidin mRNA as was demonstrated by nucleotide sequence. Moreover, its expression can be modulated by some cytokines and lipopolysaccharide.

## **QUANTITATIVE ANALYSIS OF NICOTINAMIDE N-METHYLTRANSFERASE EXPRESSION IN URINE AND TUMOUR TISSUE OF BLADDER CANCER PATIENTS AND ITS POTENTIAL RELEVANCE FOR DISEASE DETECTION.**

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Carcinomas of urinary bladder rank among the top ten most common cancers worldwide. The majority of these tumours recur and progress to muscle-invasive disease, thus needing long-term and frequent follow-up. Urinary cytology lacks sensitivity for low-grade tumours. More sensitive and non-invasive methods are therefore required, what fosters interest in identifying new bladder cancer markers. In the present study, DNA macroarrays were used to profile the gene expression of tumour and non-tumour tissues obtained from patients with transitional cell carcinoma (TCC), the most common tumour of the urinary bladder. The enzyme Nicotinamide N-methyltransferase (NNMT) was identified as highly expressed in bladder cancer. Real-Time PCR analysis, Western blot, and catalytic activity assay confirmed NNMT upregulation. Moreover, NNMT mRNA levels were significantly higher in urine specimens from patients with bladder tumour than those detected in samples from healthy controls. Our results indicate that a marked NNMT increase is a peculiar feature of TCC and suggest the potential suitability of urine NNMT expression levels determination for the diagnosis of bladder cancer.

## **CALCIUM-INDEPENDENT PHOSPHOLIPASE A2 MEDIATES GLIOMA-ENHANCED PRO-ANGIOGENIC ACTIVITY OF BRAIN ENDOTHELIAL CELLS**

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Glioma is characterized by an active production of proangiogenic molecules. We preliminarily observed that conditioned medium (CM) from C6 glioma significantly enhanced proliferation and migration of brain endothelial cells (ECs). We then evaluated PKC $\alpha$ , ERK1/2, cPLA2 expression/phosphorylation, iPLA2 protein/mRNA levels and we found that phosphorylation of cPLA2, which was significantly stimulated after 24 h CM co-incubation, was attenuated by PKC $\alpha$ , ERK and PI3K specific inhibitors. By confocal microscopy, enhancement of fluorescence signal for phospho-cPLA2, phospho-ERK1/2, phospho-PKC $\alpha$  and iPLA2 was observed. siRNAs directed against iPLA2 and cPLA2 significantly inhibited cell proliferation, and were able to reduce front migration using a wound healing assay. VEGF stimulated EC proliferation and migration. Incubation of CM- or VEGF peptide-stimulated ECs with antibodies against VEGF or VEGF receptors 1/2 strongly reduced mitotic rate, migration, and phospho-cPLA2 and iPLA2 protein levels. Our findings suggest that PLA2 activities, particularly iPLA2, are involved in stimulating EC migration and proliferation, and are positively and PI3K cascade. regulated upstream by ERK1/2, PKC $\alpha$

## **THE TRANSCRIPTION FACTOR CHOP IS CRITICAL FOR WIN-MEDIATED DR5 UP-REGULATION IN APOPTOSIS INDUCED BY WIN/TRAIL CO-TREATMENT IN HEPATOMA CELLS**

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Recently, we have demonstrated that the cannabinoid WIN sensitizes hepatoma HepG2, Hep3B and SK-Hep1 cells to TRAIL-induced apoptosis and that the combination of the two drugs exerts synergistic cytotoxic effects. The study on sensitization mechanism evidenced that WIN significantly increased the expression of TRAIL death receptor DR5. This effect was dependent on the ER-stress-related factor CHOP whose mRNA and protein levels increased after WIN treatment. Moreover, other preliminary experiments indicated that CHOP up-regulation can be dependent by p8 protein which has been demonstrated to be an essential mediator of cannabinoid antitumor action. To assess the role of p8-CHOP axis on WIN-mediated DR5 up-regulation, we transfected HepG2 cells with siRNA against CHOP. The transfection resulted in a marked decrease of both CHOP and DR5 up-regulation thus confirming that CHOP induction is required for WIN-mediated DR5 increase. The suppression of CHOP by siCHOP transfection also significantly reduced the apoptosis induced by WIN/TRAIL combined treatment. Studies are in course to better define the biochemical route activated during WIN/TRAIL-induced apoptosis in hepatoma cells.

## **KARYOTYPIC COMPLEXITY AND CHROMOSOMAL ABERRATIONS IN HUMAN EMBRYONIC CANCER STEM CELLS 3AB-OS**

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We have recently produced, by long-term treatment of human osteosarcoma MG-63 cells with 3-aminobenzamide, a novel cancer stem cell line (3AB-OS), highly expressing genes required for maintaining stem cell state (Oct3/4, hTERT, nucleostemin, Nanog) and for inhibiting apoptosis (HIF-1a, FLIP-L, Bcl-2, XIAP, IAPs, and survivin) (1). Now, we have shown by spectral karyotyping and microarray comparative genomic hybridization that the intricate chromosomal aberrations present in MG-63 cells is exacerbated in 3AB-OS cells which show about 80 chromosomes some of which with a number of focal high-level amplification. We also report that, in contrast with MG-63 cells where TP53 is inactivated by hypermethylation, 3AB-OS cells have TP53 unmethylated, and overexpresses p53 which colocalizes in the nucleus with Nanog and PCNA and which is inversely correlated with differentiation. In addition p53 shows multiple molecular forms with a small MW form appearing localized in the cytosol. 3AB-OS cells could be an important tool for studying the molecular alterations leading to aneuploidy. A possible responsibility of p53 is suggested. 1. Di Fiore R. et al (2009) J Cell Physiol. 219, 301-3

## **SYNERGISTIC INTERACTION BETWEEN PARTHENOLIDE AND TRAIL INDUCES APOPTOSIS IN HUMAN HEPATOCARCINOMA CELLS**

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Parthenolide, a natural compound used in traditional medicine for its anti-inflammatory activity, has recently shown anti-tumor and apoptotic effects. Our studies demonstrated that HepG2, Hep3B and SK-Hep1 hepatocarcinoma cells, which are resistant to human recombinant TRAIL, are potently sensitized to TRAIL-induced apoptosis by low doses of parthenolide resulting in a marked synergist effect. To clarify the mechanism that accounts for this interaction, we demonstrated that parthenolide/TRAIL combination markedly increased DR4 and DR5. These effects might be correlated with STAT proteins modifications. In fact parthenolide and parthenolide/TRAIL combination decreased STAT3 and STAT5 and their phosphorylated forms. These lowering effects could be responsible for increased expression of death receptors, as suggested by the observation that down-regulation of STAT proteins by siRNA, stimulated the expression of both DR4 and DR5. Although the anti-tumor activity of parthenolide was identified recently, this research area appears promising as it is likely that parthenolide/TRAIL combination may contribute to the rational design of novel targeted therapies.

## **LANCL2 IS THE ABSCISIC ACID RECEPTOR IN HUMAN GRANULOCYTES AND IN RAT INSULINOMA CELLS**

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Abscisic acid (ABA) is a plant hormone regulating fundamental physiological functions in plants, such as response to abiotic stress. Recently, ABA was shown to be produced and released by human granulocytes, by insulin-producing rat insulinoma cells and by human and murine pancreatic beta cells. ABA autocrinally stimulates the functional activities specific for each cell type through a receptor-operated signal transduction pathway, sequentially involving a pertussis toxin (PTX)-sensitive receptor/G-protein complex, cyclic AMP, CD38-produced cyclic ADP-ribose and intracellular calcium. Here, the ABA receptor on human granulocytes and on rat insulinoma cells is identified as the lanthionine synthetase C-like protein LANCL2. Co-expression of LANCL2 and CD38 in the human HeLa cell line reproduces the ABA-signaling pathway. Results obtained with granulocytes and CD38+/LANCL2+ HeLa transfected with a chimeric G protein (Galphaq/i) suggest that the PTX-sensitive G protein coupled to LANCL2 is a Gi. Identification of the mammalian ABA receptor will enable the screening of synthetic ABA antagonists as prospective new anti-inflammatory and anti-diabetic agents.

## **INVOLVEMENT OF AQUAPORIN 8 IN NOX-RELATED REDOX SIGNALLING ACROSS THE PLASMA MEMBRANE IN LEUKEMIC CELLS**

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Aquaporins (AQP) constitute a large family of integral membrane proteins, present in all domains of life, that form pores appearing to be designed for the selective passage of water and glycerol [1]. Since recent evidence suggests that a mammalian aquaporin homologue (AQP8) has the capacity to channel H<sub>2</sub>O<sub>2</sub> across membranes, we hypothesized that AQP8 could be a potential way through which H<sub>2</sub>O<sub>2</sub>, produced by NAD(P)H oxidase (Nox), passes rapidly the plasma membrane to act as signal molecule inside leukemic cells under study. Indeed, redox deregulation of proliferative pathways involving ROS (particularly H<sub>2</sub>O<sub>2</sub>) in cancer initiation and progression is now firmly established. Nevertheless, it still remains unclear whether Nox-generated ROS outside the cell can enter the cell by simple diffusion or protein-mediated transport. Thus, we investigated the presence of AQP8 and the effects of Ag<sup>+</sup>, a potent inhibitor of AQP [2], on intracellular ROS level and viability in leukemic cells, where a correlation between Nox-derived ROS and proliferation occurs [3]. 1 *Agre P Biosci Rep.* 24:127, 2004 2 *Niemietz CM et al. FEBS Lett.* 531:443, 2002 3 *Prata C et al. Free Radic Res.* 42:405, 2008

## **POSSIBLE INVOLVEMENT OF PLASMA MEMBRANE CAVEOLAE/LIPID RAFTS IN VEGF-MEDIATED REDOX SIGNALING IN HUMAN LEUKEMIC CELLS**

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Caveolae and lipid rafts are cholesterol and sphingolipid-rich plasma membrane microdomains; they act as platforms for compartmentalization of proteins involved in different transduction signal mechanisms, one of which is VEGF-induced redox signaling. Recent experimental evidence shows that VEGF promotes the release of VEGF-R2 from caveolae/lipid rafts and its consequent activation, possibly stimulating ROS production via activation of NAD(P)H oxidase (Nox) in endothelial cells (1). In turn, Nox-derived ROS are involved in various signaling pathways. We previously reported that ROS can protect leukemic cells from apoptosis (2). We hypothesize that, in B1647 myeloid leukemic cell line, expressing two isoforms of the Nox family, Nox2 and Nox4 (3), rafts/caveolae can represent the link among ROS generation, VEGF-R2 autophosphorylation, increase in glucose uptake mediated by Glut-1, and cell survival. 1 *Ushio-Fukai M Antioxid Redox Signal* 9, 731, 2007 2 *Maraldi T et al. Free Radic Biol Med* 46, 244, 2009 3 *Prata C et al. Free Radic Res* 42, 405, 2008

## **RIMONABANT-INDUCED APOPTOSIS IN LEUKAEMIA CELLS: ACTIVATION OF CASPASE-DEPENDENT AND -INDEPENDENT PATHWAYS**

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SR141716 (SR), a cannabinoid CB1 receptor antagonist, has more recently shown to inhibit tumor cell growth (Sarnataro et al,2006). As SR exhibited minimal if not any toxicity in normal white blood cells (Malfitano et al., 2008), we investigated its anti-tumor potential in leukaemia cells. SR was found to affect cell cycle progression by causing a marked G0/G1 arrest in U937 and a less pronounced S block in Jurkat. In addition SR was found to induce cell death, Jurkat being more susceptible than U937. SR-treated cells exhibited the morphological and biochemical features of apoptosis and to some extent of necrosis. While in Jurkat the apoptotic process was typically caspase-dependent, in U937 caspase-independent pathways were also activated. The occurrence of early protein PARylation and apoptosis reversion by PARP inhibitors suggested a key role of PARP in SR-induced U937 death. Simultaneous inhibition of caspase- and PARP-dependent pathways had an additive effect, suggesting that these pathways are independently activated in U937. In particular, sustained PARP activation was counteracted by caspase-3- and cathepsin-mediated proteolytic cleavage (negative feedback loop).

## **PROTEOMIC ANALYSIS OF FORMALIN-FIXED PARAFFIN-EMBEDDED PARATHYROID ADENOMA**

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Parathyroid carcinoma is a rare cause of parathyroid hormone dependent hypercalcaemia. Until now, no biomarkers that distinguish carcinoma from adenoma have been identified. Although fresh or frozen samples are more ideal samples for proteomic investigation, they are difficult to obtain especially for this rare kind of tumour. Therefore, we performed a proteomic analysis in formalin-fixed paraffin-embedded (FFPE) parathyroid tissue samples. Briefly, slides of FFPE adenoma parathyroid tissues were deparaffinized and rehydrated, then resuspended in buffers settled at different pH with 2% SDS and 0.2 M Glycine and heated. Finally protein fraction was precipitated prior to undergo 2D electrophoresis. Among the different protein extraction protocols tested, a major yield of spot number is obtained at pH 4 or 6 followed by protein precipitation with methanol-chloroform (220 spots). Some of these spots were identified as ATP synthase subunit beta, 40S ribosomal protein SA and cytochrome b-cl complex subunit 1. Our preliminary results provide an initial detailed framework to carry out the further protocol refinement needed to investigate parathyroid cancer.

## **PROTEOMIC PROFILE OF FINE NEEDLE ASPIRATION FLUID OF PAPILLARY THYROID CANCERS BY SELDI-TOF/MS**

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The aim of the present study was to perform a proteomic analysis of fine needle aspiration fluid (FNA) of thyroid papillary cancers with surface-enhanced laser desorption/ionization time-of-flight/mass spectrometry (SELDI-TOF/MS). FNA samples (n=31) were made on thyroid nodules of three variants of papillary cancer: classical (cPTC), tall cell (TCV) and follicular (FVC), and from ipsilateral and contralateral lobe normal tissue (controls). SELDI-TOF-MS analysis was performed using different proteins chip arrays (CM10, Q10, IMAC, H50) that selectively bind and retain whole classes of proteins from complex samples. The mass profile of the bound proteins was read by the ProteinChip reader. The analysis of the obtained spectra, allowed us to observe significant change of intensity of some peaks in FNA papillary cancer with respect to the controls. The average variability of each class was found to be in the normal experimental range (CV of 3.2 to 15.3%). This study shows the utility of FNA to obtain the protein profiles in the SELDI-TOF-MS application and demonstrates the effectiveness of this technique as a tool for discovering potential biomarkers in thyroid cancer.

## **SEMAPHORIN 3A REGULATES THE ANGIOGENIC SWITCH AND TUMOR PROGRESSION DURING SPONTANEOUS CARCINOGENESIS**

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Class 3 Semaphorins regulate tumor progression and angiogenesis. Based on these evidences we analyzed the role of Sema3A in angiogenesis during spontaneous tumorigenesis of pancreas in RipTag2 mice. Real-time RT-PCR and confocal microscopy analysis revealed that Sema3A was basal expressed in normal pancreatic islet, up-regulated in pre-malignant stages and strongly down-regulated in tumors. Sema3A was mainly localized in vessels and, to a lesser extent, in epithelial cells of pre-malignant lesions. We over-expressed Sema3A directly into pancreas of RipTag2 mice through the abdominal aorta by a new gene delivery system employing adeno-associated virus (AAV8). Re-expression of Sema3A in tumor-bearing RipTag2 led to a reduction in tumor volume, tumor blood vessel density and branching and to an increased in pericyte coverage compared to controls. Remarkably, Sema3A treatment inhibited  $\beta$ 1 integrin activation in tumor endothelial cells. On the contrary, pharmacological inhibition of endogenous Sema3A during the angiogenic switch enhanced angiogenesis and accelerated tumor progression. Finally, Sema3A over-expression at the earlier stages of carcinogenesis delayed the angiogenic switch.

## **CERAMIDE AND SPHINGOSINE-1-PHOSPHATE EXERT OPPOSITE ROLES ON AUTOPHAGIC DEATH OF MALIGNANT GLIOMA CELLS**

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Growing literature supports that the bioactive sphingoids ceramide and sphingosine-1-phosphate (S1P) act as important players in tumor biology, and emerging evidence suggests they are involved in drug resistance. We investigated the possible role of ceramide and S1P in the toxicity of the alkylating drug temozolomide (TZ) in T98G cells from human malignant gliomas. These tumors are among the deadliest cancers, being resistant to many treatments, and prone to acquire drug resistance. In T98G cells, TZ induces autophagic death. This is preceded by the increase of ceramide that, in turn, is able to mimic TZ toxicity. In TZ-resistant cells obtained from T98G, ceramide is unchanged, but S1P levels and sphingosine kinase-1 (SK) expression are higher than in T98G. The exposure of resistant cells to SK inhibitors restores cell sensitivity to TZ. Moreover, extracellularly applied S1P, but not S1P generated by caged S1P photolysis, raises the survival of T98G cells exposed to ceramide. All this suggests that ceramide and S1P exert opposite roles on glioma autophagic death, and that extracellular S1P may offer glioma cells a survival advantage, resulting in resistance to autophagic death.

## **TRANSACTIVATION OF EPIDERMAL GROWTH FACTOR RECEPTOR (EGFR) BY FORMYL PEPTIDE RECEPTOR (FPRL1) IN CALU-6 CELLS**

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Formyl-peptide receptors (FPR) are expressed in several cell types and a wide variety of agonists of FPR and of its FPRL1 variant have been identified. In nonphagocytic cells, agonist/FPR binding also induces transactivation of the membrane receptors PDGF-R, EGF-R and uPAR that in turn trigger specific intracellular signal transduction pathways. We demonstrate that CaLu-6 cells express FPRL1 and stimulation with WKYMVm results in the transactivation of EGFR and in c-Src activation. Furthermore, phosphorylation of EGFR is prevented by pertussis toxin (PTX) and by specific inhibitors of general tyrosine kinases and of c-Src, indicating that Src kinase plays a key role in bridging the signal transduction between FPRL1 and EGFR. c-Src activation and EGFR transactivation are also prevented by pretreatment with WRW4, an antagonist of FPRL1 receptor. The downstream signaling triggered by WKYMVm in CaLu-6 cells involves the phosphorylation on Tyr705 and Ser727 residues of STAT3. These events are prevented by PTX and by the EGFR specific tyrosine kinase inhibitor AG1478. Our study proposes that FPRL1 and EGFR cooperate to contribute the exacerbation of tumoral phenotype of these cells.

## NEW DIAGNOSTIC PROSPECTIVE IN HUMAN COLORECTAL CANCER

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Colorectal cancer (CRC) is often diagnosed at a late stage with concomitant poor prognosis. Early detection greatly improves prognosis but the invasive and unpleasant nature of current diagnostic procedures limits their applicability. No serum-based test is currently of sufficient sensitivity or specificity for widespread use, hence, there is great need for new biomarkers for early detection of CRC. This work presents a new approach to study serum proteome in CRC, based on bi-dimensional chromatographic separation of the proteins by ETTAN LC Purifier (GE Healthcare), a very sensitive instrument which permits the complete resolution of the proteins in less than 2 hours. We analyzed 10 patients affected by CRC, whose clinical history was well known, compared to 10 healthy controls. After the albumine depletion, the samples were loaded on MONO Q and MONO S ion exchange columns put in tandem. We evidenced a peak more than 5 times higher in all the stages of the pathology, respect to the controls. Futures studies will be directed to confirm the interesting data and to the identification of altered protein(s) by mass spectrometry, opening new diagnostic prospective in CRC.

## SEROTONIN TRANSPORTER EXPRESSION IN CONTROL AND PHORBOL ESTHER-TREATED HUMAN MEGAKARYOBLASTIC MEG-01 CELL CULTURES

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This study further investigated the effects of the phorbol ester beta-TPA on serotonin transporter (SERT) in the megakaryoblastic cell line MEG-01. Previously, we had examined SERT in control and 3 day beta-TPA-treated MEG-01 cells (1). Controversial results obtained afterwards impelled us to assess SERT by modifying Western blot and immunofluorescence procedures: primary antibodies directed against other SERT epitopes and double immunofluorescence/nuclear bis-benzimide labeling were used herein. SERT function was measured by [3H]5-HT re-uptake and MEG-01 beta-TPA stimulation was extended to 8 days. By these techniques, we reported a functional SERT isoform in untreated megakaryoblasts. Beta-TPA stimulation provoked MEG-01 differentiation together positive SERT fluorescence diffuse in cell bodies and blebs; SERT resulted 1.5-fold increased in treated cells by immunoblot and 5-HT re-uptake. These findings imply a role of serotonin in platelet differentiation and encourage to depict SERT molecular/regulatory features during megakaryocytopoiesis. References 1.Schmid L. et al, 2007, Ital. J. Biochem.56,3, 2.43. Acknowledgements: The present work was supported by a MIUR grant.

## **ROLE OF CERAMIDE TRANSFER PROTEIN CERT AS A REGULATOR OF GLIOMA CELL PROLIFERATION**

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Different lines of evidence indicate that, as in the majority of cells, in glial cells too, ceramide (Cer) represents a crucial mediator of cell death and acts as a negative regulator of cell proliferation. Stimulation of SM biosynthesis represents a crucial step in the Cer-mediated control of cell growth. Since the Cer transfer protein CERT has been identified as a key factor for the transport of Cer to the Golgi apparatus for sphingomyelin (SM) biosynthesis, in this study we evaluated the possible role of CERT in the regulation of glioma cell proliferation. Although down regulation of CERT inhibited SM biosynthesis, it resulted in an increased cell proliferation associated to an increased ERK1/2 phosphorylation. In vitro and in vivo experiments indicated that CERT is necessary for the inhibitory effect of Cer on ERK1/2. Moreover immunoprecipitation experiments suggested a role of CERT as a scaffold protein for a phosphatase activity involved in ERK1/2 inactivation. These results suggest that, besides its role on Cer metabolism, CERT participates to Cer signalling favouring the interaction of Cer with its downstream targets relevant in the control of glioma cell proliferation

## **CHARACTERIZATION OF AN HLA-A2-RESTRICTED EBNA1-DERIVED CTL EPIOTOPE**

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The Epstein-Barr virus (EBV) nuclear antigen 1 (EBNA1) is expressed in all EBV+ tumors and is therefore an interesting target for specific immunotherapy. Since alleles of the HLA-A2 family are dominantly expressed in Caucasians we sought to identify EBNA1 specific CTL responses restricted through this allele. We report on the characterization of the LQTHFAEV (LQT) epitope. LQT-specific memory CTLs responses were reactivated only in 3 of 14 healthy EBV+ donors. LQT-specific CTL clones did not lyse EBV carrying lymphoblastoid cell lines and Burkitt's lymphoma cells nor EBNA1 transfected BL cells but specifically released IFN- $\gamma$  upon stimulation with HLA-matched EBNA1-expressing cells and this response was enhanced by deletion of the Gly-Ala domain that protects EBNA1 from proteasomal degradation. The poor presentation of the endogenously expressed LQT epitope was not affected by inhibition of peptidases that trim antigenic peptides in the cytosol but full presentation was achieved in cells expressing a trojan antigen construct that releases the epitope into the ER. Thus, inefficient proteasomal processing appears to be mainly responsible for the poor presentation of this epitope.

## INVOLVEMENT OF SIALIDASE NEU4L IN NERVOUS SYSTEM TUMORS

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Dept. of Medical Chemistry, Biochemistry and Biotechnology

The aberrant expression of sialoglycoconjugates characterizes cancer. Sialidases which cleave sialic acid residues seem to be involved in these phenomena and are severely impaired during carcinogenesis. Sialidase Neu4 is coded as two forms, long (Neu4L) and short and is rarely expressed in adult tissues. Here, we demonstrated that Neu4L expression is unusually high in two nervous system tumors and is involved in their control of proliferation/self-renewal. Neuroblastoma SK-N-BE cells were transfected with Neu4L cDNA. This induced a marked acceleration of proliferation, assessed by an increase of [3H]thymidine incorporation (+ 45%) and by growth curve (+ 36%). After serum withdrawal, Neu4L over-expressing SK-N-BE cells failed to block, enhancing the expression of cyclin D1 and cyclin D2. The direct substrates of Neu4L in SK-N-BE cells are soluble glycoproteins around 60 kDa. In glioblastoma, we discovered a significantly high expression of Neu4L in stem cells isolated from U87 cell line, opposed to the bulk of the cells which constitutes the tumor, in which Neu4L is scarcely relievable. Also in this case, Neu4L seems to be related to a undifferentiated/self renewal phenotype.

## PROTEOMICS ANALYSIS OF K562 CELLS TREATED WITH INHIBITORS OF GLYCOSYLATION

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The sugar moieties of mammalian glycoproteins show significant changes in their structures and relative occurrences during growth, development and differentiation and have been involved in many biological functions. Our study essentially focuses on the analysis of the glyco(phospho) conjugated, a family of biomolecules whose altered expression is often correlated to various pathologies. Human myelogenous leukemia (K562) cells have been treated with some antiretroviral drugs, namely 2',3'dideoxyinosine (ddI), 2',3' dideoxycytidine(ddC) and 5-azacytidine (5-AzaC). The concentrations tested (20 mM ddI, 5 mM ddC, 3 mM 5-AzaC) induced a block of the cell cycle in phase S. Among the various glyco(phospho) proteins, our attention has been attracted on those containing residues of N-acetylglucosamine, or phosphate, both groups able to link serine, tyrosine or threonine. K562 cells treated with ddC showed an increase of glyco(phospho) conjugated, with respect to Control untreated cells, as evidenced by ELISA and FACS analysis. On the contrary, K562 cells exposed to 5-AzaC, displayed a decrease of glyco(phospho) conjugated, while ddI treatment did not induce any significant alteration.

## **TEMOZOLOMIDE AND METALLOPROTEINASES INHIBITORS: NEW INSIGHTS FOR HUMAN MALIGNANT GLIOMA THERAPY**

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Malignant glioblastomas (GBM) are one of the most difficult tumors to treat, characterized by rapid cell proliferation and high invasiveness into the surrounding brain. Despite modern advances in chemotherapy and surgery, most patients die of tumour or related complications, in the first year from diagnoses Among chemotherapeutic compounds, temozolomide (TMZ) has shown activity against glioblastoma, however many patients develop resistance to the drug. For this reason, recent studies look for new multitarget therapy (1). Since matrix metalloproteinases (MMP) are important in glioma cell invasion, the combination of TMZ and MMP inhibitors (MMPi) has been proposed in GBM. In the present study, we investigated the effects of new selective MMP-2 inhibitors on viability, and invasiveness of a human glioma cell line. Moreover, we evaluated the co-treatment of cells with specific MMP2 inhibitors and TMZ, as multi-target approach. Our data revealed that the cell treatment with selective MMPi resulted in a remarkable inhibition of glioma cell invasion. Interestingly, such inhibition was amplified in the cells co-treated. Such studies produced encouraging results.[1] Stern and Raizer, 200

## **NOVEL ISOPENTENYLADENOSINE ANALOGUES: SYNTHESIS AND EVALUATION OF ANTIPROLIFERATIVE ACTIVITY**

**Roberta Ottria<sup>1</sup>, Silvana Casati<sup>1</sup>, Erika Bandoli<sup>1</sup>, Jeanette A.M.Maier<sup>1</sup>  
Ada G. Manzocchi<sup>2</sup>, Pierangela Ciuffreda<sup>1</sup>**

1. DISP LITA-Vialba 2. Dip.Chim.Bioch.Biotec.per Med., Facoltà Medicina e Chirurgia, Università degli Studi di Milano

N6-isopentenyladenosine (iPA) is able to inhibit protein prenylation and competes for nucleoside transport. It has been shown that iPA exerts a potent in vitro anticancer activity while it has a slight effect on tumour growth in rodents. This lack of in vivo activity could be due to the short plasma half-life of iPA as it is well known for other nucleosides. To identify compounds endowed with in vitro and in vivo antiproliferative activity, we have investigated structural modifications of iPA. We synthesized acyclonucleosides characterized by the presence of an acyclic component, structurally resembling part of the ribose moiety in iPA. Then we synthesized iPA analogues in which the hydroxyl group were replaced by an hydrogen group in order to verify the importance of each hydroxyl group of the furanosidic moiety for the activity of iPA. Lastly we also synthesized iPA analogues in which the N6-position was differently substituted with the aim of verify the importance of the isopentenyl chain for the activity of the molecule. All compounds were in vitro tested using different cell lines and the proliferation assay was carried out in the presence and absence of serum.

## AN EXOGENOUS INDOLE-DERIVATIVE INDUCES SYNCHRONIZATION IN OVARIAN CANCER CELLS

**C. Cappadone, N. Calonghi, J. Hysomema, C. Parolin, G. Sartor, L. Masotti**

Department of Biochemistry "G.Moruzzi", Alma Mater Studiorum, Università di Bologna, Bologna, Italia

Recently the antitumor activity of a new indole-derivative, 3L, towards IGROV1, an ovarian cancer cell line, has been reported (1). Further studies have demonstrated that this compound induces a synchronization of cells in G<sub>0</sub>, after 72 hours of treatment. It is known that serum-starved as well as tumour cells can be induced to reenter the cell cycle by treatment with either serum or individual growth factors. 3L-treated cells instead are unable to reenter the cycle upon treatment with EGF. 3L exhibits fluorescence properties with excitation at 488nm and emission at 540nm. Dual-dye labeling with Propidium Iodide, followed by colocalization analysis, has revealed that 3L can move to the nuclei. This result correlates to LC-ESI MS analysis, which has shown that the molecule is able to reach the nucleus already 6 hrs after administration. The aim of this work is to identify the molecular targets and mechanism of action of 3L: the fluorescent characteristics of the molecule can provide a useful tool for linking biochemical investigation with optical visualization methods. 1. Andreani A, et al. (2007) J Med Chem, 50(14):3167-72.



## **Drug, receptors and metabolism**

**Poster session:  
26/09/2009 (h. 12.00-13.00)**



## **FROM CRYSTAL STRUCTURE TO BIOLOGICAL ACTIVITY: DESIGN AND CHARACTERIZATION OF A NOVEL DUAL PPAR $\alpha$ /GAMMA AGONIST WITH POTENT ANTIDIABETIC AND ANTI-OBESITY ACTIVITY**

**M. Crestani, F. Gilardi, M. Giudici, F. Loiodice<sup>1</sup>, G. Pochetti<sup>2</sup>, A. Lavecchia<sup>3</sup>, U. Guerrini, G. Rando, A. Maggi, N. Mitro**

Università degli Studi di Milano, <sup>1</sup>Università degli Studi di Bari, <sup>2</sup>CNR Rome, <sup>3</sup>Università degli Studi di Napoli

Peroxisome proliferator-activated receptors (PPARs) are nuclear receptors playing a key role in the regulation of lipid, glucose and energy metabolism. Recently we identified a new compound, LT175, that binds and activates both PPAR $\alpha$  and  $\gamma$  and reported the 3D structures in the complex with this ligand. Coregulator recruitment by FRET assay reveals that LT175 is a full PPAR $\alpha$  and a partial PPAR $\gamma$  agonist. In transcription assays with PPRE-Luc reporter mice LT175 switches on the PPAR program in typical target tissues. LT175 ameliorates the metabolic profile and insulin sensitivity and reduces visceral fat as assessed by *in vivo* NMR imaging in high fat fed mice. Interestingly, LT175 increases brown fat contributing to decrease body weight via fat burning. Real time qPCR shows that LT175 enhances the expression of target genes in the liver and adipose tissue. Altogether, we demonstrate that the structure-based design of a dual PPAR $\alpha$ / $\gamma$  ligand allowed to obtain a lead compound with favorable effects on glucose and lipid metabolism that could be exploited to treat diabetes, obesity and their cardiovascular complications. Funded by FP6 LSHM-CT-2006-037498 and Università degli Studi di Milan

## **STUDY ON THE EFFICACY OF RAPAMYCIN AS AN INDUCER OF FETAL HEMOGLOBIN IN PRIMARY ERYTHROID CULTURES FROM PATIENTS WITH HEMOGLOBINOPATHIES**

**R. Di Marzo<sup>1</sup>, A. Pecoraro<sup>1</sup>, A. Troia<sup>1</sup>, C. Scazzone<sup>2</sup>, R. Calzolari<sup>1</sup>, B. Spina<sup>1</sup>  
V. Motta<sup>1</sup>, R. Di Stefano<sup>1</sup>, A. Maggio and A. Bono<sup>2</sup>**

<sup>1</sup>U. di Ric. "P. Cutino" Div. Ematologia II A.O. V. Cervello <sup>2</sup>Dip. di Biotec. Mediche e Med. Legale, Università di Palermo

Among the various drugs proposed to increase fetal hemoglobin (HbF) production and to improve the clinical course in sickle cell disease and beta-thalassemia, hydroxyurea (HU) seems to be the most effective. However the increases in HbF and the patient's response are variable: some patients are responders, while others exhibit little or no change in HbF level. New pharmacological agents could be useful for patients who are not responders to HU treatment or who show a reduction of response during long-term treatment. The main goal of our project was to evaluate the efficacy of Rapamycin as inducer of HbF production in primary erythroid cell culture system from beta-thalassemia and sickle cell patients. The combined use of quantitative RT-PCR technique, flow cytometric analysis and HPLC allowed us to determine the increase on HbF and on gamma globin gene expression in human erythroid cells treated with Rapamycin. The results of our study, carried out on a population of 38 patients, demonstrated an increase of gamma globin gene expression in 16 of them indicating that Rapamycin could be a good candidate to be used "in vivo" as an inducer of HbF for the treatment of hemoglobinopathies.

## DRUG-DRUG AND DRUG-FOOD INTERACTIONS OF CYTOCHROME P450 3A4

**S. Sadeghi, C. Bologna, S. Ferrero, G. Di Nardo and G. Gilardi**

Department of Human and Animal Biology, University of Turin

Inhibition of CYP-mediated (cytochrome P450) drug metabolism by a concomitantly administered second drug is one of the major causes of drug–drug interactions in humans and can lead to serious adverse reactions or toxic side effects. Although less publicised, drug-food interactions can also cause an increase or decrease in the oral drug bioavailability when co-administered, the most well known case being that of grapefruit juice and the short-acting calcium channel blocker, nifedipine. One major limitation of these types of studies is the lack of fast and reliable tests for measuring such phenomena. Here we report the first in vitro characterisation of drug-drug and drug-food interactions of CYP enzymes using an electrochemical platform devised in our group. The use of in vitro data to predict the CYP inhibition by a co-administered drug/food is attractive because of the rapid and simple experimental procedures involved. Data will be presented on CYP3A4 inhibition by both strong and weak inhibitors of this enzyme; ketoconazole, cimetidine, grapefruit juice, curcumin (curry spice turmeric) and resveratrol (red wine). Fluridone as a new anti-inflammatory drug.

## FLURIDONE AS A NEW ANTY-INFLAMMATORY DRUG

**1 Mirko Magnone, 1 Lucrezia Guida, 1,2 Santina Bruzzone, 1,2 Sonia Scarfi, 1Annalisa Salis  
1Antonio De Flora and 1Elena Zocchi.**

1 DI.ME.S section of Biochemistry and CEBR, University of Genova, Italy;2 Advanced Biotechnology Center of Genova, Italy

Fluridone is an herbicide largely utilized in agriculture for its documented absence of adverse effects on animals. The mechanism of Fluridone toxicity in plants involves inhibition of the biosynthetic pathway of  $\beta$ -carotene, the precursor of the plant hormone abscisic acid (ABA). Our group recently discovered that ABA is also synthesized in human inflammatory cells, where it stimulates cellular pro-inflammatory activities. Thus, we investigated whether Fluridone could represent a new anti-inflammatory compound in human cells. Results obtained show: 1) the increase of the intracellular ABA concentration ([ABA]<sub>i</sub>) in stimulated human monocytes, granulocytes and lymphocytes is inhibited when cells were pre-incubated with Fluridone (ranging between 0.5-50  $\mu$ M) in a dose-dependent manner; 2) Fluridone inhibits the release of pro-inflammatory mediators (MCP-1, TNF- $\alpha$ , PGE<sub>2</sub>) and the increase of COX-2 expression in stimulated human inflammatory cells. These results suggest that Fluridone could represent the prototype of a new anti-inflammatory drugs active on human ABA biosynthesis. Hepatic effect of HDAC inhibitors (HDACi) on the regulation of CYP7A1 by bile acids and FGF19.

## HEPATIC EFFECT OF HDAC INHIBITORS (HDACI) ON THE REGULATION OF CYP7A1 BY BILE ACIDS AND FGF19

**M. Giudici, F. Gilardi, N. Mitro, A. Galmozzi, E. Scotti, E. De Fabiani, D. Caruso, M. Crestani**

Lab "Giovanni Galli" Biochim Biol Mol-Spet Massa, Dipartimento Scienze Farmacologiche, Università degli Studi di Milano

Bile acid (BA) synthesis is regulated through mechanisms that target the transcription of cholesterol 7 $\alpha$ -hydroxylase (CYP7A1), the rate-limiting enzyme of cholesterol degradation. Mouse fibroblast growth factor 15 (FGF15) and human ortholog FGF19 have been identified as the BA-induced intestinal factors that mediate feedback inhibition of CYP7A1 transcription in liver. However, the mechanism underlying the FGF19 inhibition of BA synthesis remains unclear. Investigating the role of histone deacetylases (HDACs), we found that HDACs are key regulators that repress CYP7A1 gene transcription in response to BA. In fact, BA sequentially recruit HDAC7, 3, 1 and corepressor SMRT $\alpha$ . Moreover, HDAC inhibitors stimulate CYP7A1 expression by preventing the BA negative feedback. We treated cultures of hepatic cells with recombinant hFGF19 (100ng/ml) in the absence or presence of the HDAC-i VPA (1.5mM) or TSA (200 nM) for 16h. The expression of CYP7A1 mRNA and FGF19 target genes was monitored by real time qPCR. Our preliminary results suggest that HDACs could be involved in downregulation of CYP7A1 in the FGF19-mediated pathway. GRANTS: EC SOUTH LSHM-CT2006-037498 & FONDAZIONE CARIPLO 2008.2511

## IDENTIFICATION OF A SUB-POPULATION OF PITUITARY LACTOTROPH CELLS REQUIRED FOR THE NORMAL LUTEOTROPHIC FUNCTION OF PROLACTIN IN MICE

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Sub-populations of rodent pituitary lactotroph cells have been described based on morphological and functional heterogeneity, but it is not clear whether this reflects different stages of cell maturity or distinct lineages of lactotrophs with specific physiological functions. Two mouse models have either DsRed or Cre-recombinase expression driven by the same prolactin (PRL) promoter, leading to expression of both specifically in a fraction of lactotrophs. Their expression is overlapped, with DsRed reflecting current transgene activity whilst Cre activity detected with a ROSA-YFP reporter permanently marks cells that have ever been Cre-positive, suggesting that transgene-positive and negative cells represent distinct sub-populations of PRL cells. Ablation of all cells expressing Cre using ROSA26-DT induces a severe reduction of the pituitary PRL content and of the fertility, with 80% of matings not resulting in pregnancy. GRF-M2 mice with a similar reduction in pituitary PRL content have normal phenotype, suggesting that the reduced fertility is not a result of reduced pituitary PRL but that the transgene-expressing lactotrophs are required for the luteotrophic function of PRL.

## **A SMALL SYNTHETIC PURINE, REVERSINE, INCREASE CELL PLASTICITY OF DIFFERENTIATED CELLS AND OF ADULT STEM CELLS**

**E. Conforti<sup>1,2</sup>, E. Arrigoni<sup>3</sup>, M. Piccoli<sup>1,2</sup>, L. de Girolamo<sup>4</sup>, B. Venerando<sup>1,2</sup>, G. Tettamanti<sup>1</sup>  
A. Brini<sup>3</sup> and L. Anastasia<sup>1,2\*</sup>**

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Stem cells have received an increasing attention by the scientific community for their potential applications in regenerative medicine. In recent years, a new approach has surfaced, which is the possibility of re-programming somatic cells (adult cells) into stem-cell-like progenitors. In fact, a growing body of evidence suggests that lineage-restricted somatic cells are capable of gaining increased plasticity, comparable to that of stem cells. Along this line, a synthetic purine, reversine, has been shown to increase cell plasticity of differentiated cells, including murine myoblasts and human dermal fibroblasts (1, 2). Moreover, initial results seem to point out that reversine may also increase cell plasticity of adult stem cells, that often can be induced to differentiate into the desired cell types with yields that are too low to be practical for their therapeutic use. Herein we show that reversine treatment improves the differentiation capability of dermal fibroblasts, mesoangioblasts, and mesenchymal stem cells. [1] Anastasia L., Sampaolesi M, et al. *Cell Death Differ.* 2006, 12, 2042-51. [2] Fania, C, Anastasia L, et al. *Electrophoresis.* 2009 Jun;30(12):2193-206.

## **USE OF SMALL SYNTHETIC MOLECULES FOR IN-SITU TRANSDIFFERENTIATION OF MYOCARDIAL FIBROBLASTS TO CARDIOMYOCITES**

**Marco Piccoli<sup>1,2</sup>, Andrea Garatti<sup>1</sup>, Lorenzo Menicanti<sup>1</sup>, Bruno Venerando<sup>1,2</sup>  
Guido Tettamanti<sup>1</sup> and Luigi Anastasia<sup>1,2\*</sup>**

1IRCCS Policlinico San Donato, San Donato M.se (Milan); 2Dep. of Med. Chem. Biochem. and Biotech, Univ. of Milan

Myocardial scar formation is marked by fibroblast proliferation and scar tissue deposition. This process impairs heart function by inducing cardiac remodelling and decreasing myocardial compliance. Several therapeutic modalities promoting regeneration have been developed, modulating various aspects of the healing process. However, the conversion of myocardial scar fibroblasts into cardiomyocytes may be an effective alternative treatment to limit loss of cardiac performance after myocardial injury. Recently, a new class of small molecules, called sulfonyl-hydrazones (Shz), has been identified as potent activators, in human PBMCs, of Nkx2.5, one of the earliest lineage-restricted genes expressed in cardiovascular progenitor cells [1]. On these bases, we decide to investigate the differentiation effects of Shz treatment on fibroblasts, isolated from postmyocardial infarction scars of human left ventricles. In the preliminary phase of this study, we demonstrated a 28-fold increase of the expression of the cardiac troponin T (TNNT2) in fibroblast treated with Shz for 14 days as compared to control cells. [1] H. Sadek et al. *PNAS* 105(16); 2008; 6063-8

## DESIGN OF O-ACETYL SERINE SULFHYDRYLASE INHIBITORS BY MIMICKING NATURE

**F. Spyrakis, E. Salsi, A. Bayden, G. E. Kellogg, B. Campanini, S. Bettati, P. Cozzini  
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O-acetylserine sulfhydrylase-A (OASS) catalyzes the last step of L-cysteine biosynthesis and is inhibited by serine acetyltransferase (SAT) that inserts part of its C-terminal decapeptide into OASS active site. By exploiting the available structure of *Haemophilus influenzae* OASS complexed with SAT C-terminal pentapeptide (MNLNI), 400 MNXXI pentapeptides were generated and docked into OASS active site using GOLD and scored by the HINT force field. HINT analysis indicates that, in agreement with experimental findings, the terminal Ile accounts for about 50% of the binding interaction in most pentapeptides, and Glu or Asp at position P4, and to a minor extent at position P3, were found to significantly contribute to the binding interaction. The HINT scores for fourteen pentapeptides were found to well correlate with the experimentally determined dissociation constants. Moreover, the docked poses for three high affinity pentapeptides were compared with the conformations determined by x-ray crystallography of the OASS-pentapeptide complexes. Results defines the structure of a pharmacophore suitable for the design of inhibitors with potential antibiotic activity.

## ANTIMICROBIAL PEPTIDES: INTERACTION STUDIES AND MECHANISM OF ACTION WITH MODEL MEMBRANES

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Antimicrobial peptides (AMPs) are effector molecules of the innate immune response of pluricellular organisms. Temporins and bombinins H are two families of cationic peptides, isolated from the anurans skin. The peptides used in this work were temporins A and B, bombinins H2 and H4. Experiments with lipid monolayers, have shown a significant increase in monolayer surface pressure ( $\Delta\pi$ ), strictly related to the degree of the peptide insertion and to disturbance of the monolayer hydrophobic core. These peptides have shown to induce the leakage and the release of the liposome-entrapped low molecular mass (622 Da) fluorescent probe calcein. In addition, the same peptides were found to cause the release of FITC-dextran of various size from liposomes of different composition as a function of the size of the entrapped marker. Experiments give with trypsin loaded-liposomes, demonstrated that these peptides are translocated inside the liposome where they undergo hydrolysis by trypsin. In these liposomal samples incubated with SBTI (soybean trypsin inhibitor), the generation of pores caused by the various peptides, can allow the entry of SBTI inside liposomes thus blocking trypsin activity.

## HIGHLY POLYMERIZED HYALURONAN BY INTERACTING WITH TOLL-LIKE RECEPTORS REDUCED INFLAMMATION IN EXPERIMENTAL ARTHRITIS

**Giancarlo Nastasi, Angela Avenoso, Salvatore Campo, Angela D'Ascola, Dario Samà  
Alberto Calatroni, Giuseppe M. Campo.**

DBPNS, section of Medical Chemistry, School of Medicine, University of Messina

Low molecular mass and highly polymerized hyaluronan (HA) elicited pro- or anti-inflammatory responses by modulating the toll-like receptor 4 (TLR-4) and TLR-2 or by activating/inhibiting the nuclear factor kappa B (NF- $\kappa$ B). Collagen-induced arthritis (CIA) mediated activation of TLR-4 and TLR-2 complexes induces the myeloid differentiation primary response protein (MyD88) and the tumor necrosis factor receptor-associated factor 6 (TRAF6), and the activation of the NF- $\kappa$ B that, in turn, stimulates pro-inflammatory cytokine production. The aim of this study was to investigate the influence of high polymerized HA at different concentrations on TLR-4 and TLR-2 modulation in CIA in mice. CIA increased TLR-4, TLR-2, MyD88 and TRAF6 mRNA expression and the related protein production in the cartilage of arthritic joints, as , IL-17, MMP-13 $\beta$ , IL-1- $\alpha$  as well as mRNA and related protein levels of TNF- and inducible nitric oxide synthase (iNOS). Intraperitoneal daily treatment of CIA mice with highly polymerized HA significantly limited CIA incidence and decreased all the parameters up-regulated by CIA. The improvement of biochemical parameters was also supported by histological analysis.

## CELL PROLIFERATION AND APOPTOSIS ARE DIFFERENTLY REGULATED BY NF $\kappa$ B SIGNALING IN AUTOSOMAL DOMINANT AND RECESSIVE POLYCYSTIC KIDNEY DISEASE

**Gianluca Aguiari\*, Alessandra Mangolini\*, Marco Bogo\*, Paolo Pinton\$  
Luigi Catizone# and Laura del Senno\***

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A common factor that unifies dominant (ADPKD) and recessive (ARPKD) Polycystic kidney diseases (PKD) is the abnormal cell growth and apoptosis. We have found that in both ADPKD and ARPKD cell models, cell proliferation and apoptosis are regulated by NF $\kappa$ B signaling, but in an opposite way. In fact, results of biochemical, molecular and cellular studies indicate that NF $\kappa$ B activity and its nuclear localization are increased in ADPKD or ARPKD human renal cells obtained by depletion of either polycystin-1 (PC1) or fibrocystin-1 by siRNA expression and by overexpressing a dominant negative PC1 terminal tail. NF $\kappa$ B activity contributes to increased cell proliferation and survival in PC1-defective cells, whereas it contributes to increased apoptosis in FC1-defective cells. Moreover, in the former cells NF $\kappa$ B leads to increased expression of adenosine receptors type 3 (A3AR), a negative modulator of adenylyl cyclase. Consistently, Cl-IB-MECA, a specific A3AR agonist, markedly reduces cAMP levels and consequently cell proliferation in PC1-depleted cells. Agonists and antagonists of NF $\kappa$ B should be therefore investigated for the control of disease progression in both ADPKD and ARPKD forms.

## **INTERLEUKIN-1 RECEPTOR-ASSOCIATED KINASE 1 (IRAK1) AND HEAT SHOCK PROTEIN 90 KDA ALPHA B1 (HSP90AB1) ARE UP-REGULATED BY AF4**

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AF4 is a transcriptional activator implicated in the early lymphoid development. Its gene frequently translocates with mixed-lineage leukemia (MLL) gene in childhood acute leukemia, and forms the MLL-AF4 oncogenic chimera. AF4 has a central role in the transcriptional elongation and in H3-K79 histone methylation by recruiting Dot1 to RNA Pol II. Although AF4 lacks DNA-binding domains, it might have a gene-specific transactivity. In fact, the MLL-AF4 leukemia phenotype differs from other MLL-associated leukemias, and the spectrum of the MLL-AF4 target genes does not overlap with that of other MLL-fusion proteins. In order to reveal AF4-specific gene expression changes, we analyzed an AF4 over-expression cellular system by suppression subtractive hybridization. Forty-two genes were up-regulated by AF4. Eleven of these showed from 2- to 7-fold higher expression than control, as determined by qRT-PCR. The most up-regulated genes are IRAK1 and HSP90AB1. We found that these two novel AF4 target genes are also novel putative targets of the MLL-AF4 chimeras. (Regione Campania-Conv. CEINGE G.R.27/12/07 N 2495;L.R.5/2002,Es. 2005; MIUR-Rome PS35-126/IND, PRIN 2007).

## **ANALYSIS OF EXPRESSION OF CLASSICAL ESTROGEN RECEPTORS AND GPR30 DURING MEGAKARYOBLASTIC DIFFERENTIATION**

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Megakaryocytes are myeloid cells which release platelets through long cytoplasmatic processes. Growing evidence suggest that estrogens and their receptor ER $\alpha$  and ER $\beta$  may play a crucial role during megakaryopoiesis. However, the role of GPR30, a novel estrogen receptor, ER $\alpha$  and ER $\beta$  have not yet been investigated. In this context, we evaluate the expression of ER $\alpha$ , ER $\beta$  isoforms and GPR30 during differentiation of megakaryoblastic leukemia (MEG-01) and human erythroleukemia (HEL) cell lines stimulated with TPO and 17 $\beta$ -estradiol as well as during megakaryopoiesis by Real Time PCR. Our findings demonstrate that ER $\alpha$  is not present in HEL and in MEG01. On the other hand ER $\beta$ 1, ER $\beta$ 4, ER $\beta$ 5 are expressed in HEL and MEG01 cells, but their expression decreases with estrogen stimulation and increases with TPO stimulation. Moreover ER $\alpha$ , GPR30 and ER $\beta$  isoforms expression decreases strongly during differentiation of human CD34+ stem cells and in mature megakaryocytes only ER $\beta$  isoforms are detectable. These findings lead to conclude that GPR30 and ER $\alpha$  are not involved in megakaryocyte maturation. Moreover, ER $\beta$  isoforms may be the main actors of the potential role of estrogens during megakaryopoiesis and platelet release. Transcriptional regulatory networks in familial combined hyperlipidemia (FCHL)

## **TRANSCRIPTIONAL REGULATORY NETWORKS IN FAMILIAL COMBINED HYPERLIPIDEMIA (FCHL)**

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Familial combined hyperlipidemia (FCHL) is the most frequent dyslipidemic syndrome in our population. In order to understand the basis of FCHL, we have undertaken the search for regulatory "nodes" or "modules" that are altered in this multifactorial and genetically complex syndrome. In our transcriptome analysis we find 1913 genes hyper- or hypo-expressed in FCHL patients, and 688 genes significantly altered after statins (the elective drugs for FCHL) treatment. 97 genes are present in both lists; the majority of them are hypo-expressed in FCHL patients and become normo- or hyper-expressed after statin treatment. These genes have been selected for promoter analysis. In silico analysis of the promoter regions of these genes identifies several significantly enriched motifs. Many promoters contain two or more motifs. Network analysis reveals a transcriptional regulatory circuitry connecting these genes, a specific "node" of a wider transcriptional network of genes involved in FCHL syndrome. The possibility to interfere with this regulatory connection is presently under investigation.

## **NEW INSIGHTS INTO 3-IODOTHYRONAMINE (T1AM) CARDIAC METABOLISM BY LC/MS/MS**

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T1AM is an endogenous compound derived from thyroid hormone (T4) through decarboxylation and deiodination, which showed unique biological properties. At the molecular level it is a potent agonist of the TAAR1, and an agonist ligand against the  $\alpha$ -2A adrenergic receptor. When administered pharmacologically it induced hypometabolic state opposite to that induced by excess of T4. In isolated working rat heart, T1AM produced a reversible dose-dependent negative inotropic effect. In the present study we investigate the catabolism and uptake of exogenous T1AM in rat cardiac tissues, biological fluids and H9C2 cells, using HPLC-ESI-MS-MS. Preliminary data indicated that T1AM undergoes oxidative deamination and is metabolized to 3-iodothyroacetic acid (TA1), both in working rat hearts and in H9C2 cells. Accordingly, the pretreatment of H9C2 cells with 100mM or 200mM iproniazide, a known MAO and SSAO inhibitor, significantly inhibited T1AM conversion to TA1. In conclusion LC/MS/MS proved to be the technique of choice for investigating the catabolism of T1AM in rat hearts. Further studies are in progress to identify T1AM metabolism products in rat tissue homogenates and biological fluids.

## **MUTUAL INTERFERENCE AMONG THREE ENERGETIC ROUTES: HISTIDINE DECARBOXYLATION, MALATE DECARBOXYLATION AND ADI PATHWAY, IN A WINE ISOLATED LACTOBACILLUS HILGARDII.**

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Lactobacillus hilgardii ISE5211 was isolated from an Italian red wine spontaneously undergoing malo-lactic fermentation. Besides performing malate decarboxylation, this strain is also able to produce histamine and to perform ADI pathway. The bacterial strain was grown in MRS medium with and without excess of histidine and/or arginine and/or malate. In control conditions no histamine is produced. When malate is added to the histidine fortified medium, no significant reduction or delay in histamine accumulation occurs, suggesting that the two pathways proceed in parallel without any interference. At the same time, also the presence of histidine has no effects on the malo-lactic fermentation. On the contrary when histidine and arginine are simultaneously present in the medium a slight but significant delay in histamine accumulation takes place. Moreover, a biosynthetic control exerted by arginine over the histidine decarboxylase enzyme has been proved by means of 2DE analysis. The biosynthesis of some ADI pathway enzymes is also attenuated in histidine plus arginine medium, so these two pathways seem to display some competition.

## **IN VIVO AND IN VITRO EFFECTS OF CONJUGATED LINOLEIC ACIDS (CLA) ON HEPATIC LIPID METABOLISM**

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Conjugated linoleic acids (CLA) refer to positional and geometric isomers of linoleic acid (LA). In different animal models dietary CLA exhibit positive effects, particularly for body composition. However, mice fed CLA-supplemented diet develop lipodystrophy. Aim of the present study was to investigate in rats, by in vivo and in vitro experiments, the effect of CLA on plasma lipids and on the activities of a number of key enzymes involved in hepatic lipid metabolism. Dietary CLA induced a decrease of cholesterol and phospholipid levels in total plasma and in lipoproteins. In vitro analysis revealed that CLA were incorporated to a lesser extent than LA into VLDL lipids. The activity of acetyl-CoA carboxylase, regulatory enzyme of lipogenesis, resulted inhibited when measured in isolated hepatocytes whereas no effect was observed in vivo. Moreover, CLA decreased the activity of diacylglycerol acyltransferase, pace-setting step of triacylglycerol synthesis, only in the in vivo experiments. This study, by comparing the in vitro and in vivo results, allowed to understand the hepatic short-term control events preceding long-term CLA-induced changes.

## **N-3 POLYUNSATURATED FATTY ACID REGULATION OF CARDIAC GENE TRANSCRIPTION. A NEW LINK BETWEEN PPAR ACTIVATION AND FATTY ACID COMPOSITION**

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Different PPAR activation in different tissues have been often attributed to the prevalence of a specific isoform. In this work we demonstrated that in neonatal rat cardiomyocytes the selective activation of PPAR beta/delta by eicosapentaenoic (EPA) and docosahexaenoic (DHA) acid is not related to a different abundance of PPAR proteins. An increased EPA and DHA concentration was observed in the nuclear fraction of supplemented cells. Since fatty acids are trafficked to the nucleus by FABPs, which bind fatty acids mainly as NEFAs, and long-chain acyl-CoA esters act as antagonists while long chain NEFAs as agonists for the PPARs (1), the NEFAs/acyl-CoAs ratio is an important determinant in fatty acid control of gene transcription. We evidenced that the activity of acylCoA thioesterase (ACOT), catalysing the reaction leading to NEFAs from acyl-CoAs, increased in n-3 PUFAs supplemented cells. Since ACOT gene transcription is modulated by PPAR, it seems that EPA and DHA create a self-maintaining loop which guarantees their high intracellular concentration in the not esterified form, which acts as PPAR activator in the nucleus. 1. Murakami, K et al Biochem. J. 353, 231-238, 2001

## **ROLE OF HOMOCYSTEINYLATED ALBUMIN IN THE ENDOTHELIAL DAMAGE INDUCED BY HYPERHOMOCYSTEINEMIA.**

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**BACKGROUND.** Hyperhomocysteinemia is a cardiovascular risk factor. Homocysteine (Hcy) in circulation is covalently bound to proteins, mainly serum albumin, to form homocysteinylated proteins (HcyProt). HcyProt are potential mediators of Hcy toxicity. **AIMS.** Identification of the role of HcyProt in determining endothelial damage and the relevant mechanisms and effectors involved. **METHODS.** Evaluation of Hcy-albumin treatment on monocyte adhesion onto an endothelial monolayer. Identification and characterization of genes (microarray, real time PCR) and proteins (immunoblot, ELISA, FACS), involved in the endothelial response to HcyProt treatment. **RESULTS.** Endothelial treatment with Hcy-albumin increased monocyte adhesion through upregulation of specific adhesion molecules (VCAM1, ICAM1), inflammatory chemokines and other mediators (MCP1, Hsp60, ADAM17). **CONCLUSIONS.** Results support the role of Hcy-albumin in monocyte and endothelial activation, promoting monocyte adhesion. HcyProt are a trigger factor of the inflammatory modifications occurring in the vasculature during development of the early atherosclerotic lesions.

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