53rd National Meeting of the Italian Society of Biochemistry and Molecular Biology (SIB)

and

National Meeting of Chemistry of Biological Systems Italian Chemical Society (SCI - Section CSB)

Palazzo dei Congressi di Riccione
23rd - 26th September 2008

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Programma

Tuesday 23rd September

8:30 *Registration of SCI-DCSB and whole-conference attendants*

9:00 – 12:45

SALA POLISSENA

SYMPOSIUM C1 (SCI-DCSB)
MOLECULAR SYSTEMS BIOLOGY

Chairmen: Mauro Fasano (Varese), Lucia Banci (Firenze)

09:00 **Katrin Marcus (Ruhr-Universität Bochum, Germany)**
Proteomics in neurodegenerative diseases

09:45 **Pierluigi Reschiglian (Università di Bologna)**
Field-flow fractionation for the analysis and biophysical characterization of biological systems

10:10 **Maurizio Simmaco (Università di Roma La Sapienza)**
An integrated platform for clinical analysis of gene expression

10:35 *Coffee Break*

11:00 **Andreas Zanzoni (Institute for Research in Biomedicine, Barcelona)**
Network biology: towards the comprehension of complex biological systems

11:45 **Fulvio Magni (Università di Milano Bicocca)**
Role of mass spectrometry in protein biomarkers discovery: the case of renal clear-cell carcinoma

12:10 **Cecilia Gelfi (Università di Milano)**
The proteome of skeletal muscle in neurodegenerative conditions

13:05 – 14:30 *Lunch and hanging up of SCI-DCSB posters*

14:30 – 16:00

SALA POLISSENA

SYMPOSIUM C2 (SCI-DCSB)
CHEMISTRY OF NUCLEIC ACIDS

Chairmen: Marco Franceschin (Roma), Roberto Purrello (Catania)

14:30 **Pasquale De Santis (Università di Roma La Sapienza)**
Sequence-dependent collective properties of DNA and their role in biological systems

15:00 **Aldo Galeone (Università di Napoli Federico II)**
Quadruplex DNA: structural features and potential applications

15:30 **Gianmaria Bonora (Università di Trieste)**
PEG-addition to oligonucleotides: a story with a future?

16:00 – 17:30 *Poster session with coffee break*

17:30

SALA POLISSENA

"GASTONE DE SANTIS" AWARD

Chairmen: Gianluca Fossati (Milano), Mauro Fasano (Varese)

18:20 *Meeting of SCI-SCSB members*

Parallel meetings of the following SIB groups:

SALA VIOLANTE – PALAZZO DEI CONGRESSI

14:30 – 17:00 **NEUROCHEMISTRY**
Chair: Tommaso Russo

17:00 – 18:30 **BIOGENIC AMMINES**
Chair: Antonio Toninello

SALA GINEVRA - PALAZZO DEI CONGRESSI

14:30 – 16:30 **NUTRITIONAL BIOCHEMISTRY**
Chair: Francesco Bonomi

16:30 – 19:30 **NUCLEOTIDES, NUCLEIC ACIDS, GENOMES**
Chair: Enrico Marinello

SALA COSTANZA - PALAZZO DEI CONGRESSI

15:30 **MEMBRANES AND BIOENERGETICS**
Chair: Paola Palestini

SALA DANTE - HOTEL CRISTALLO

16:00 – 18:30 **INDUSTRIAL BIOCHEMISTRY AND BIOTECHNOLOGY**
Chair: Gennaro Marino

SALA VESPUCCI - HOTEL MEDITERRANEO

15:30 **CELLULAR BIOCHEMISTRY**
Chair: Giovanni Raugei

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Wednesday 24th September

8:30 *Registration of SIB attendants*

9:00 – 12:30
SALA POLISSENA
SYMPOSIUM S1
(SIB AND SCI COMBINED SYMPOSIUM)
PROTEIN AND DRUG STRUCTURE
Chairmen: Martino Bolognesi (Milano), Mauro Fasano (Varese)

09:00 **Alessandra Topai (Università di Roma Tor Vergata)**
New inhibitors of metalloproteases via Structure/Ligand based design-case study

09:35 **Claudio Luchinat (Università di Firenze)**
NMR in structural biology and drug discovery

10:10 **Menotti Ruvo (Università di Napoli Federico II)**
Peptide antagonists of protein-protein interactions identified by screening protein fragments

10:30 *Coffee Break*

11:00 **Giuseppe Zanotti (Università di Padova)**
Structural and functional characterization of H. pylori proteins. Identification of new pharmacological targets

11:35 **Rolf Hilgenfeld (University of Lubecca, Germany)**
Structure-based discovery of new antivirals

12:10 **Pierfausto Seneci (Università di Milano)**
Smac Mimics-XIAP Binders: Protein-Protein Interaction Inhibitors from Structure-Based Drug Design

12:30 – 13:30 *Lunch*

12:30 – 13:30 *removal of SCI-DCSB posters and hanging up of the first set of SIB posters*

13:30 – 16:00
SALA POLISSENA
SYMPOSIUM S2
SIGNAL TRANSDUCTION AND BIOMOLECULAR TARGETS
Chairmen: Antonio de Flora (Genova), Paola Chiarugi (Firenze)

13:30 **Marcello Allegretti (Dompé, L'Aquila)**
Allosteric modulators of CXCL8 receptors

14:00 **Elena Zocchi (Università di Genova)**
The functional effects of the new human hormone abscisic acid on animal cells are mediated by the second messenger cyclic ADP-ribose

14:30 **Silvia Giordano (Università di Torino)**
Targeting MET in tumor and metastases: a matter of addiction and sensitivity

15:00 **Lorenzo Pinna (Università di Padova)**
Targeting CK2 as a general strategy in Signal Transduction Therapy

15:30 **Giovanni Melillo (National Cancer Institute-Frederick, Maryland, USA)**
Targeting hypoxic cell signaling for cancer therapy

16:00 – 16:30 *Coffee break*

16:30 – 18:00
SALA POLISSENA
SYMPOSIUM S3
(SIB AND SCI COMBINED SYMPOSIUM)
ENVIRONMENTAL BIOTECHNOLOGY
Chairmen: Gennaro Marino (Napoli), Massimo Coletta (Roma)

16:30 **Christopher C. R. Allen (Queen's Univ. Belfast, Northern Ireland)**
Dioxygenases from arene-degrading bacteria: Can we develop their potential for application in biocatalysis?

17:00 **Alberto Di Donato (Università di Napoli Federico II)**
Metabolic Engineering: a tool for bioremediation strategies

17:25 **Fabio Fava (Università di Bologna)**
Microbial reductive dehalogenation for the sustainable remediation of PCB-contaminated sediments

17:50 **Luigi Campanella (Università di Roma La Sapienza)**
Ecological Risk assessment by sensoristic approach

18:15 **G. Fiorentino (Università di Napoli Federico II) – Selected Poster 6.1**
Stress by aromatic compounds in sulfolobus solfataricus: detoxification, regulation and biomonitoring

18:30 – 19:30 *Poster session 1*

20:00 **Get together dinner**
"Riccione City Eye", panoramic terrace restaurant of Palazzo dei Congressi

Thursday 25th September

08:30 – 11:00
SALA POLISSENA
SYMPOSIUM S4
PROTEIN MISFOLDING AND ITS RELATIONSHIP WITH DISEASE
Chairmen: Fabrizio Chiti (Firenze), Giampaolo Merlini (Pavia)

08:30 Introduction by **Giampaolo Merlini** (Policlinico San Matteo, Pavia)

08:40 **Christopher M. Dobson (University of Cambridge)**
Life on the Edge: The Nature and Origins of Protein Misfolding Diseases

09:05 **Gennaro Esposito (Università di Udine)**
β2-microglobulin refolding intermediates and fibrillogenesis

09:30 **Vittorio Bellotti (Università di Pavia)**
Misfolding and tissue localization of amyloidogenic proteins: complexities and breakthroughs

09:55 **Fabrizio Tagliavini (Istituto Carlo Besta, Milano)**
Prion protein, prion diseases and tetracyclic compounds

10:20 **Roberto Sitia (Istituto Scientifico San Raffaele, Milano)**
Protein folding and signaling in the early secretory apparatus

10:45 **P. Polverino de Laureto (Università di Padova) – Selected Poster 15.60**
Characterization of oligomeric species on the aggregation pathway of human lysozyme

11:00 – 11:30 *Coffee break*

11:30 – 16:30 *Election of 5 Members of the SIB Executive Board*

11:30 – 13:00
SALA POLISSENA
SYMPOSIUM S5
EMERGING TECHNIQUES IN BIOCHEMISTRY
Chairmen: Niccolò Taddei (Firenze), Gennaro Marino (Napoli)

11:30 **Annalisa Relini (Università di Genova)**
"Seeing" at the nanoscale with the atomic force microscope

11:55 **Piero Pucci (Università di Napoli Federico II)**
Orthogonal strategies in Proteomics

12:20 **Francesco Saverio Pavone (LENS, Firenze)**
Manipulation and imaging of single bio-molecules

12:45 **E. Dainese (Università di Teramo) – Selected Poster 15.6**
Small angle x-ray scattering studies reveal important clues for membrane binding and activity of fatty acid amide hydrolase (faah)

13:00 – 14.10 *Lunch*

13:00 – 14:10 *Removal of first set of SIB posters and hanging up of second set of SIB posters*

14:10 – 15:00
SALA POLISSENA
ANTONINI LECTURE
Rino Rappuoli (Novartis, Siena)
The bacterial pan-genome and the global gene pool

15:00 – 18:00
SALA POLISSENA
SYMPOSIUM S6
GENE SILENCING
Chairmen: Tommaso Russo (Napoli), Antonio De Flora (Genova)

15:00 **John M. Denu (University of Wisconsin, Madison, Wisconsin, USA)**
Molecular functions of sirtuins

15:30 **Gerry Melino (Medical Research Council, Leicester, UK)**
miR, "stemness" & skin

16:00 *Coffee break*

16:30 **Roberto Gherzi (Ist. Naz. per la Rierca sul Cancro, Genova)**
KSRP, a RNA binding protein with many talents

17:00 **Tommaso Russo (Università di Napoli Federico II)**
RNA interference-based screening of genes involved in embryonic stem cell self-renewal and differentiation

17:30 **Massimo Zollo (Università di Napoli Federico II)**
miRNAs in paediatric cancer

18:00 *Premio Zanichelli e Premio Medaglia SIBBM*

18:15 *Assemblea dei Soci*

20:30 *Social Dinner*

Friday 26th September

08:30 – 10:30
SALA POLISSENA
SYMPOSIUM S7
REDOX SIGNALLING AND OXIDATIVE STRESS
Chairmen: Paola Chiarugi (Firenze), Luciana Avigliano (Roma)

08:30 **Fatima Mechta-Grigoriou ("Stress and Cancer" Lab. Institut Curie - Inserm U830, Paris)**
The role of Oxidative stress in insulin secretion and aging

08:55 **Enrico Avvedimento (Università di Napoli Federico II)**
Oxidation-coupled transcription: histone H3 demethylation generates an oxidation wave that drives estrogen-induced transcription

09:20 **Franca Esposito (Università di Napoli Federico II)**
Identification and functional characterization of a novel antiapoptotic gene involved in adaptation to oxidative stress and chemoresistance

09:45 **Vittorio Calabrese (Università di Catania)**
Redox regulation of cellular stress response in aging and neurodegenerative disorders: role of vitagenes

10:10 **M. Donadelli (Università di Verona) – Selected Poster 2.33**
Intracellular zinc increase inhibits p53$^{-/-}$ pancreatic adenocarcinoma cell growth by ros/aif-mediated apoptosis

10:30 – 11:00 *Coffee break*

11:00 – 13:00

SALA POLISSENA

SYMPOSIUM S8
LIPIDS IN CELL COMMUNICATION AND SIGNAL TRANSDUCTION
Chairmen: Paola Bruni (Firenze), Bruno Venerando (Milano)

11:00 **Laura Riboni (Università di Milano)**
Cell communication in the nervous system: the mediator role of sphingosine-1-phosphate

11:25 **Andrea Graziani (Università Piemonte Orientale, Novara)**
Diacylglycerol kinases as switch regulators from diacylglycerol to phosphatidic acid-mediated signaling: implication in cell signaling

11:50 **Alessandro Prinetti (Università di Milano)**
Sphingolipids as modulators of membrane signaling complexes

12:15 **Daniela Corda (Mario Negri Sud, Chieti)**
Cellular targets and activities of the glycerophosphoinositols

12:40 **N. Mitro (Università di Milano and Scripps Res. Institute) – Selected Poster 2.43**
Regulation of hepatic lipid and carboidrate metabolism by the liver X receptor

13:00 – 14:00 *Lunch*

14:00 – 15:00 *Poster Session 2*

15:00 – 19:45

SALA POLISSENA

SYMPOSIUM S9
MITOCHONDRIAL FUNCTION AND DYSFUNCTION
Chairmen: Giorgio Lenaz (Bologna), Sergio Papa (Bari)

15:00 **Shinya Yoshikawa (University of Hyogo, Japan)**
Reaction mechanism of bovine heartcytochrome c oxidase

15:45 **Paolo Bernardi (Università di Padova)**
The mitochondrial permeability transition in pathophysiology

16:15 **Paolo Sarti (Università di Roma La Sapienza)**
Nitric oxide and control of mitochondrial respiratory chain electron flux

16:45 **Alessandra Baracca (Università di Bologna)**
Mitochondrial substrate-level phosphorylation improves viability of human cells with severe oxidative phosphorylation impairment

17:15 *Coffee break*

17:45 **Ferdinando Palmieri (Università di Bari)**
Mitochondrial membrane transport proteins in health and disease

18:15 **Domenico De Rasmo (Università di Bari)**
Regulation by cAMP dependent phosphorylation of mitochondrial protein import

18:45 **Giorgio Casari (Istituto Scientifico San Raffaele, Milano)**
The mitochondrial protease AFG3L2 is essential for development and maintenance of motor axon and cerebellum d

19:15 **F. Pallotti (Università di Varese) – selected poster 12.20**
Plasma Coenzyme Q10 levels in patients with hypercholesterolemia and high serum creatine kinase levels

19:30 *Meeting end*

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Società Chimica Italiana

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Presentazione

Il 53° Congresso Nazionale della Società Italiana di Biochimica e Biologia Molecolare che si tiene a Riccione dal 23 al 26 Settembre si distingue per l'alto livello scientifico e l'interesse interdisciplinare delle numerose sessioni nelle quali è strutturato.

Il Programma scientifico vede tre Simposi congiunti della SIB con la Sezione della Chimica dei Sistemi Biologici della Società Italiana di Chimica (SCI) su Molecular Systems Biology, Chemistry of Nucleic Acids, Protein and Drug Structure, Environmental Biotechnology.

Questi Simposi, riguardano argomenti di avanguardia per i quali fa piacere l'interesse condiviso delle due Società, che per la prima volta organizzano dei Simposi congiunti a significare l'intento di procedere insieme negli scambi scientifici. Gli argomenti delle altre sessioni sono stati scelti dal comitato scientifico in base alla loro rilevanza e attualità scientifica, con particolare cura nella individuazione dei relatori. Le sessioni SIB spazieranno da Signal Transduction and Biomolecular Targets, Protein Misfolding and its Relationship with Diseases, Emerging Techniques in Biochemistry, Gene Silencing, Redox Signalling and Oxidative Stress, Lipids in Cell Communication and Signal Transduction, Mitochondrial Function and Dysfunction.

Sono tempi difficili per l'esiguità degli stanziamenti pubblici per l'Università e la ricerca scientifica, ma la perseveranza, dedizione e qualificazione degli addetti alla ricerca e il sopravvivere a tutte le difficoltà dell'entusiasmo, dei piu' giovani in particolare, lasciano ancora aperta la speranza per un immediato futuro migliore.

Con questo spirito viviamo insieme queste belle giornate del nostro Congresso.

Sergio Papa
Presidente SIB

Speakers’ presentations

R.1

PROTEOMICS IN NEURODEGENERATIVE DISEASES

Thorsten Müller, Florian Tribl, Katrin Marcus*

Department of Functional Proteomics, Ruhr University Bochum, Germany

Proteomics emerged as a key technology to identify novel proteins, to characterize their isoforms based on numerous post-translational modifications, and to unravel protein regulation, e.g., in healthy and diseased states. Brain affecting diseases such as Alzheimer's (AD) and Parkinson's disease (PD) most commonly affect elderly people and give rise of severe and slowly progressing neurodegeneration. Since there is neither an effective pharmaceutical treatment available to cure these disorders, nor to reliably diagnose them at a pre-symptomatic stage, proteomics may contribute to the understanding pathomechanisms of underlying pathomechanisms as well as the identification of new diagnostic biomarkers and novel therapeutic targets in the future.

As with other tissues, two strategies can be distinguished in brain proteomics: "Mapping" approaches with the aim to identify the entire set of proteins within the sample of interest and "comparative/differential" proteomics aiming at the identification of key players for development, plasticity or disease-related changes of the nervous system. Examples include mapping studies in individual brain areas or neuronal cell types or comparative analysis of cerebral disorders like AD and PD which are thought to dramatically disturb the cellular proteome.

In this talk I will focus on the application of proteomics on the investigation of AD and PD especially underlining advantages as well as limitations of some of the existing strategies.

R.2

FIELD-FLOW FRACTIONATION FOR THE ANALYSIS AND BIOPHYSICAL CHARACTERIZATION OF BIOLOGICAL SYSTEMS

P. Reschiglian, S. Casolari, D.C. Rambaldi, B. Roda, A. Zattoni

Dipartimento di Chimica "G. Ciamician", Università di Bologna, Italia; *pierluigi.reschiglian@unibo.it*

A technique able to separate in 5-20 minutes a broad range of analytes of biological interest, from proteins to whole cells, over a molar-mass range of 10^{14}, and with relevant biophysical information obtained from retention parameters. This is field-flow fractionation (FFF)[1], for long considered to be "the best-kept secret" in bioanalytical chemistry. In fact, over more than 30 years FFF has slowly evolved from a research-oriented technique to a well-assessed methodology. Today's FFF is applied to solve real analytical problems in various biological niches. The key point for using FFF with biological systems is the "gentle" fractionation mechanism that makes "delicate" analytes like non-covalent protein complexes or living cells be separated in their native, functional state. This makes FFF particularly useful if the fractionated analytes need to be reused or further characterized. The development of hyphenated methods based on coupling FFF with uncorrelated techniques for biophysical (size, shape and/or mass) characterization, such as multi-angle laser scattering or soft-impact mass spectrometry, today shows to be particularly promising.

We overview FFF basic principles and technology, and show applications from top-down proteome analysis[2] of biological fluids and subcellular units[3] to serum lipoprotein profiling[4], from monitoring protein aggregation processes in prions[5], amyloidic proteins or viruses to stem cell sorting[6]. These are just some examples that indicate the analysis under native conditions as one of the most representative application niches to make FFF a booming methodology in the study of biological systems.

1. P. Reschiglian, A. Zattoni, B. Roda, E. Michelini, A. Roda. (2005) Trends Biotechnol. 23, 475-83.
2. P. Reschiglian, M.H. Moon. "Flow field-flow fractionation: a pre-analytical method for proteomics J. of Proteomics, *in press.*
3. D. Kang, S. Oh, P. Reschiglian, M.H. Moon. (2008) Analyst 133, 505-15.
4. D.C. Rambaldi, A. Zattoni, S. Casolari, P. Reschiglian, D. Roessner, C. Johann. (2007) Clin. Chem. 53, 2026-8.
5. J.R. Silveira, G.J. Raymond, A.G. Hughson, R.E. Race, V.L. Sim, S.F. Hayes, B. Caughey. (2005) Nature 437, 257-261.
6. P. Reschiglian, B. Roda, A. Zattoni, G.P. Bagnara. Patent pending PCT/EP2007/054226.

R.3

AN INTEGRATED PLATFORM FOR CLINICAL ANALYSIS OF GENE EXPRESSION

V. Annibali, L. Aimati, M.Borro , G. Gentile, C. Ialongo, O. De Luca, L. Lionetto , R. Mechelli, A. Provenza M.S. Torre A. De Blasi and M. Simmaco

Dipartimento Scienze Biochimiche – Diagnostica Molecolare Avanzata – II Facoltà di Medicina – Azienda Ospedaliera Sant'Andrea Università Sapienza di Roma (w3.uniroma1.it/biocmed2/dima)

A farsighted interaction between the 2nd Faculty of Medicine and the Department of Biochemical Sciences "A. Rossi Fanelli" of "Sapienza" University and Sant'Andrea Hospital allowed to realize, in 2001, an integrated system biology laboratory named "Diagnostica Molecolare Avanzata" (Di.M.A.), a pioneering structure whose mission is to promote the development of new diagnostic and therapeutic strategies applying genomics and proteomics to clinical research.

The wide range of activities of Di.M.A. Laboratory is supported by the strong synergy among different resources (both human and financial).

The recent advances in molecular biology and biochemistry strongly influence the request of diagnostic laboratory analyses by clinicians and patients. For this purpose, several platforms are used in a way to produce the more complete overview in order to offer a service of Personalized Medicine.

In particular, it will be described the evaluation of fluoropyrimidime metabolism using a combined approach based on SNPs analysis by pyrosequencing and the effective rate of drug degradation by LC-MS/MS.

A similar strategy is used for monitoring antipsychotic drugs combining genomic analysis based on an Affymetrix platform with the LC-MS/MS pharmacokinetic assay. Furthermore, the latter techniques offer a simple way for the evaluation of the intestinal permeability in pediatric and oncological patients[1]. Finally, proteomic techniques are proposed as a tool for the use of T lymphocytes as biosensors capable of a sensitive discrimination of different pathological states[2].

1. Lostia AM, Lionetto L, Principessa L, Evangelisti M, Gamba A, Villa MP, Simmaco M. (2008) Clin Biochem. 41 887-92.
2. Borro M., Gentile G., Stigliano A., Misiti S., Toscano V. and Simmaco M. (2007) Clin. Exp. Immunol. 150, 494-501.

R.4

NETWORK BIOLOGY: TOWARDS THE COMPREHENSION OF COMPLEX BIOLOGICAL SYSTEMS.

A. Zanzoni

Structural and Computational Biology Programme, Institute for Research in Biomedicine (IRB), Barcelona, Spain

Much of systems biology aims to predict the behaviour of biological systems on the basis of the set of molecule involved. Understanding the interactions between these molecules (proteins, DNA, RNA and small molecules) is therefore crucial to such efforts.

Several genome-wide experiments have provided large protein-protein interaction datasets. More recently, given the increase in the availability of human protein interaction data, protein networks are progressively serving as implements to unveil the molecular basis of disease.

A current major challenge for biology is to apply an integrated computational and experimental approach to chart, comprehend and model the properties of molecular networks that control the physiological and the pathological behaviour of the cell.

R.5

ROLE OF MASS SPECTROMETRY IN PROTEIN BIOMARKERS DISCOVERY: THE CASE OF RENAL CLEAR CELL CARCINOMA.

F. Magni, C. Chinello, E. Gianazza, V. Mainini, C. Galbusera, M. Kienle

Dipartimento di Medicina Sperimentale, Università di Milano-Bicocca, Milano, Italia

Mass spectrometry (MS), in combination with 2DE, is widely used to identify a proteome profile in biological fluids which results to be specific for a particular disease. Unfortunately this approach is laborious and time consuming. A different approach based on the combination of active surface prepurification with MALDI-TOF analysis has been described. Only a group of proteins present in the biological sample can be retained on the active surface target and detected by MALDI-TOF analysis (SELDI-TOF). A suitable alternative is based on the use of magnetic beads with activated surface (ClinProt) which allows pre-purification of the sample before mass spectrometry analysis. An example of the application of this approach to the renal cell carcinoma (RCC) will be described .

Renal Cell Carcinoma (RCC) is one of the major cause of cancer death and is radio- and chemioresistant. Many renal masses remain asymptomatic and non-palpable until late in the natural course of the disease. Therefore more than 30% of patients have metastasis when symptoms become evident. Biomarkers for early detection of RCC, for the differential diagnosis of RCC from benign renal lesions when imaging is not helpful, for prognosis and follow up are urgent.

Serum and urine of healthy subjects (n=29) and clear cell RCC patients (n = 33-39) were analysed by ClinProt technique aimed to search possible biomarkers for early RCC diagnosis.

We found in serum a cluster of three signals (marker A, B and C) able to discriminate patients from control subjects with 100 % and 90 % of specificity and sensitivity, respectively, in the training test and 100 % and 92.3 % in the external validation. Moreover, this cluster showed 100 % sensitivity for patients at pT2 (n=5) and pT3 (n=8) and of 85 % for pT1 patients (n=20). Markers A and C signals area continuously decreased from pT1 to pT3 while marker B increased in pT1 and pT2 reaching a plateau for pT3.

In urine, a cluster of three different signals (marker A, B and C) was also able to discriminate patients from controls. Receiver Operating Characteristic (ROC) curve analysis showed values of AUC higher than 0.9 for marker A and B, corresponding to sensibility of 85-90 % and specificity of 90 %, while marker C gave a lower AUC (0.84) corresponding to sensibility of 70% and specificity of 100%. Combination of three markers allows an improvement of diagnostic efficacy with specificity and sensibility of 100 % and 95 %, respectively, in the training test and of 100 % and of 85 % in the testing experiment. Efficacy of this cluster of signals to distinguish RCC patients grouped on tumour stage showed a sensibility of 100% for patients at pT1. Moreover one of the signal present in the cluster was identified as a fragment of Tamm-Horsfall Protein (THP).

In conclusion, our data, although needing further validation with more patients and with different renal pathologies, are promising in the possibility to be used as multiple biomarkers for ccRCC diagnosis.

R.6

THE PROTEOME OF SKELETAL MUSCLE IN NEURODEGENERATIVE CONDITIONS

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Muscular atrophy is a common feature in a neurodegenerative disease such as Amyotrophic Lateral Sclerosis (ALS). Transgenic mice overexpressing human mutant SOD1 gene develop progressive loss of spinal motor neurons with a consequent muscle atrophy and paralysis. The loss of muscle proteins, the differential changes in muscle proteome could act as signalling events inducing progressive damage both at the muscular and motoneuronal levels.

The aims of this study was to detect markers of primary events in animal models and identify changes induced in muscle proteome in humans. In the present study we examined the proteomic profile of the gastrocnemious muscle from hSOD1^{G93A} transgenic (Tg) mutant mice in the pre symptomatic and symptomatic stage of the disease by two dimensional difference in gel electrophoresis (2D-DIGE) and mass spectrometry. The same proteomic approach was performed on gastrocnemious of 7- and 14-week-old wild type (NTg) mice to identify the physiological changes induced in muscle by ageing and by crush of sciatic nerve in 14 weeks old animals.

The same proteomic investigation was applied to 4 patients presenting mutations in a SOD1 gene in a symptomatic stage of the disease; healthy age-matched subjects were adopted as controls.

Approximately 3500 spots were detected in a 3-10 non linear pH gradient. Differential analysis of muscles at the early pre symptomatic age compared to NTg, revealed that 82 spots were significantly and differentially expressed in Tg hSOD1^{G93A} versus NTg mice, 24 of them identified by mass spectrometry. At 14 weeks of age, quantitative differential analysis of Tg hSOD1^{G93A} vs. NTg generated 153 differentially expressed spots, 55 were common to the two different disease stages, and 16 of them were identified.

Among identified proteins, 8 were differentially expressed at 7 weeks and these changes were absent 7 weeks later. All the proteins were mitochondrial. A total of 7 proteins maintained the same up or down regulation suggesting their role as disease markers.

Ageing induced a significant change in 95 spots, while 153 were changed after denervation, 28 of them were common between ageing and crush. Only spots exclusively changed during disease progression or presenting differential behaviour from ageing and crush were taken into consideration. Overall, 23 proteins were identified, 12 of them were not influenced by the ageing and denervation processes indicating these changes were exclusively related to disease progression. The remaining 11 proteins showed differential behaviour between pre symptomatic and symptomatic transgenic mice compared to ageing and denervation.

R.7

SEQUENCE-DEPENDENT COLLECTIVE PROPERTIES OF DNA AND THEIR ROLE IN BIOLOGICAL SYSTEMS.

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In the present post-genomic era, DNA sequences with billions of informational elements are currently accumulating in the data banks and need to be translated into functional elements. The classic image of DNA, commonly represented as a straight rigid rod, is presently just an icon. The DNA chain, which was in a recent past considered as a simple repository of the gene information, is now recognized as a very complex polymorphic macromolecule, which plays a relevant part in the management of its informational content. Inside the cell, DNA continuously interacts with the proteins involved in replication, transcription, repair, and regulation processes. During these processes, the DNA transforms between packed and unpacked architectures, like that of chromatin or of other higher-order structures morphing into shapes with structural spikes alternative to the canonical B-form in connection with biological events. The base sequence encodes the dynamics of these transformations from the atomic to the nanometer scale length, and over higher spatial scales. Therefore, an important part of the DNA information content is not localized on the codon regions but is related to collective features of relatively large tracts of sequence. Since some years, we are investigating the effects of the sequence on modeling the superstructural properties of DNA by integrating over nano-scale the theoretically evaluated slight structural and electronic features of the different nucleotide steps along the sequence. Our theoretical model appears to be fully suitable to translate the base sequence in superstructural properties of DNA. It allows the prediction of the thermodynamic constants of the sequence dependent circularization reactions of DNA tracts and their writhing transitions from relaxed to super-coiled circular forms as well as the stability constants and positioning of nucleosomes along eukaryotic genomes in excellent agreement with the experiments. We have found that collective properties of DNAs are generally involved in nano-structural features and functional elements in genomes as well as in possible chemical evolution of nucleic acid informational content in prebiotic processes as can be speculated from the ability of inorganic surfaces to recognize and stabilize DNA tract of sequence. In fact, sequences strongly adsorbed on surface could be more protected against the physical-chemical injuries by the environment and therefore selected as "DNA phenotypes".

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R.8

QUADRUPLEX DNA: STRUCTURAL FEATURES AND POTENTIAL APPLICATIONS.

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G-quadruplexes are higher-order DNA and RNA structures formed by G-rich sequences based on tetrads of hydrogen bonded guanine bases.[1] The important role of these structures in biological systems relies mainly on three features, since they are probably (in several cases definitely) involved in: (i) the architecture of telomeres of many organisms;[2] (ii) G-rich sequences that are present within a wide range of genes;[3] (iii) the scaffolds of several oligonucleotide aptamers.[4] G-quadruplexes are structures characterized by a great variability.[5] Their structural differences involve several aspects, which are often mutually interconnected. For example, the relative orientation of the strands affects the glycosidic conformation (*syn* or *anti*) of the guanosine residues forming the scaffold of the quadruplex structure. In fact, each of the four possible strand arrangements, namely A_4 (all strands parallel), A_3B (three strand parallel and one antiparallel), A_2B_2 (two adjacent strands parallel and the other antiparallel) and $(AB)_2$ (each strand running in the opposite direction respect to the adjacent ones),[6] is characterized by the presence of different type of tetrad: all G-*anti* or all G-*syn* for A_4, *syn-syn-syn-anti* or *anti-anti-anti-syn* for A_3B, *syn-syn-anti-anti* for A_2B_2 and *syn-anti-syn-anti* for $(AB)_2$. The structural variability of the quadruplexes has been further increased by introducing modification on both bases and sugar-phosphate backbone. In the latter frame, we have synthesized quadruplex forming oligonucleotides containing 8-methyl-2'-deoxyguanosine being able to affect the *syn-anti* conformational equilibrium. Furthermore, we have prepared and studied quadruplex structures containing a 3'–3' or 5'–5' inversion of polarity site in the G-stretches and quadruplexes in which four strands are interconnected by a tetra-end linker. The structural features of these modified quadruplexes and their possible applications will be described and discussed.

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R.9.

PEG-ADDITION TO OLIGONUCLEOTIDES: A STORY WITH A FUTURE?

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The biological properties of synthetic oligonucleotides can be modified by their conjugation with polymeric moieties to get better cellular uptake, in vivo stability and, in general, more favourable pharmacological properties. Among the different biocompatible polymers, the poly(ethylene glycol) (PEG) was extensively investigated on the basis of its success with many proteins.

The introduction of a large PEG chain by classical solid-phase procedures, as commonly used for much post-synthetic oligonucleotide modification, suffers from the phase heterogeneicity of the process that implies poor reactivity and unpredictable kinetic effects. To solve this drawback, different procedures for the PEGylation of synthetic oligonucleotides have been evaluates to ascertain the optimum level of modification achievable especially with large polymeric chains. The same procedure was applied to non-natural nucleic acid sequences as the PNAs.

The presence of high-molecular weight PEGs has showed a minimal effect on the hybridization behaviour of the conjugated oligonucleotides, while a clear enhancement of in vivo stability and cellular permeation, without any adverse toxic effect, was observed. Recently, a PEGylated synthetic DNA sequence has been successfully commercialized, but other are expected to follow in a next future, in particular as new RNA-based derivatives.

R.10

NEW INHIBITORS OF METALLOPROTEASES *VIA* STRUCTURE/LIGAND BASED DESIGN: A CASE STUDY.

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Matrix metalloproteinases (MMPs) are a family of approximately 27 Zn^{2+} ion-dependent endopeptidases which are involved in the proteolytic processing of several components of the extracellular matrix, such as collagens, proteoglycans and fibronectin as well as several cytokines and chemokines[1].

As MMPs are involved in several pathological processes (cancer, arthritis, multiple sclerosis, cardiovascular diseases, etc), the development of powerful and selective inhibitors might represent an important therapeutic goal[2].

However, recent failures of MMP inhibitors in cancer clinical trials have raised a question on MMPs druggability, mostly because of their multiple and often contradictory role in cancer disease[3].

Even though the relevance of the physiopathological role of MMPs is unquestionable, we need to understand which (and when) a MMP is actually involved in a specific pathological process to address the design of inhibitors toward a higher selectivity for different MMPs (the target MMP)[4].

C4T carried out a drug discovery MIUR-financed project whose main aim was the exploitation of high-throughput virtual screening, parallel synthesis of chemical libraries and *in vitro* screening, in order to discover new classes of MMP inhibitors.

We adopted a challenging approach, studying a set of 8 different MMPs representatives of shallow/deep-pocket S1' as well as cancer target/anti-target proteases, namely MMP-1, MMP-2, MMP-3, MMP-8, MMP-9, MMP-12, MMP-13 and MMP-14.

The aim was to identify a new series of compounds able to potently inhibit gelatinases and in particular MMP-2.

An active site detailed *in silico* analysis, a pharmacophore design and a library screening from our virtual database were followed by rational compound selection based on *criteria* as docking scoring, physical-chemical properties and ADMET predictions.

This research approach let us identify different innovative scaffolds: a new inhibitors class active in nanomolar range is being patented (PCT: EP2008/055563) and a second group of NCEs containing a new Zinc Binding Group (ZBG) is being explored.

Furthermore C4T is carrying on a novel approach for the identification of a potential class of MMP-13 inhibitors, whose main feature is an innovative no-Zinc-chelating mechanism of action.

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R.11

NMR IN STRUCTURAL BIOLOGY AND DRUG-DISCOVERY

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ABSTRACT NOT RECEIVED

R.12

PEPTIDE ANTAGONISTS OF PROTEIN-PROTEIN INTERACTIONS IDENTIFIED BY SCREENING PROTEIN FRAGMENTS.

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Protein tridimensional structure is the complex recapitulation of local and distant intramolecular forces that cooperatively contribute to maintain finely tuned energetic equilibria. Secondary structure motifs and small protein domains might act as building blocks that often can be isolated and investigated by several techniques to gain structural insights on the protein global structure and to modulate interactions with external partners.
While a lot of progresses have been made in this field by using de novo designed synthetic peptides, the idea of using protein fragments obtained by chemical or enzymatic methods has been only rarely pursued. Enzymatic degradation of folded proteins under mild conditions can provide shorter polypeptides with preserved secondary and tertiary structures that can be "reminders" of the original structure and can be utilized as probes for structural studies and as useful tools in in vitro assays to identify protein-protein interaction contacts. Active fragments can also be used as agonists or antagonists of the original protein or as starting scaffolds for the design of more potent and selective ligands. This simple but effective methodology has been successfully applied to find out peptide antagonists of several protein-protein interactions, allowing the identification of inhibitors having high efficacy and specificity. The systems studied include the complex between PED/PEA-15 and PLD1, believed to play a relevant role in the insulin resistance mechanism in PED/PEA-15-overexpressing tissues[1,2], the self association of the CARD of BCL10 that mediates a protein oligomerisation event responsible of NF-κB activation and cell proliferation and the self association of Gadd45β, a major player of the endogenous NF-κB-mediated resistance to apoptosis in a variety of cell lines[3,4].

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R.13.

STRUCTURAL AND FUNCTIONAL CHARACTERIZATION OF HELICOBACTER PYLORI PROTEINS. IDENTIFICATION OF NEW PHARMACOLOGICAL TARGETS.

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H. pylori is a Gram-negative bacterium which establishes a life-long chronic infection in more than half of the human population. Infections are often asymptomatic, but, in an important minority, *H. pylori* causes a range of pathologies, including gastritis, peptic ulcers, adenocarcinomas and MALT lymphomas[1]. Several important factors involved in the bacterial survival and in the persistence of infection have been identified[2]. We have selected a pull of these *H. pylori* proteins, cloned, expressed in *E. coli* and purified for crystallization trials. In particular we have purified in high yield HP0175, a secreted toxin with PPIase activity that causes apoptosis of gastric cells[3], HP1287, a homologue of *Bacillus subtilis* TenA, a protein involved in the salvage of the thiamine [4]. Crystals of HP1287 have been obtained and the three-dimensional structure determined at 2.8 Å resolution. The molecular model, a homotetramer with 222 symmetry, shows that the protein is structurally similar to the enzymes belonging to the TenA family. Activity tests are in progress. Very recently, diffraction quality crystals of the putative toxin HP1028 have been obtained and the corresponding SeMet derivative is under purification and crystallization trials.

Other *H. pylori* relevant targets are under investigation, first of all some of the Cag secretion system major components and accessory proteins. Indeed, the presence of the genomic locus codifying for the Cag proteins, the so called *cag*-Pathogenicity Island (*cag*-PAI), constitutes one of the main features characterizing the most virulent strains[1]. These proteins define a peculiar secretion apparatus which is devoted to the major toxin CagA export, but also triggers a relevant proinflammatory response in the gastric epithelial cells. The CagZ, CagS and CagD structures have been solved, while crystals of two truncated fragments of CagV have been obtained[5,6]. The structure determination is currently underway.

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R.14

STRUCTURE-BASED DISCOVERY OF ANTIVIRALS

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The majority of recent viral outbreaks has been caused by RNA viruses. Yet, for most viral infections, no causative therapy is available. Hence, there is an urgent need for discovering and developing new antivirals. For the past few years, my laboratory has been working on the structure-based design and the chemical synthesis of inhibitors of proteases from coronaviruses and picorna/enteroviruses.

Coronaviruses have been in the spotlight since the SARS outbreak of the year 2003. These viruses feature the largest genome of all plus-stranded RNA viruses (27-31 kb of ssRNA). Open reading frame 1 covers 2/3 of the entire genome and codes for two polyproteins, pp1a and pp1ab, the latter of which arises through a ribosomal frameshift during translation. These polyproteins are processed by two or three viral proteases, yielding the non-structural proteins (Nsp1-16) required for virus replication. In most coronaviruses, these cleavage reactions are performed by three cysteine proteases, two of which are of the papain type ($PL1^{pro}$ and $PL2^{pro}$) and one of the chymotrypsin type (M^{pro}). In SARS-CoV, there is only one PL^{pro}. The main protease of Transmissible Gastroenteritis Virus (TGEV) was the first protein of any coronavirus to have its three-dimensional structure determined[1]. This was followed by the structures of the homologous enzyme from human coronavirus 229E[2] and from the SARS coronavirus[3-5]. Soon it became clear that the main protease is an attractive target for the design of anticoronaviral inhibitors. The latest inhibitors will be discussed, and a number of their complexes with the M^{pro} will be presented[2,6-8]. Also, new methodology will be presented for converting peptidic hits into non-peptidic lead compounds[9].

Some of our inhibitors also exhibit an interesting activity against coxsackievirus B3 (CVB3) and caliciviruses. Hence, we have determined the crystal structure of the 3C protease of CVB3 and designed inhibitors against this target. We also characterized calicivirus 3C-like proteases[10]. Latest developments will be discussed.

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R.15

ALLOSTERIC MODULATORS OF IL-8/CXCL8 RECEPTORS.

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Several allosteric modulators have been shown to contribute to physiological or pathophysiological processes, and allosteric sites represent attractive targets for small molecule drugs able to specifically inhibit signal transduction pathways induced by endogenous agonists rather than their interaction with the receptor as for orthosteric receptor antagonists. The allosteric modulation concept represents a largely unexploited region of the therapeutic chemical space, but it also poses new questions that invest different phases in drug discovery and design process, being the characterization of the binding mode only one of the first steps to the full comprehension of the specific mechanism underlying drug action.

The chemokine family is a group of low molecular weight, multifunctional cytokines which play a primary role in the inflammatory response, finely regulating the leukocytes recruitment in the inflamed tissue and exerting additional functions in physiological and pathological conditions. Among the chemokine family, CXCL8 plays a key role in the activation and recruitment of neutrophils at the site of inflammation. CXCL8 binds two membrane receptors, CXCR1 and CXCR2, and in the last decade the physiopathological role of CXCL8 in several inflammatory conditions has been widely investigated. Up to date, a limited number of small molecular weight (SMW) CXCL8 inhibitors have been disclosed in the literature but only in recent times the first drug candidates have entered clinical studies aimed at the assessment of the therapeutic potential of this class.

Reparixin (formerly repertaxin) and meraxin, identified in Dompé laboratories, are the first examples of neutral allosteric modulators of IL-8/CXCL8 receptors, CXCR1 and CXCR2, with proved efficacy in several experimental models of IL-8/CXCL8-related acute and chronic pathologies. Reparixin is presently under evaluation in phase II clinical trials for early graft failure prevention in lung and kidney transplantation. The investigation on their mechanism of action has demonstrated a selective inhibition of IL-8/CXCL8-induced neutrophil migration, with no effect on agonist-induced receptor intracellular trafficking, possibly unravelling novel opportunities for optimized pharmacological modulation.

Recent advances in the characterization of allosteric modulators of CXCL8 receptors will be discussed.

R.16

THE FUNCTIONAL EFFECTS OF THE NEW HUMAN HORMONE ABSCISIC ACID ON ANIMAL CELLS ARE MEDIATED BY THE SECOND MESSENGER CYCLIC ADP-RIBOSE.

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Abscisic acid (ABA) is a hormone involved in pivotal physiological functions in higher plants, such as response to abiotic stress and control of seed dormancy and germination. Recently, ABA was demonstrated to be autocrinally produced by human granulocytes and to stimulate granulocyte function through a signalling pathway involving the second messenger cyclic ADP-ribose (cADPR)[1].

Functional effects of ABA on human cells are not limited to granulocytes. ABA also stimulates the proliferation of human mesenchymal stem cells (MSC) and of uncommitted hemopoietic progenitors *in vitro*, through a cADPR-mediated increase of the intracellular calcium concentration ($[Ca^{2+}]_i$).

On MSC, ABA stimulates several functional activities, including cyclooxygenase 2-catalysed production of PGE_2, release of several cytokines known to mediate the trophic and immunomodulatory properties of MSC and chemokinesis. ABA is produced and released by MSC stimulated by specific growth factors, by inflammatory cytokines and by lymphocyte-conditioned medium. Lymphocyte-stimulated human MSC produce and release ABA at concentrations sufficient to exert growth-stimulatory effects on co-cultured $CD34^+$ cells.

On $CD34^+$ cells, micromolar ABA induces transcriptional effects, which include NF-κB nuclear translocation and transcription of genes encoding for several cytokines.

These results provide a remarkable example of conservation of a stress-hormone and of its second messenger from plants to humans and identify ABA as a new paracrine signal between MSC, inflammatory/immune cells and hemopoietic progenitors.

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R.17

TARGETING MET IN TUMOR AND METASTASIS: A MATTER OF ADDICTION AND SENSITIVITY

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In spite of the established knowledge of the genetic alterations responsible for cancer onset, the genes promoting and maintaining the invasive/metastatic phenotype are still elusive. The *MET* proto-oncogene, encoding the tyrosine kinase receptor for Hepatocyte Growth Factor (HGF), senses unfavorable micro-environmental conditions and drives cell invasion and metastasis. *MET* overexpression, often induced by tumor hypoxia, leads to constitutive activation of the receptor and correlates with poor prognosis. To establish the role of *MET* in the different phases of tumor progression we developed an inducible lentiviral delivery system of RNA interference. Silencing the expression of the endogenous MET gene in tumor cells resulted in impairment of the execution of the full invasive growth program in vitro, and of the generation of metastases *in vivo*. Notably, silencing *MET* in already established metastases led to their almost complete regression. This indicates that persistent expression of the *MET* oncogene is mandatory till the advanced phases of cancer progression. These findings can be explained by the known ability of *MET* to switch-off the apoptotic program and by its recently postulated contribution to the maintenance of stemness properties, required for the growth of metastatic colonies.

R.18

TARGETING CK2 AS A GENERAL STRATEGY IN SIGNAL TRANSDUCTION THERAPY

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In recent years a breakthrough has taken place in our understanding of molecular alterations underlying human diseases whereby most of pathological conditions are related to intra- and inter-cellular communication disorders. This gave rise to the concept of "Signal Transduction Therapy" (STT), a neologism coined to indicate a variety of approaches useful to identify and treat "signalling disorders". The first choice targets in STT are protein kinases, for a number of reasons, notably the implication of these enzymes in nearly all signalling pathways, their deregulation often related to global diseases, and their catalytic nature, conferring susceptibility to low molecular weight active site-directed specific inhibitors displaying pharmacological potential. Not surprisingly therefore the largest subset of the "druggable genome" (22%) is provided by the individual family of protein kinases (either Ser/Thr or Tyr specific)[1], which is encoded by less than 2% of the human genome (collectively referred to as the human "kinome"). Such a "druggable kinome" currently includes more than 150 members of the family[2] with inhibitors of many of these already in clinical practice or in advanced clinical trials.

It is generally held that the pathogenic potential of protein kinases is conferred by their unscheduled activation, due to mutations and/or other genetic alterations responsible for the onset of specific diseases. Notable exceptions however are provided by a number of protein kinases whose pathogenic potential is not the outcome of obvious genetic alterations, being caused instead by their abnormally high cellular level, due to as yet not deciphered epigenetic events. The most remarkable example in this respect is provided by CK2, a highly pleiotropic, constitutively active Ser/Thr protein kinase whose level is invariably elevated in tumour cells, where it promotes a global anti-apoptotic and pro-survival response[3], defective susceptibility to anti-cancer agents[4] and an accentuated multidrug resistance phenotype[5]. As a consequence cells with abnormally high CK2 levels appear to be positively selected by the tumour, a circumstance which makes CK2 an attractive target in a variety of neoplastic diseases where its down-regulation may abrogate a number of general devices exploited for tumour progression, even if the molecular alteration underlying the onset of the disease is not residing in CK2 itself.

Based on these premises the development of selective cell permeable inhibitors able to attenuate CK2 activity may represent a "master key" useful for the treatment of a wide spectrum of different neoplastic disorders , rather than tailored for combating a specific type of cancer.

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R.19

TARGETING HYPOXIA INDUCIBLE FACTOR 1 (HIF-1) FOR CANCER THERAPY.

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Most solid tumors develop regions of low oxygen tension because of a tissue imbalance between oxygen supply and consumption. Hypoxic cancer cells are more invasive and metastatic, resistant to apoptosis and ultimately to chemo- and radiation-therapy. Failure to eliminate the hypoxic fraction of solid tumors is eventually associated with poor prognosis. Hence, the identification of survival pathways utilized by hypoxic cancer cells is important for the potential therapeutic implications and strategies to target hypoxic cell signaling are being actively pursued.
HIF-1α is an attractive target for development of novel cancer therapeutics, in particular for its involvement in angiogenesis, tumor metabolism and metastasis. HIF-1 is not only expressed in cancer cells but also in stromal infiltrating cells and endothelial cells, thus representing a common molecular determinant of the tumor microenvironment. HIF-1 expression in macrophages regulates genes that may promote tumor growth, including the inducible nitric oxide synthase gene. HIF-1 alpha expression in endothelial cells is essential for angiogenesis and response to growth factors. Therefore, HIF-1 may represent a novel target of the tumor microenvironment.

Despite the increasing number of HIF-1 inhibitors described in the literature it is unclear which is the most effective way to inhibit HIF-1 and how these agents should be developed. Over the last few years the Developmental Therapeutics Program of the National Cancer Institute has identified several novel HIF-1α inhibitors, including topotecan, a topoisomerase I poison that inhibit HIF-1α expression by a DNA-damage independent mechanism and echinomycin, which blocks HIF-1 DNA binding activity. In an effort to validate HIF-1 inhibition in the clinic, we are conducting a clinical trial of oral topotecan on a 2-week schedule in patients whose cancers over-express HIF-1α, as assessed by IHC. Since inhibition of HIF-1α alone may be insufficient to generate meaningful therapeutic responses we have tested the hypothesis that combination of HIF-1 inhibitors with antiangiogenic agents may have increased therapeutic efficacy. Results of the combination of topotecan with Avastin®, an anti-VEGF antibody, in xenograft models will be discussed.

In conclusion, efforts should be aimed at validating HIF-1 inhibition in relevant animal models and in early clinical studies. Combination of HIF-1 inhibitors with novel molecular targeted agents should be explored to maximize the potential therapeutic effects of HIF-1 inhibition.

R.20

DIOXYGENASE ENZYMES FROM ARENE-DEGRADING BACTERIA: CAN WE DEVELOP THEIR POTENTIAL FOR APPLICATION IN BIOCATALYSIS?

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Arene-dioxygenase (or ring-hydroxylating dioxygenase) enzymes are typically found in bacteria that degrade aromatic hydrocarbons – including BTEX, PAHs and PCBs – under aerobic conditions. With these substrates they destabilise the aromatic ring, through incorporation of molecular oxygen, to yield arene *cis*-dihydrodiol metabolites. However, another significant property of these enzymes is their relaxed substrate specificity, and it has been shown that they can facilitate the biotransformation of a wide range of substrates to produce chiral alcohols of various types that can then be used as chiral precursors in organic synthesis (Boyd *et al*., 2001).

Recently, we have explored the biochemical properties of these enzymes further, and have discovered a wider range of unexpected biotransformations (Boyd *et al.,* 2006). Here we discuss the implications of these findings – with special focus on the combination of dioxygenase biocatalysis with other forms of chemical catalysis, and on their application in 'second generation' biotransformations, where an initial biotransformation product is subjected to further rounds of biocatalysis with these enzymes.

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R.21

METABOLIC ENGINEERING: A TOOL FOR BIOREMEDIATION STRATEGIES.

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Bioremediation strategies exploit the metabolic potential of microorganisms able to degrade pollutants. This is the case of *Pseudomonas sp.* OX1, which is able to use aromatic compounds like benzene, toluene, *o*-xylene and other phenols as the sole sources of carbon and energy. To unravel the molecular basis of this ability we have cloned, expressed and characterized toluene-*o*-xylene monooxygenase (ToMO) and phenol hydroxylase (PH), which activate aromatic hydrocarbons, and catechol 2,3 dioxygenase (C2,3O), the "gateway" enzyme to the pathway that leads to molecules which enter CAC. We have discovered that ToMO and PH posses wide substrate specificity producing dihydroxylated aromatic compounds, which are not always substrates of C2,3O. For example ToMO and PH convert *o*-xylene, to 3,4-dimethylcatechol - which is a good substrate of C2,3O - and *m*- and *p*-xylene, to 3,6- and 3,5-dimethylcatechols (DMC) which are not. Thus, production of these products is responsible for the inability of *Pseudomonas sp.* OX1 to grow on *m*- and *p*-xylene, hence impairing the "bioremediation potential" of the microorganism.

Mutagenesis experiments indicate that changing residues 100, 103, 107, 176, 180 of ToMO A subunit allows to finely tune its regioselectivity. For example, mutant ToMO A-E103G is able to increase 3,4-DMP production, which is transformed by PH into 3,4-DMC, which can be further transformed by C2,3O. As for C2,3O, we have discovered that reducing the hindrance of residue T249 leads to a mutant which is able to bind 3,6- and 3,5-DMC, whereas changing residues at positions 267 and 270 allows to increase the catalytic efficiency on these substrates.

Another research line we are pursuing is the rapid identification, using bioinformatics tools, of new strains useful for bioremediation strategies. Presently, more than one thousand microbial genomes are being, or have been, sequenced. Microbial genomic databases are searched for proteins homologous to enzymes which initiate the catabolism of xenobiotics in already-known pathways. This allowed for the selection of four potentially useful strains: *Ralstonia metallidurans* CH34, *Rhodobacter sphaeroides* 2.4.1, *Azotobacter vinelandii* OP and *Silicibacter poymeroi* DSS-3. A preliminary characterization of *R. metallidurans* CH34 and *R. sphaeroides* 2.4.1 has shown that they are able to metabolize aromatic compounds and tetrahydrofuran, respectively, a previously unknown ability.

At the same time we have tried also to isolate new useful strains from polluted environments. Incubation of samples of surface seawater collected in the harbor of Pozzuoli - heavily polluted by fuels - with naphthalene, anthracene and phenanthrene, allowed to isolate several strains able to grow on complex mixtures of hydrocarbons. One of these strains, named PP1Y, is able to degrade methyl, ethyl, dimethyl, and trimethyl-benzenes, biphenyl and several polyciclic aromatic hydrocarbons.

R.22

MICROBIAL REDUCTIVE DEHALOGENATION FOR THE SUSTAINABLE REMEDIATION OF PCB-CONTAMINATED SEDIMENTS.

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Due to their high recalcitrance and hydrophobicity, PCBs are extensively accumulating in sub-surface sediments of freshwater and marine habitats, through which they enter the food chain. PCBs occurring in such sediments may undergo microbial reductive dechlorination, which results in their conversion into lower chlorinated less toxic and bio-accumulable congeners. The occurrence of such processes has been mostly observed in slurry microcosms of freshwater sediments suspended in mineral media and ascribed to bacteria of the phylum *Chloroflexi*. Recently, we detected microbial-mediated PCB reductive dechlorination processes in 5 marine sediments of Brentella Canal (Venice lagoon) suspended in their own site water, i.e. under geobiochemical conditions close to those *in situ*[1-3].

To gain deeper insights on PCB dechlorination under such conditions, native microflora was sub-cultured 7 times in sterile slurry microcosms containing decreasing amounts of sediment suspended in site water in the presence of 5 exogenous coplanar PCBs (100 mg/kg dry sed. each) and then of Aroclor 1254 (1 g/kg dry sed.). In the last two transfers, H_2, short chain organic acids (formate, acetate, propionate and butyrate) and antibiotics (vancomycin, ampicillin) were monthly added to parallel cultures to study their effects on the dechlorination process. Additional microcosms without PCBs were also set up for each culture condition. Microbial populations selectively enriched under the different conditions in the presence and in the absence of PCBs were subjected to 16S rDNA-DGGE analysis with universal Bacterial primers and primers specific for the dehalogenating members of the *Chloroflexi* phylum.

Increasing PCB-dechlorination rates along with remarkable increases in sulfate-reduction and a progressive decline in methanogenic activity were observed throughout subculturing. The culture resulting from the last transfer displayed a marked dechlorination activity towards penta- through octa-chlorinated biphenyls of Aroclor 1254, that were bioconverted by more than 70% into di-, tri- and tetra-chlorinated congeners after 30 weeks of incubation. DGGE analysis of 16S rRNA genes revealed the occurrence of a *Dehaloccoccoides*-like microorganism, having high sequence similarity with the putative PCB dechlorinating bacterium m-1, in all the actively PCB dechlorinating cultures, that was absent in all the corresponding PCB-free microcosms. Taken together, these findings suggest that reductive dehalogenation widely occur *in situ* in the Brentella Canal and that native *Dehalococcoides*-like bacteria are involved in the process. Thus, natural attenuation seems to be a feasible option for the sustainable remediation of the sediments of the Canal and suitable substrate mixtures able to selectively stimulate *Dehaloccoides* sp. *in situ* are currently sought.

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R.23

SENSOR FOR CHEMICAL RISK ASSESSMENT

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The traditional chemical analysis is not alway the most suitable way to assess the chemical risk from the environment where one is living.

Generally a risk can be evaluated by integral indexes able to give informations not about this or that compound but about the integral effect of such contemporary presence.

A description is here given of a preliminary approach to the use of a new generation solid state sensor based on the capacity of the sensor element to catalyze the photodegradation of various kinds of organic compounds and to monitor the consequent pH variation. The electron holes present in the TiO_2 structure are able to trigger an oxidative process involving substances present in the environment, in particular those ones that can be adsorbed on it. These characteristics make titanium dioxide a suitable material to be used as a sensor for measuring environmental persistance and consequent risk.

The informations are obtained by the delay to observe photodegradation starting from the initial irradiation and by the slope of the pH decrease following the beginning of the mineralization process announced by the acidification after the delay time.

To the question about the contribution of Chemistry to the evaluation of human and environmental risk from anthropic activity , also a radical approach answer can be given. When we produce or use energy some of the energy remains segregated within secondary pathways molecules called radicals highly energetic and reactive due to the presence in their electronic structure of unpaired electrons which can be so or lost or coupled. As consequence the radicals act as reducing or oxidising agents. Radicals are able to attack any substrate or tissue so behaving as a very dangerous chemical species.

Radicals particularly attack proteins and produce oxidative stress.

All amino acid residues of proteins are potential targets for attack from reactive oxygen species (ROS) produced in the radiolysis of water; however, in only a few cases have the oxidation products been fully characterized. Moreover, under most physiological conditions, cysteine, methionine, arginine, lysine, proline, histidine, and the aromatic amino acids are primary targets for ROS-mediated oxidation.

Exposure of proteins to reactive oxygen species can lead to the formation of protein-protein cross-linkages.

Respiromeytric biosensor can also be successfully applied to chemical risk evaluation basing on the comparison between the respiratory capacity of different cells in reference and actual conditions.

R.24

LIFE ON THE EDGE: THE NATURE AND ORIGINS OF PROTEIN MISFOLDING DISEASES

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Because proteins are involved in every chemical process taking place within living systems, the failure of proteins to fold, or to remain correctly folded, can give rise to serious cellular malfunctions that frequently lead to disease. One particularly important group of such diseases is associated with the aggregation of misfolded proteins into remarkable thread-like structures known as amyloid fibrils[1], and includes disorders ranging from Alzheimer's disease to late-onset diabetes, conditions that are becoming increasingly common in our aging populations. The manner in which the normal soluble forms of peptides and proteins can convert into these pathogenic amyloid structures is being uncovered by a wide variety of *in vitro* experimental studies along with theoretical simulations and bioinformatics studies[2]. As with folding, these studies are increasingly being linked to events occurring *in vivo* using a variety of strategies. Of particular interest are experiments an simulations designed to link the principles of misfolding and aggregation to the effects of such processes in model organisms such as *Drosophila* (the fruit fly). This talk will draw together some of the ideas that are emerging from recent work in our laboratory including evidence for the extremely narrow boundary between normal and aberrant behaviour[4] and how this concept sheds light on the origin, current proliferation and potential means of prevention of many of the diseases associated with misfolding.

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R.25

β_2-MICROGLOBULIN REFOLDING INTERMEDIATES AND FIBRILLOGENESIS

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β2-microglobulin (β 2-m) is the nonpolymorphic light chain of the class-I major histocompatibility complex. The deposition of β 2-m fibrils is associated to dialysis-related amyloidosis (Gejyo et al., 1985), a disease which arises in individuals with chronic renal failure following long-term haemodialysis treatment. Stopped-flow kinetic analysis (Chiti et al., 2001) could clearly reveal the occurrence of a long-lived intermediate of full-length β 2-m, I_2, along the refolding pathway characterized by a kinetic constant of about 10^{-3} s^{-1}. The recent characterization of W60G β 2-m, a mutant of β 2-m where the replacement of Trp60 with a Gly is responsible for reduced conformational flexibility and dynamic association as well as for the loss of fibrillogenicity in TFE (Esposito et al., 2008), prompted us to elucidate comparatively the refolding kinetics of wild type and mutant–proteins at atomic definition level by NMR. Our analysis was thus conducted by the band Selective Flip Angle Short Transient (SOFAST) real time 2D NMR method with a time resolution of few tenths of second. The results show that the refolding process is more complex than previously reported and seems to involve more than a single intermediate species with novel insights into the fibrillogenic conformation(s).

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R.26

MISFOLDING AND TISSUE LOCALIZATION OF AMYLOIDOGENIC PROTEINS: COMPLEXITIES AND BREAKTHROUGHS

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Amyloidosis caused by β2-m offers elements of general interest for understanding the general mechanism of fibrils deposition in systemic amyloidosis. In this type of disease the tissue specificity of the amyloid deposition that is a generic clinical-pathological feature, of all the amyloidosis is particularly prominent. Bones, ligaments and synovial membranes are in fact the inescapable prominent target of the amyloid deposits. These peculiar hystopathological evidences should provide important clues on the elucidation of the real events occurring *in vivo* when the protein looses the monomeric globular structure and self aggregates into the typical cross-beta amyloid polymers. Combination of proteomic and ultramicroscopic studies provides interesting clues on the identification of tissue factors and characterisation of β2-m modifications that might affect the kinetics of amyloid deposition. Natural amyloid fibrils accumulate in the close proximity of collagen fibres and in particular of those with the largest section. The pathognomonic cleavage at the sixth residues is only present when the deposit of β2-m is organized in the fibrillar structure. Taking into account these findings we have established a method of fibrillogenesis in which β2-m is incubated with physiologic concentrations heparin, δN6-truncated β2-m and collagen fibres. By this method we obtain the globular to fibrillar transformation in a physiologic-like environment. Structural transitions occurring in these conditions are unknown and difficult to disclose, but we have robust evidences suggesting that in these conditions the kinetics of fibrils formation is dictated by the concentration of soluble oligomers. The discovery of this method highlights the possibility that, *in vivo*, multiple factors might concur cooperatively to the protein amyloidogenesis. Availability of this method represents a significant step forward the accomplishment of research strategy aimed to identify new amyloid interactors suitable for pharmacological exploitation.

R.27

PRION PROTEIN, PRION DISEASES AND TETRACYCLIC COMPOUNDS

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The prion diseases are fatal neurodegenerative disorders for which no effective treatment is available. A large number of molecules have been tested in an attempt to find therapeutic agents, but only a few classes of compounds showed any beneficial effect in in vivo and/or in vitro models. Studies from our group led to recognize that some tetracyclic compounds, such as iododoxorubicin and tetracyclines, are able to (i) interact with and revert the protease-resistance of PrPSc extracted from brain tissue of patients with all forms of CJD, cattle with BSE and rodents with experimental scrapie, (ii) reduce the infectivity titer in prion-contaminated material, and (iii) prolong survival of prion-infected animals. On this ground, a series of CJD patients observed at "Carlo Besta" Institute in the last five years received compassionate treatment with doxycycline at a daily oral dose of 100 mg from the time of diagnosis to death. The choice of this drug among others tetracyclic compounds effective in experimental models was based on the observation that doxyclycline has favorable kinetics, relatively good capacity to cross the blood-brain barrier, low toxicity and good tolerability even for prolonged administration. Indeed, no CJD patient chronically treated with this drug showed adverse secondary effects. The retrospective analysis revealed that the subjects treated with doxycycline (n=21) survived significantly longer than untreated patients (n=78); in particular, the survival time (median ± SE) was 13.0 ± 4 months in the former and 6.0 ± 0.7 months in the latter (Log Rank test: $p<0.001$). A significant difference was still present when the doxycycline-treated group was compared to an untreated group equivalent for sex, age at onset and codon 129 PRNP polymorphism (treated: 13.9 ± 3.8 months, untreated: 6.1 ± 0.5 months, $p<0.01$), which are major predictors of survival of CJD patients. This result, based on an open observation, is under verification in a randomized, double-blind study of doxycycline versus placebo that has been approved by the Italian Agency of Drug (AIFA). A positive outcome of this trial would activate similar studies in other neurodegenerative disorders due to protein misfolding such as Alzheimer's disease, since the effects of doxycycline seem to be dependent upon a direct interaction with abnormal protein conformers with an extensive beta-sheet conformation rather than with a specific amino acid sequence.

R.28

PROTEIN FOLDING AND SIGNALING IN THE EARLY SECRETORY APPARATUS.

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The endoplasmic reticulum (ER) is a multifunctional organelle integrating diverse physiological tasks. Efflux of Ca^{2+} via IP_3R1 is fundamental for many signalling processes. Moreover, suitable redox conditions must be maintained for oxidative protein folding and signal generation and tuning[1]. ERp44 mediates thiol-dependent retention in the early secretory pathway, forming mixed disulfides with Ero1α and Ero1β, the two main ER resident oxidases, and other substrate proteins through its conserved CRFS motif[1-3]. ERp44 also binds non-covalently ERGIC-53 and IP3R1, thus concurring in the regulation of redox homeostasis, ER to Golgi protein transport and calcium signalling. Its crystal structure at a 2.6 Å resolution reveals three thioredoxin domains, a, b and b', arranged in a clover-like structure. A flexible C-terminal tail turns back from the b' to the a domain, shielding a hydrophobic pocket in domain b' and a hydrophobic patch around the CRFS motif in domain a. Mutational and functional studies indicate that the C-terminal tail gates the CRFS area and the adjacent hydrophobic pocket, dynamically regulating protein quality control[2]. The peculiar structure and subcellular localization of ERp44 suggest a key role of this conserved molecule in integrating protein transport and signalling in the early secretory compartment.

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R.29

"SEEING" AT THE NANOSCALE WITH THE ATOMIC FORCE MICROSCOPE

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Since its invention in 1986[1], the atomic force microscope (AFM) proved to be a powerful, non-invasive tool in the structural characterization of biological samples at the nanoscopic scale. This instrument employs a microscale cantilever with a sharp tip which probes the sample surface. The AFM allows operation both in air and in fluid environment; sample integrity can then be maintained under hydrated conditions. Under appropriate conditions, very high resolution can be attained, and, in favorable cases, even single biomolecules can be imaged. There is no need of fixation or staining procedures, which may affect sample morphology. Even very soft samples, which would be damaged by a steady contact with the tip, can be successfully imaged using the tapping mode, in which the AFM probe is oscillated, lightly tapping the sample surface. As samples can be maintained under controlled hydrated conditions, it is possible to perform in situ studies of processes such as assembly, polymerization, dissolution. Finally, the AFM can be used not only for imaging purposes, but also to perform force spectroscopy measurements, which can give information on single molecule rupture or stretching forces, such as those involved in ligand-receptor interaction or protein unfolding.

Applications of this technique to the study of amyloid aggregation, protein crystals and protein/lipid membrane interaction will be presented.

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R.30

ORTHOGONAL STRATEGIES IN PROTEOMICS

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Following the end of the Human Genome Project, the biological challenge has again shifted from gene to the protein side, giving rise to the so-called "proteomic era". Proteomics (the Science of Proteome) is a totally new way to look at the protein kingdom, aiming to investigate very complex protein mixtures within their biological context. Proteomic research is focused on two major areas, differential proteomics aimied to measure the up- and down-regulation of protein expression levels and functional proteomics, addressed to investigate protein functions *in vivo*.

In both cases, proteomic samples consist of complex mixtures of components that cannot be directly analysed without pre- fractionation by high resolution and sensitive techniques. Several separation methodologies have been developed most of which are based on two distinct physico-chemical properties of the sample, the so-called bidimensional ("orthogonal") techniques. Among these, 2D gel electrophoresis and 2D chromatography are commonly used since years.

Besides these classical bidimensional approaches, new emerging mass spectrometry methodologies have evolved, together with high performance instruments capable of carrying out sophisticated experiments. These approaches give rise to a sort of "instrumentally driven" orthogonal selection of ions able to simplify the sample mixtures, leading to selective identification of specific peptides.

Ion Mobility Spectrometry (IMS) was recently proposed to provides additional stages of ion separation before MS analysis. IMS is like a "Gas Phase Electrophoresis" in which the mobility of an ionised peptide is dependant on its Size (Shape) and Charge State. Since IMS separation is based on a different physical property from both HPLC and MS, it can provide an extra dimension of ion separation for the analysis of very complex mixtures.

The RIGhT (Reporter Ion Generating Tag) strategy is based on the labelling of target residues with reagents capable of generating reporter ions in MS2/MS3 experiments, such as Dansyl chloride. Fragmentation of dansyl derivatives shows the typical 170 m/z and 234 m/z ions in MSMS experiments and a diagnostic 234-170 m/z transition in MS3 mode. Experiments combining a precursor ion scan with a MS3 scan mode can then be tailored to selectively analyse derivatised peptides.

Isolation of protein complexes in functional proteomics experiments mainly rely on (immuno)affinity procedures, followed by protein fractionation by SDS-PAGE and identification by LCMSMS. These procedures require extensive pre-cleaning steps to reduce false positives. Orthogonal methods have been proposed that integrate the (immuno)affinity steps with pre-fractionation of native complexes, as achieved by ultracentrifugation in glycerol or sucrose gradients, Blue Native Electrophoresis and/or gel filtration chromatography.

R.31

MANIPULATION AND IMAGING OF SINGLE BIO-MOLECULES

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We will show some experiments in which biochemistry of molecule interaction is investigated at the single molecule level. In particular, we will show experiments where single DNA-protein interaction and single molecular motor dynamics and kinetics are investigated by means of optical tools. Some manipulation operations based on optical tweezers and magnetic tweezers will be shown, together with the possibility to visualize single molecules. Finally, an experimental approach combining trapping and fluorescence detection will demonstrated the capability of single molecule localisation with nanometer spatial co-localisation and 10 microsecond time resolution, allowing to follow in real-time single protein conformational dynamics.

R.32

MOLECULAR FUNCTIONS OF SIRTUINS.

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The Sir2 protein deacetylases (or Sirtuins) are an evolutionarily conserved family of enzymes that utilizes co-substrate NAD^+ to deacetylate the ε-amino group of lysine from acetylated proteins, yielding two additional products nicotinamide and *O*-acetyl-ADP-ribose (Fig 1). Sirtuins have been implicated in a wide range of cellular processes, including pathways that affect diabetes, cancer, lifespan, and Parkinson's disease. Seven mammalian sirtuins (SIRT1-7) display diverse sub-cellular localization and have been implicated in cellular processes including cell survival, cell cycle regulation and genomic stability, fatty acid synthesis, and glucose and insulin homeostasis. To understand the molecular basis for sirtuin action, recently we have *a*.) probed the mechanism of catalysis using acetyl-lysine analog peptides, *b*.) identified metabolic enzymes as targets of regulation by sirtuins, and *c*.) determined sirtuin-specific changes to the mouse metabolome. The results indicate that mechanism-based chemical probes can be utilized to further our understanding of the biologial processes controlled by sirtuin activity[1-2]. The ability of SIRT1 and SIRT3 to directly regulated the activity of key metabolic enzymes was demonstrated through the regulation of acetyl-CoA synthetases by SIRT1 and SIRT3[3], and the metabolome changes identified in the livers of $Sirt3^{-/-}$ mice.

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R.33

miR, "STEMNESS" & SKIN

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Although p63, similarly to its homologues p73 and p53, regulate apoptosis during DNA damage, shows a crucial role in regulating epidermal development. The activity of p63 depends on its steady state protein levels, and a number of evidence suggest that post-transcriptional regulation rather than transcriptional control plays a major role in p63 function. While p63 is important for epithelia formation (Candi et al. CDD 2006. 13: 1037), including the thymus (Candi et al. PNAS 2007. 104: 11999), p73 is also involved in neurodegeneration and immune responses. Although the tumor suppressor protein PML modulates p63 half-life by recruiting them to the PML-nuclear bodies to regulate their transcriptional activities and thus inhibiting their degradation, the molecular mechanisms underlying the regulation of p63 protein stability remain largely unknown.

We have identified the Hect-containing E3 (Nedd-4-like) ubiquitin-protein ligase Aip4/ITCH as responsible for the proteosomal degradation of both p73 (Rossi et al. Embo J 2005. 24: 836) and p63 (Rossi et al. PNAS 2006. 103: 12753). The PY motif-containing C-terminal region of p73/p63 binds to the WW domain of Itch, resulting in ubiquitination and degradation of p73/p63. We have also identified a regulator of ITCH activity, N4BP1, able to regulate the function of ITCH's substrates (Oberst et al. PNAS 2007. 104: 11280). On these bases, we are developing small molecular inhibitor of Itch able to regulate p73/p63 degradation and therefore their function in DNA damage, finely regulating apoptosis and chemosensitivity.

Here, we describe the ability of p63 mutants, found in genetic diseases, to be regulated by Itch. We also report the identification of miR-203 able to target and repress p63, thus repressing "stemness" and allowing epithelial differentiation. During keratinocyte's differentiation the sudden upregulation of miR-203 causes the drastic reduction of DNp63 proteins, as miR-203 binds the 3'UTR of the *trp63* transcript to regulate its translation. This relationship is concerved in ES cell commitment in vitro. miR203 inhibit ES clonogenicity, while antagomiR-203 enhances clonogenicity, in keeping with a role of DNp63 in "stemness" proliferation potential (Candi et al. PNAS 2007. 104: 11999). miR-203 seems to be at the interfece regulating the transit of epithelial cells from the "stemness" compartment to the differentiation compartment.

R.34

MANY FACES OF THE RNA BINDING PROTEIN KSRP

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The interaction of different protein factors with target RNA sequences is essential for the intricate series of events that determine post-transcriptional control of gene expression. KSRP is a single strand RNA-binding protein that interacts with distinct RNA sequences and is involved in different steps of post-transcriptional RNA life from splicing to transport, from cellular localization to decay. Many studies from our and other laboratories demonstrated that KSRP binds to inherently unstable mRNAs and target them for rapid degradation recruiting the ribonucleolytic enzymes. Now, we have found that KSRP is also implicated in an additional and unexpected step of post-transcriptional regulation of gene expression. KSRP is a component of both Drosha and Dicer complexes and binds with a high affinity to the terminal loop of a subset of miRNA precursors, critically regulates their maturation and, in turn, the ability of the mature forms to appropriately inhibit expression of target mRNAs. These findings reveal an unexpected mechanism that links KSRP to the machinery regulating maturation of a cohort of miRNAs, that, coupled to its role in promoting mRNA decay, serves to integrate specific regulatory programs of protein expression.

This work has been supported by AIRC, ISS Italia-USA, e CIPE 2007 Regione Liguria.

R.35

RNA INTERFERENCE-BASED SCREENING OF GENES INVOLVED IN EMBRYONIC STEM CELL SELF-RENEWAL AND DIFFERENTIATION

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Loss-of-function genetics is a very powerful and effective tool to explore the functions of genes. In contrast to gene knock out obtained by genetic recombination, RNA interference offers a simple, rapid and quite effective approach to analyze the effects of gene suppression in cultured cells.

We developed an assay based on the ability of mouse embryo stem cells (ESC) to differentiate in vitro leading to various neuronal populations. ESC clones, stably transfected with EGFP under the control of the neuron-specific alpha1 tubulin gene promoter, show a robust expression of GFP starting from day 4 of differentiation.

These clones were transfected with single plasmids each encoding an shRNA in 96 well plates and grown in differentiation conditions. At day four of differentiation the absence of GFP expression indicated that the suppression of the gene target of a specific shRNA was interfering with ESC differentiation.

We have examined shRNAs targeting about 12,000 genes and found out 80 of them that block ESC differentiation. 23 of these candidates have been validated by at least two independent shRNAs. They belong to three categories. The first one is that of mRNAs expressed in undifferentiated ES cells, whose concentration decreases upon differentiation. The second group is represented by genes that are expressed in ES cells, are downregulated upon the induction of differentiation and then reach the highest levels in fully differentiated cells. The last group contains several genes whose expression is actually unmodified before, during and after differentiation.

R.36

"miR GENES IN PAEDIATRIC CANCER"

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The aim of my presentation is to overview the biogenesis of miRNAs and apply our knowledge on their expression in two main embryonal paediatric tumors. The first is derived from cerebellum in the Central Nervous System (MedulloBlastoma) and the second from neural crest cells colonizing the adreanal medulla in the Peripheral Nervous System (Neuroblastoma).

MicroRNAs (miRNAs) are small RNA of approximately 22 nucleotides that play important regulatory roles by targeting mRNAs for cleavage or translational repression. Recent data showed that altered expression of specific miRNA contributes to the initiation and progression of cancer.

Embryonal central nervous system tumors incidence is constant from infancy to 3 years of age, medulloblastoma (MB) accounts for 15%–20% of all childhood brain tumors. Children with nonmetastatic MB has improved considerably their survival, otherwise the outcomes for infants and for those with metastatic MB remain poor. Importantly miRNAs have been suggested in developmental timing, cell death, cell proliferation, and the patterning of the nervous system. We are applying a systematic analyses pipeline, including a bioinformatic target identification, a proof of inhibition of transcription and translation of target genes in vitro by luciferase assays and an overexpression and interference methods in MB cell lines, mostly identifying miRNAs with targets involved into specific pathways: Notch, WNT and SHH. We identified some miRNAs (from http://microrna.sanger.ac.uk/) potentially regulating target genes participating to these three pathways, using the TaqMan (Applera, miRNA card) technology in a screening of 47 miRNAs in two MB cell lines, Daoy and D283Med. A quantitative expression analyses by Real Time PCR is allowing us to identify miRNAs differentially expressed during MB cell lines differentiation upon phenylbutirrate treatment. We found that several miRNAs which recognizes a specific region in 3'UTR sequence of important target genes already known to be involved in MB are good candidate for the validation step *in vitro*. One miRNA identified is targeting HES1 protein, an important activator of NOTCH pathway. We highlight these results and speculate on their potential involvement in MB development. Additionally a second miRNA appertaining to a family of miRNAs has been found regulating DDL1, translating "*in vitro*" into an inhibition of this protein expression, and then either Notch1 activation or Notch2 signaling inhibition by its over-expression.

Neuroblastoma (NB) is a childhood tumour derived from the sympathetic nervous system. It is suggested that it arises as a consequence of perturbed differentiation during the development of the sympathetic nervous system. In order to identify and characterize new miRNAs involved in NB we based our analyses on the isolation of new miRNAs from two independent approaches, one by isolating and characterizing an embryonic cDNA library of murine genes (Bulfone et al. 2005, Journal of Neuroscience), while the second strategy by analyzing

the presence of potential miRNAs in repetitive elements into the genome. Following this latest approach, a novel miRNA, was characterized for its involvement in differentiation and apoptosis of human NB SH-SY5Y cells line, thus regulating the expression of target genes including IAPs (Inhibitors of Apoptosis proteins, BIRC1, BIRC4). We will present its functional correlation to neurodevelopmental processes such as neural differentiation and apoptosis, being a new controlling gene potentially involved in brain paediatric tumours. These approaches are then followed with "antago-mir" and 0-2 Methyl chemical synthesis technology thus resulting into impairing *in vitro* the miRNA function in cell lines, and *in vivo* and in *ad hoc* animal models (xenograft/orthotopic, adrenal medulla NB model).

This study highlights the identification of a new miRNAs and their involvement in MB and NB cancer progression, and its potential use for future therapeutic applications.

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R.37

THE ROLE OF OXIDATIVE STRESS IN INSULIN SECRETION AND AGING

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Reactive oxygen species (or ROS) are implicated in the pathophysiology of various diseases, including cancer. JunD, a member of the AP-1 family of transcription factor, regulates genes involved in anti-oxidant defence and protects cells against ROS. Interestingly, by inhibiting anti-oxidant defence, *junD*-inactivation promotes chronic oxidative stress and stimulates angiogenesis. Indeed, we have recently uncovered a new mechanism linking persistent oxidative stress to enhanced angiogenesis (Gerald et al., 2004 ; Pouysségur and Mechta-Grigoriou, 2006).

Nearly sixty years ago, D. Harman proposed the "free radical theory of aging" correlating ROS production to aged-related pathologies and premature death. This theory suggested that endogenous ROS accumulate with aging and result in cumulative irreversible damages. More recently, several laboratories have also demonstrated that Insulin/IGF-1-mediated transduction pathways reduce lifespan in various species by stimulating energy storage and growth. This new theory can be referred to as "the endocrine theory of aging". In agreement with its protective role against oxidative stress, *junD*-deficient mice (*junD*$^{-/-}$) exhibit features of premature aging (such as alopecia, cachexia, cataract and sterility), develop cancers and die before the control littermates. *junD*-deficient mice are then a model of choice for studying the molecular link between oxidative stress, aging and oncogenesis. Using these mice, we have established the pro-angiogenic effect of ROS in the systemic regulation of insulin that affects lifespan. We have then demonstrated that angiogenesis as a new link between two major theories of aging "the free radical theory" and the "endocrine theory".

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R.38

OXIDATION-COUPLED TRANSCRIPTION: DEMETHYLATION OF HISTONE H3 LYSINE 9 INDUCES DNA OXIDATION AND DRIVES TRANSCRIPTION INITIATION BY ESTROGENS

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Covalent modifications at the N-terminal tails of nucleosomal histones, induced by protein complexes that assemble on chromatin, are required for gene transcription. We have analyzed the mechanism by which H3 histone methylations control expression of the estrogen-responsive *bcl-2* and *pS2* genes by chromatin immunoprecipitation analysis. DNA-bound estrogen receptor drives transcription by bending chromatin to contact the RNA polymerase II assembled 1.5 kilobases apart. This process is driven by receptor-targeted demethylation of H3 lysine 9 at both the enhancer and promoter sites, achieved by activation of resident LSD1 demethylase.

Localized demethylation produces hydrogen peroxide, which modifies surrounding DNA by oxidazing guanine. Oxidized base induces recruitment of the 8-oxoguanine-DNA glycosylase 1 (OGG1) and topoisomerase IIβ, triggering DNA cleavage and relaxation, essential for the assembly of the productive transcription initiation complex.

By using chromatin immunoprecipitation we have screened promoter arrays with chromatin DNA immunoprecipitated with antibodies specific to estrogen receptor, OGG1 (8-oxoG glycosilase), Topoisomerase II*b* and LSD1. We have isolated several human promoters-ERE regions that simultaneously recruit these proteins in the presence of estrogens.

These data highlight the role of mismatch repair enzymes in transcription initiation and suggest a common evolutionary pathway between repair and transcription initiation.

Cuozzo *et al*., 2007, PLoS Genet. 2007, 3, 1144-1162
Perillo *et al*., 2008, Science;319, 202-6

R.39

IDENTIFICATION AND FUNCTIONAL CHARACTERIZATION OF A NOVEL ANTIAPOPTOTIC GENE INVOLVED IN ADAPTATION TO OXIDATIVE STRESS AND CHEMORESISTANCE.

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TRAP1 (also known as heat shock protein 75) is a mitochondrial molecular chaperone homologous to HSP90 family members. Our previous studies demonstrated that: i) TRAP1 expression is increased in Saos-2 human osteosarcoma cells adapted to a mild oxidative stress by diethylmaleate, a GSH depleting agent[1]; ii) high TRAP1 levels yield cells highly resistant to DNA damage induced by oxidants and chemotherapeutic agents; iii) TRAP1 protects cells from apoptosis by platin derivatives[2]. Several observations suggest specific roles for this chaperone in mitochondrial physiology and indicate that TRAP1 may inhibit some key apoptogenic events in mitochondria, whose loss of regulation could contribute to different pathological conditions and phenotypes. However, molecular mechanisms involved in TRAP1 antiapoptotic functions have not been fully characterized.

We have modulated the expression levels and sub-cellular localization of TRAP1. TRAP1 stable knock down sensitized Saos-2 cells to cell death inducers acting both on the intrinsic pathway (oxidants, arachidonate) and on the extrinsic pathway to apoptosis (TNFα). Deletion of 100 aminoacids from the N-terminus of the TRAP1 protein (δNTRAP1) abrogated its mitochondrial localization, prevented the mitochondrial import of endogenous TRAP1, and favored apoptosis via the intrinsic pathway through sensitization of the mitochondrial permeability transition pore (PTP) to opening. The finding that both transient and stable transfectants expressing the δNTRAP1 mutant yield cells highly sensitive to apoptotic stimuli, suggests that the truncated protein, which differently from the wild type does not undergo phosphorylation, behaves as a "dominant negative" over the endogenous protein. In addition, we observed that TRAP1 stable transfectants, whose resistance to different apoptotic stimuli was widely demonstrated[2], loose their cytoprotective function upon transfection with δNTRAP1 mutant.

Taken together our observations demonstrate that mitochondrial TRAP1 exerts its antiapoptotic activity by desensitizing the permeability transition pore and define TRAP1 itself as a potential interactor of the PTP, thus expanding the range of possible combinatorial effects of drugs targeted to mitochondria for the treatments of human neoplasias and PTP-dependent diseases.

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R.40

REDOX REGULATION OF CELLULAR STRESS RESPONSE IN AGING AND NEURODEGENRATIVE DISORDERS: ROLE OF VITAGENES

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Oxidative damage plays a crucial role in the brain aging process[1]. Induction of heat shock proteins (HSPs) is critically utilized by brain cells in the repair process following various pathogenic insults[1,2]. When appropriately activated, the heat shock response has the capability to restore cellular homeostasis and rebalance redox equilibrium. Activation of antioxidant pathways is particularly important for neural cells with relatively weak endogenous antioxidant defenses[2]. We have recently focused our research on the role of acetylcarnitine (LAC) in the defense mechanisms against cellular stress and neurodegeneration[3]. In the present study we investigated mRNA expression and protein synthesis of Hsps and the oxidant status in adult (12 months) and senescent (28 months) rats, and the effect of LAC treatment in senescent rats. mRNA and protein synthesis of Hsps increased in senescent rats compared to adults in all brain regions examined; the maximum increase was observed in the hippocampus followed by cerebellum, cortex, and striatum. Hsps increase was associated with increased expression and activity of carnosinase and thioredoxin reductase, with decreased expression of thioredoxin and with significant oxidative changes in glutathione redox state, as well as carbonyls and HNE contents. Interestingly, treatment with LAC resulted in a marked increase of heme oxygenase-1 (HO-1), Hsp70, and mtSOD expression, of GSH content, and a decrease of HNE and protein carbonyl contents in the hippocampus, striatum, cortex and cerebellum. These results were also confirmed by in situ hybridization experiments. We used also a parallel redox proteomic approach[4-8] to identify the proteins that are oxidized in aged rat brain and those proteins that are protected by LAC treatment. Redox proteomics analysis in HP and CX, brain regions in which all indices of oxidative modification are elevated in brain aging showed that the specific carbonyl levels of three proteins, hemoglobin (HMG), cofilin 1 (COF 1) and beta-actin (ACT), are significantly increased in HP of senescent rats. Carbonyl levels of these proteins are reduced by LAC administration in old rats brains. In the CX of senescent rats, the specific carbonyl levels of eight proteins were increased. These proteins are heat shock protein 70 (Hsp70), heat shock protein 90, (Hsp 90), glyoxylase 1 (GOL 1), beta-actin (ACT), 3-mercaptopyruvate sulfurtransferase (MPST), peroxiredoxin 1 (PDX), phosphoribosyl pyrophosphate synthetase 1 (PPRPS1), and fumarase (FUM). LAC administration reduced the specific carbonyl levels of all these protein in the CX of senescent rats. The proteins identified in our study are involved in three processes which are impaired in aged brains: antioxidant defence, mitochondrial function and plasticity. LAC treatment might improve the decline of these functions. We posit that LAC should be considered as a potential therapeutic contributor for the treatment of cognitive decline in aging and age-related neurodegenerative disorders. In conclusion our results sustain a role for a redox-dependent modulation of Hsp expression occurring in aging brain. Notably, increased Heat shock protein expression, by promoting the functional recovery of oxidatively damaged proteins, protects brain cells from progressive age-related cell damage[5-6]. Therefore, therapeutic strategies focussing on acetylcarnitine treatment, by up-regulating HO-1 and Hsp70 signal pathways may represent a crucial mechanism of defence against free radical-induced damage of specific proteins occurring in aging brain and in neurodegenerative disorders[7-8]. This findings are relevant to potential pharmacological strategy pointing to maximize cellular stress resistance in vulnerable tissues such as the brain and thus providing neuroprotection[6-10].

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R.41

CELL COMMUNICATION IN THE NERVOUS SYSTEM: THE MEDIATOR ROLE OF SPHINGOSINE 1-PHOSPHATE

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Sphingosine-1-phosphate (S1P), a metabolite of membrane sphingolipids, has emerged as a potent biomediator that has important roles in the regulation of vital cellular processes. S1P is biosynthesized through the phosphorylation of sphingosine in a reaction catalyzed by sphingosine kinase (SK). A peculiarity of S1P as a signaling molecule lies in its dual action mechanism since it may act as a second messenger, through intracellular targets, and as an intercellular mediator, after interaction with specific subtypes of the G-protein-coupled receptor family (S1PR). In the nervous system, S1P metabolism and S1PRs expression are regulated throughout development and show distinct features within specific cell types. A variety of extracellular stimuli, as growth factors, pro-inflammatory cytokines, and Ca^{2+} mobilizing stimuli, activate intracellular SK and lead to a concomitant rise of S1P in various neural cells. Although S1P has been shown to act as an intracellular second messenger, its actions in the nervous system appear meanly mediated through interaction with specific S1P receptors, thus implicating it as a key mediator in cell communication. In fact, after binding to specific receptors, S1P can regulate a wide variety of biological processes in neurons, glial cells and brain endothelial cells. In the different cells of the nervous system, S1P has been shown to regulate cellular proliferation, shape regulation, migration, differentiation, neurogenesis, angiogenesis and survival. A key question on the role of S1P in the nervous system is how this bioactive sphingoid is produced in the extracellular milieu. In recent studies, we obtained evidence that different cells from the nervous system, including neurons, astrocytes and glioma-derived cells, can constitutively export S1P in the extracellular milieu, and various exogenous stimuli, as growth factors, phorbol esters and depolarizing agents, can enhance this secretion. Among the different cells of the nervous system, brain endothelial cells appear the most efficient in S1P secretion. In contrast to neurons and glial cells, these cells can also constitutively export an enzymatically active SK, which may play a role in producing S1P outside the cell. SK expression and S1P secretion by endothelial cells was found to be potently induced after conditioning with human glioma cells.

Overall, the current data strongly support S1P as a crucial mediator in the dialogue among different cells in the nervous system, and implicate this sphingolipid in the regulation of physiological and pathological events in the nervous tissue.

R.42

DIACYLGLYCEROL KINASES AS SWITCH REGULATORS FROM DIACYLGLYCEROL TO PHOSPHATIDIC ACID-MEDIATED SIGNALING: IMPLICATION IN CELL SIGNALING

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Diacylglycerol kinase enzymes (DGKs) convert diacylglycerol (DG) into phosphatidic acid (PA), thus acting as molecular switches between DG- and PA-mediated signalling. Consistently down-regulation of DGKs function enhances DG-mediated signalling in several cell signalling system. Conversely growth factors activate DGKa in different cell types. We showed that HGF-, VEGF- and chemokines-induced biological activities, including cell proliferation, migration and invasion, are mediated by activation DGKa (Cutrupi et al EMBO J 2000; Baldanzi et al. *Oncogene* 2004; Bachiocchi et al. *Blood* 2005; Chianale et al. *Mol. Biol. Cell.* 2007; Baldanzi et al. *Oncogene*, 2008).

Growth factors-induced activation of DGKa occurs through tanslocation to the plasma membrane driven by its association with Src tyrosine kinase, requires $PI(4,5)P_2$ hydrolysis and is independent by PI-3kinase.

Finally we investigated the signalling pathways regulated by DGKa. We show that upon growth factors stimulation, DGKa provides a signal which is necessary and sufficient to recruit to the membrane Protein Kinase C zeta (PKCz), an atypical PKC, and to activate it. Then activated membrane-bound PKCz activates several downstream signalling pathways, leading to Rac activation through regulation of RhoGDI function, and activation of metalloproteases.

R.43

SPHINGOLIPIDS AS MODULATORS OF MEMBRANE SIGNALLING COMPLEXES.

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(Glyco)sphingolipids at the cell surface interact with plasma membrane receptors (e.g., integrin receptors and growth factor receptors) and adapter molecules forming signaling complexes that are able to influence the activity of signal transduction molecules oriented at the cytosolic surface of the plasma membrane (mainly the Src kinases pathway members). The function of these signaling complexes appears to be strictly dependent on their (glyco)sphingolipid composition, and likely on specific sphingolipid-protein interactions. From this point of view, particularly intriguing is the connection between (glyco)sphingolipids and caveolin-1, a membrane protein that plays multiple roles as a suppressor of tumor growth and metastasis in ovarian, breast and colon human carcinomas[1].

In particular, it has been suggested a role for GM3-regulated complexes and for GM3 synthase (sialyltransferase-1, SAT-1) in the control of integrin-mediated tumor cell motility. We stably overexpressed SAT-1 in A2780 human ovarian carcinoma cells. All SAT-1 transfectants were characterized by higher ganglioside levels and by a markedly reduced cell motility when compared to wild type or mock transfected A2780 cells. A similar reduction of cell motility has been observed in the phenotypic variant A2780/HPR, characterized by high levels of GM3 synthase. On the other hand, pharmacological treatments able to increase the cellular ganglioside levels in A2780 cells strongly inhibited their motility. Caveolin-1 mRNA and protein levels were markedly up-regulated in SAT-1 transfectants. Immunoprecipitation experiments showed that caveolin-1 was associated with glycosphingolipids, c-Src and integrin receptor subunits. Moreover, c-Src was in a less active state in SAT-1 transfectants, as indicated by its increased phosphorylation at the carboxy-terminal tail at Tyr 527.

These results suggest that decreased motility could be linked to the function of a signaling complex between GM3, caveolin-1 and integrins, leading to inactivation of c-Src.

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R.44

CELLULAR TARGETS AND ACTIVITIES OF THE GLYCEROPHOSPHOINOSITOLS.

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The glycerophosphoinositols (GPIs) are cellular products of phospholipase A_2 and lysolipase activities on the membrane phosphoinositides. Their intracellular concentrations is in the micromolar range and can vary upon oncogenic transformation, cell differentiation and hormonal stimulation. Our studies into the formation of glycerophosphoinositol (GroPIns) in intact epithelial thyroid cells have indicated that its levels depend on hormonal (adrenergic and purinergic) stimulation of phospholipase A_2IVα via a MAPK-dependent pathway. A glycerophosphodiester phosphodiesterase (GDE1/MIR16) is involved in their catabolism, which, as with their formation, is under hormonal regulation.

The GPIs have diverse effects in multiple cell types. For instance, they induce cell proliferation in thyroid cells; inhibition of inflammatory responses in lymphocytes; changes in actin cytoskeleton organization in fibroblast, and reduction of the invasive potential of some tumour cell lines such as melanomas and breast carcinomas.

The mechanism of action of the glycerophosphoinositol 4-phosphate (GroPIns4*P*, when added exogenously), at least in fibroblasts, involves the activation of a signalling cascade that acts through the tyrosine kinase Src, PLCγ and Ca^{2+}/calmodulin kinase II. This results in TIAM1 translocation to the plasma membrane, and thence to the formation of membrane ruffles via Rac1 activation. Attempts to identify molecular targets of the GPIs (through a proteomic approach) have led to the identification of ß-actin, cortactin, ERK1/2, phospholipase A_2 α and α Purą all proteins that support the proposed roles of the GPIs in the regulation of the actin cytoskeleton and various signalling enzymes. Interestingly, the direct interaction of GroPIns with purified Pura, leads to the modulation of the activity of this transcription factor, indicating that these compounds may also act in the nucleus.

Recent studies have focussed on inflammatory and immune responses, as some of the cells involved in these functions have potent phospholipase A_2 activities that generate GPIs. We found that GroPIns4*P* enhances CXCR4-dependent chemotaxis in T lymphocytes. This activity results from the capacity of GroPIns4*P* to activate the Rho GTPase exchange factor Vav, through an Lck-dependent pathway which also results in activation of the stress kinases JNK and p38. GroPIns4*P* was also found to activate with a delayed kinetics the Lck-dependent activation of ZAP-70, Shc and Erk1/2. These activities of GroPIns4*P* were found to be dependent on its capacity to inhibit cAMP production and PKA activation. In addition, a potent modulatory effects on the proliferation of lymphocyte proliferation induced by the T cell receptor activation was also observed. These data provide the first evidence of a role of GPIs as modulators of T-cell signaling and T cells responses.

R.45

REACTION MECHANISMS OF BOVINE HEART CYTOCHROME C OXIDASE

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ABSTRACT NOT RECEIVED

R.46

THE MITOCHONDRIAL PERMEABILITY TRANSITION IN PATHOPHYSIOLOGY

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Ullrich congenital muscular dystrophy (UCMD) is due to mutations in the genes encoding collagen VI, an extracellular matrix protein forming a prominent microfibrillar network in the endomysium of skeletal muscle. Myoblasts from UCMD patients display mitochondrial alterations and increased apoptosis due to inappropriate opening of the permeability transition pore (PTP), a mitochondrial inner membrane channel that can be desensitized by cyclosporin A (CsA). We will report the results of a pilot trial with CsA in five patients with collagen VI myopathies. Prior to treatment, all patients displayed mitochondrial dysfunction and increased frequency of apoptosis. Both these pathological signs were largely normalized after 1 month of oral CsA administration, which also increased muscle regeneration. Together with results obtained by genetic crossing of mouse models of muscular dystrophy with mice lacking cyclophilin D these findings indicate that mitochondrial dysfunction plays a critical role in human muscle diseases *in vivo*; and represent an important proof of principle that hereditary muscle diseases can be cured with proper drugs downstream of the genetic lesion. This is a useful example of how translational medicine can rapidly move from animal models to treatment of human diseases; and how mitochondrial medicine may be useful beyond the cure of primary mitochondrial diseases.

R.47

NITRIC OXIDE AND CONTROL OF MYTHOCONDRIAL REXPIRATORY CHAIN ELECTRON FLUX

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Nitric oxide (NO) controls mitochondrial respiration via the rapid (milliseconds) reversible inhibition of cytochrome *c* oxidase (CcOX) [1, 2 and ref therein]. Two reaction mechanisms have been described leading to formation of either a relatively stable nitrosyl-derivative (CcOX-NO) or a more labile nitrite-derivative ($CcOX\text{-}NO_2^-$). Both adducts are inhibited though with different K_I, and one mechanism may prevail on the other depending on the turnover conditions and substrates concentration (cytochrome *c* and O_2). We have shown that under normal cell-culture O_2 tension (~ 270 μM) neuroblastoma as well as lymphoid cells mitochondria degrade NO to nitrite, switching to nitrosylation upon rising the electron flux level of the respiratory chain at the level of CcOX [3]. Findings are discussed with respect to cell availability of NO and its effect on the control of electron flux through the respiratory chain, as well as to the putative patho-physiological relevance of the reaction of NO with mitochondria.

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R.48

MITOCHONDRIAL SUBSTRATE LEVEL PHOSPHORYLATION IMPROVES VIABILITY OF HUMAN CELLS WITH SEVERE OXIDATIVE PHOSPHORYLATION IMPAIRMENT

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The aim of the present study was to evaluate whether enhanced substrate-level phosphorylation catalyzed by the TCA cycle succinyl-CoA synthetase increases the survival of cells with impaired oxidative phosphorylation under different conditions of energy demand. We used both experimental in vitro models, in which cell lines are "poisoned" with specific inhibitors, and "natural" models, including transmitochondrial human cell lines (cybrids) harboring four different pathogenic mutations[1]. Moreover, we have used different cell types to better simulate the vulnerability of the nervous system to mitochondrial dysfunction. We found that human fibroblasts cultured in glucose-free medium and energy-challenged by oligomycin inhibition, had a 5 % survival at 72 h, which increased to 70 % when the medium was supplemented with α-ketoglutarate/aspartate. Similarly, homoplasmic cybrids carrying the 8993T→G NARP mutation were also protected from death (75 vs 15% survival at 72 h) by the supplemented medium. Significantly, the ATP content of cells protected by the α-ketoglutarate/aspartate supplement was similar to controls. In contrast, cells with impaired mitochondrial protein synthesis (MERRF 8356T→C and MELAS 3243A→G) or with rotenone-induced complex I deficiency were not protected by the supplement, as shown by unaffected viability and ATP content. These results indicate that α-ketoglutarate/aspartate supplementation to cells is especially beneficial when the impaired enzyme of OXPHOS is ATP synthase[2], and cells are under conditions of high energy demand. This suggests potential dietary or pharmacological therapeutic approaches based on the supplementation of α-ketoglutarate/aspartate to patients with impaired ATP production and functional respiratory chain.

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R.49

MITOCHONDRIAL TRANSPORTERS IN HEALTH AND DISEASE

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The internal membrane of mitochondria contains numerous nuclear-coded proteins belonging to the mitochondrial carrier protein family that transport metabolites, nucleotides and coenzymes from the cytoplasm in the mitochondrial matrix and vice versa. The functional information obtained in studies with mitochondria and/or the reconstituted system has helped to gain insight into the physiological role of mitochondrial carriers in cell metabolism, as have tissue distribution, the use of knock-out mice (and/or yeast) and over-expression in human cell lines (or yeast) of individual carriers and isoforms. Furthermore, the cloning and functional identification of many human genes encoding mitochondrial carriers have led to the discovery of genetic defects responsible for disease and to understanding the symptoms of the disorders. Mutations of mitochondrial carrier genes involved in mitochondrial functions other than oxidative phosphorylation are responsible for carnitine/acylcarnitine carrier deficiency, HHH syndrome, aspartate/glutamate isoform 2 deficiency, Amish microcephaly, and neonatal myoclonic epilepsy; these disorders are characterized by specific metabolic dysfunctions, depending on the physiological role of the affected carrier in intermediary metabolism. Defects of mitochondrial carriers that supply mitochondria with the substrates of oxidative phosphorylation, inorganic phosphate and ADP are responsible for diseases characterized by defective energy production. With the exception of progressive external ophthalmoplegia (PEO), which is an autosomal dominant disorder, all the other currently known diseases are autosomal recessive. Particular emphasis will be given to the molecular basis and pathogenetic mechanism of these inherited disorders.

R.50

REGULATION BY cAMP DEPENDENT PHOSPHORYLATION OF MITOCHONDRIAL PROTEIN IMPORT

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Most of mitochondrial protein are encoded by nuclear genes, translated in the cytosol and imported into mitochondria. It has been reported that cAMP signalling (via PKA) is able to promote the level of some proteins in mitochondria through transcriptional (Gopalakrishnan et al., 1994), post-transcriptional (Ginsberg et al., J. Mol. Biol, 2003) and post-translational (Robin et al., JBC, 2003) regulation.

Work is presented here on the effect of PKA mediated phosphorylation of the NDUFS4 and CREB proteins on their import, accumulation and activity in mitochondria.

The human NDUFS4, a subunit of complex I of respiratory chain, has a conserved serine phosphorylation consensus site for PKA in the carboxy-terminal tail. The bovine ESSS, another subunit of complex I, has also a consensus serine phosphorylation site which, however, is absent in the human subunit. The results show that the accumulation in mitochondria of the mature NDUFS4 protein is specifically promoted by PKA-catalysed phosphorylation of the serine at the C-terminal and depressed by alkaline phosphatase (AP). PKA and AP had no effect on the import/maturation of the ESSS subunit. The PKA promoting effect on the mitochondrial accumulation of NDUFS4 protein appears to be due inhibition of its retrograde diffusion into the cytosol, through interaction of the phosphorylated form with the cytosolic Hsp70 (De Rasmo et al., Cell. Sign., 2008). Phosphorylation of the NDUFS4 subunit can play a functional role in vivo in maintaining the intramitochondrial concentration of the mature NDUFS4 subunit at the level required for its assembly in complex I. This promoting effect of PKA can be involved in the stimulation of the NADH-ubiquinone oxidoreductase activity and prevention of mitochondrial oxygen free radical production exerted in vivo by cAMP in murine and human fibroblast in cultures (Papa et al., BBA, 2008).

The human CREB has a PKA phosphorylable serine. The results indicate that CREB protein enters in isolated mitochondria by a classic mechanism $\Delta\Psi$ dependent. In this case PKA and AP had no effect on the import/accumulation of the CREB protein but cytosolic PKA increased the transcriptional factor activity of CREB protein in mitochondrial matrix which resulted in an enhancement of mitochondrial encoded protein synthesis.

In addition to those present in the cytosol, evidence is available showing the existence in the inner mitochondrial membrane of a pool of PKA and protein phosphatases, which can phosphorylate/dephosphorylate respectively the mitochondrial proteins in the inner mitochondrial compartment. Cytosolic and mitochondrial PKA and phosphatases can contribute to modulate the phosphorylation state and activity of the mitochondrial proteins.

R.51

THE MITOCHONDRIAL PROTEASE AFG3L2 IS ESSENTIAL FOR DEVELOPMENT AND MAINTENANCE OF MOTOR AXON AND CEREBELLUM

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Mitochondria harbour a conserved proteolytic system mediating the complete degradation of organellar proteins. Among the proteins composing this system there are paraplegin and AFG3L2, ubiquitous nuclear-encoded mitochondrial proteins belonging to the AAA metalloproteases family. These proteins form high molecular weight hetero-oligomeric paraplegin/AFG3L2 and AFG3L2 homo-oligomeric complexes known as *m*-AAA proteases. Mutations of paraplegin cause a specific axonal degeneration of the upper motoneuron and, therefore, hereditary spastic paraplegia. We charaxcterized two *Afg3l2* murine models: a newly-developed null and a spontaneous mutant that we found carrier of a missense mutation. Contrasting with the mild and late onset axonal degeneration of paraplegin-deficient mouse, *Afg3l2* models display a marked impairment of axonal development with delayed myelination and poor axonal radial growth leading to lethality at P16.

The increased severity of the *Afg3l2* mutants is explained by two main molecular features that differentiate AFG3L2 from paraplegin: its higher neuronal expression and its versatile ability to support both hetero- and homo-oligomerization. Our data assign to AFG3L2 a crucial role by linking mitochondrial metabolism and axonal development.

Abstracts

1.1

FULL-LENGTH TRANSGLUTAMINASE 2 AND ITS SHORT ISOFORM ARE OVER EXPRESSED IN HUMAN ASTROCYTOMAS

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Astrocytomas are the most common glioma, accounting for about half of all primary brain tumors. Astrocytoma is graded as pilocytic (WHO grade I), diffuse (WHO grade II), anaplastic (WHO grade III), and glioblastoma multiforme (WHO grade IV). The progression from low- to high-grade astrocytoma is associated with distinct molecular changes that vary with other patient status (age, side), yet the prognosis of high-grade tumors remain dismal. Transglutaminase 2 (TG-2) is involved in the physiological regulation of cell growth, but has also been associated with a number of cancer-associated features, such as cell adhesion, metastasis and extracellular matrix modulation. Several studies undertaken with primary tumors suggest that TG-2 expression and activity in the tumor body and surrounding matrix generally decreases with tumor progression, favoring matrix destabilization, but supporting angiogenesis and tumor invasion. Elevated expression and alternative splicing, resulting in a short isoform of TG-2, TG-S, with more active crosslinking activity, are associated with increased neuronal loss in affected regions in the demented brain and more neurodegenerative disorders.

The aim of this work is to investigate expression of TG-2 full-length protein and TG-S isoform and their role in different grade of human astroglial brain tumors. A cohort of 10 flash-frozen surgical specimens, obtained in adult patients who underwent craniotomy for microsurgical tumor resection, were used. These cerebral tumors has been histologically verified, according to the revised World Health Organization (WHO) classification, as 3 glioblastoma (grade 4 WHO), 3 Anaplastic Astrocytoma (grade 3 WHO), and 4 Low grade Astrocytoma. We studied TG-1,2,3,5 and TG-S by RT-PCR, Western Blot and Immunohistochemistry analysis.

Our results showed that TG-2 and TG-S are expressed in all grade of human astroglial brain tumors. In tumor/normal area TG-S may be have a crucial role in apoptosis phenomena inside of tumoral mass wich characterize astrocytomas. The up-regulation of TG2 may play an important role in the astrocytoma progression, favoring matrix destabilization, supporting angiogenesis and tumor invasion.

1.2

THE POLYAMINE ANALOGUE N^1,N^{11}-DIETHYLNORSPERMINE CAN INDUCE CHONDROCYTE APOPTOSIS INDEPENDENTLY OF POLYAMINE METABOLISM AND LEVELS

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Chondrocyte survival is linked to cartilage integrity and forms of chondrocyte apoptotic death can contribute to cartilage degeneration in articular diseases, such as osteoarthritis (OA). Since growing evidence implicates polyamines in the control of cell death, we have been investigating the role of polyamine metabolism in chondrocyte survival and apoptosis by using specific polyamine biosynthesis inhibitors and, more recently, the symmetrically alkylated polyamine analogue N^1,N^{11}-diethylnorspermine (DENSPM)[1,2]. Actually, synthetic polyamine analogues are currently tested as anticancer agents, but little information is available in literature about the effects of DENSPM and other polyamine analogues in non-tumour cells. Treatment of human C28/I2 chondrocytes with DENSPM for up to 24 h did not reduce cell viability nor increase caspase activity significantly when given alone, but caused a caspase-3 and -9 dependent apoptosis in chondrocytes exposed to cycloheximide (CHX). Previous reports indicate that polyamine depletion and polyamine catabolic pathways involving spermidine/spermine N^1-acetyltransferanse (SSAT) and N^1-acetylpolyamine oxidase (PAO) or spermine oxidase (SMO) may play a role in DENSPM-induced cell death of some cancer cell lines. However according to the present study, DENSPM/CHX can cause apoptosis in chondrocytes independently of these pathways and polyamine levels. The addition of CHX together with DENSPM caused an increased uptake of the polyamine analogue by the cells and was associated with altered expression and/or phosphorylation of signalling proteins, i.e. MAPKs and Akt.

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1.3

INVOLVEMENT OF TRANSGLUTAMINASE IN CELL STRESS RESPONSE EVOKED BY HOMOCYSTEINE IN SH-SY5Y NEUROBLASTOMA CELLS

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Transglutaminases catalyze a Ca^{2+}-dependent transamidating reaction leading to protein cross-linking or polyamine incorporation into protein substrates. Tissue transglutaminase (TG2) not only acts as a transamidating enzyme but is also a multifunctional protein, showing a complex role in cell response to different stress agents. Further, its involvement in apoptosis, cell differentiation, and inflammation, has been reported in different pathological conditions, such as neurodegenerative diseases[1]. However, conclusive evidence for a TG2 role in cell death is still lacking. Previous reports indicated that induction of TG2 expression by retinoic acid (RA) treatment or stable transfection with TG2 cDNA constructs did not produce any change in the rate of spontaneous apoptosis in human neuroblastoma SH-SY5Y cells[2]. Considering that TG2 is expressed at different levels depending on cell differentiation state, we evaluated the cell response to stress conditions induced by homocysteine (Hcy) in undifferentiated or RA-differentiated SH-SY5Y neuroblastoma cells.

SH-SY5Y cells were grown in MEM/Ham's F-12 (1:1), and differentiated by RA (10 μM) treatment for 7 days *in vitro* (DIV). Then, undifferentiated and differentiated cell cultures were exposed to a sub-toxic concentration of Hcy (250 μM) for 4 h. RT-PCR and Western blot analyses demonstrated that TG2 was almost undetectable in SH-SY5Y undifferentiated cells. Instead, RA treatment resulted in a strong TG2 induction accompanied by the formation of extended neurites. Hcy exposure led to a limited TG2 *de novo* synthesis in undifferentiated cells, and only slightly increased TG2 levels in RA-differentiated ones.

Further, Hcy largely increased ROS production in both cell culture types. This effect was associated to NF-κB activation. In particular, the NF-κB activated levels were more significantly increased in undifferentiated cells compared with RA-differentiated ones. Notably, the inhibition of TG activity, with a site specific inhibitor of TG2, produced a reduction in NF-κB levels increased by Hcy in differentiated cells. Conversely, the specific NF-κB inhibition by SN50 suppressed Hcy-induced TG2 up-regulation in undifferentiated cells. In addition, Hcy treatment produced a more relevant increase in Hcy-induced endoplasmic reticulum protein (Herp) levels in RA-differentiated cells compared with undifferentiated ones. These data suggest that Hcy triggered cytosolic calcium alterations, which were in turn responsible for TG2 activation.

Based on these results, TG2 up-regulation and NF-κB activation seem to be strongly involved in cell response to Hcy. Further research efforts towards the identification of proteins modified by TG2-mediated cross-linking will be helpful for a better understanding of TG2 role in these stress conditions.

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1.4

CARDIOPROTECTION AND ENERGY METABOLISM MODULATION BY 3-IODOTHYRONAMINE, A NEW ENDOGENOUS CHEMICAL MESSENGER

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3-Iodothyronamine (T_1AM) is a novel endogenous thyroid hormone derivative that induces bradycardia and hypothermia in mouse and a dose-dependent negative inotropic effect in rat heart with IC_{50} on the order of 27 μM[1]. T_1AM hemodynamic effects are remarkably increased by perfusion with genistein, a tyrosine kinase inhibitor, and attenuated by vanadate, a tyrosine phosphatase inhibitor, while no changes are observed after treatment with inhibitors of other kinases[2]. In the present study, using the isolated and perfused rat heart as a model, we evaluated changes upon treatment with T_1AM in ischemic injury, in glycogen phosphorylase (GP) activity, and in energy metabolism.

Isolated rat heart were subjected to 20 min of aerobic perfusion, followed by 30 min of global normothermic ischemia and 120 min of retrograde reperfusion. Irreversible tissue injury was evaluated by triphenyltetrazolium chloride (TTC) staining. Under control conditions tissue injury averaged 25±2%. T1AM did not modify the susceptibility to ischemic injury at 12 nM concentration (33±4%), while it produced a significant protection at 120 nM and 1.2 μM concentration (15±2% and 9±4%, respectively $P<0.05$ in both cases). The cardioprotective effect was blunted at higher concentrations, since ischemic injury averaged 18±2% and 21±3% with 12 μM and 25 μM T1AM, respectively. Notably, T1AM did not produce any significant change in cardiac output nor in heart rate, if used at 120 nM or 1.2 μM concentration. The effects of T1AM on cardiac metabolism were also studied. Glucose uptake was determined by measuring changes in glucose concentration during ricirculating perfusion. Oxygen consumption was assessed by measuring oxygen tension with an oxygen electrode in the perfusate collected from the aortic and the pulmunary cannula. Glycogen phosphorylase activity was carried out spectrophotometrically in the cytosolic fraction, Although contractile performance was significantly depressed at 16 μM and 25 μM T_1AM, glucose uptake was not modified while oxygen consumption was decreased only at 25 mM. In addition, perfusion with 25 μM T_1AM increased myocardium glycogen phosphorylase a activity.

In conclusion, we obtained evidence of a cardioprotective effect of T1AM with a biphasic response, at concentrations which did not produce any negative inotropic or chronotropic action. Furthermore our findings suggest that T1AM may modulate energy metabolism, increasing oxygen consumption, if related to contractile performance, and glycogen phosphorylase a activity.

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1.5

EFFECT OF SPERMIDINE ON DNase I ACTIVITY

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The natural polyamines, putrescine, spermidine and spermine are ubiquitous cellular components that are involved in a variety of cellular functions. Polyamines are aliphatic molecules with amine groups distributed along their structure and are fully protonated at physiological pH. They are, therefore, polycations, a physicochemical feature involved in their biological effects through bonding by strong cation–anion interactions with different polyanionic macromolecules such as DNA and RNA and it is described that spermine and spermidine induce the condensation and aggregation of DNA in aqueous solution.

With the aim to test if polyamines were able to protect DNA from digestion by endonucleases, we have incubated a commercial polydeoxyribonucleotide (PDRN) with different concentrations of spermidine. The spermidine-treated PDRN was then subjected to digestion by DNase I. Due to the molecular weight heterogeneity of our DNA, it was difficult to evaluate the enzyme activity by electrophoresis. Thus, we analyzed the activity of DNase I by the Kunitz assay measuring the increase of light absorbance induced by the DNA hydrolysis.

The results demonstrate that at physiological concentration (μM) spermidine is not able to protect DNA from the hydrolysis by DNase I, whereas a protective effect was observed at 5 mM spermidine. In order to evaluate if in our experimental conditions the binding of spermidine to DNA occurs, we have analyzed the binding of the polyamine by measuring the formation of DNA-aggregates and by analyzing the binding of ethidium bromide to DNA in the presence of spermidine.

Our results confirm the behaviour of DNA in the presence of spermidine described in the literature[1]: the polycation induces first the formation of aggregates that disappear after a further addition of spermidine. The binding of spermidine was also confirmed by the ethidium bromide assay. At 5 mM spermidine, DNA is totally aggregated and the enzymatic activity of DNase is inhibited. It is notably that also at 1.5 mM spermidine DNA is totally aggregated, but at this concentration the polyamine is not able to inhibit the DNase activity. It is therefore possible that the inhibitory effect of 5 mM spermidine is due to an action of the polyamine on the enzyme, rather than to a protective role on the DNA molecule. This hypothesis is supported by the observation that the pretreatment of DNase with spermidine before the enzymatic assay, increases the inhibitory effect.

On the basis of our results we can conclude that the binding of spermidine to DNA is not able to protect the macromolecule from the hydrolysis catalyzed by DNase I, also at concentrations of polyamine that induce the total aggregation of DNA.

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1.6

DNA AGGREGATION INDUCED BY SPERMIDINE: A TECHNICAL NOTE

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DNA condensation into compact structures has received considerable attention in recent years due to its biological implications in DNA packaging in virus heads and chromatin as well as to understand the mechanism of uptake of gene vectors in living cells. Several studies have demonstrated the efficacy of internal proteins, neutral polymers, and polyamines in condensing DNA. The natural polyamines, putrescine, spermidine and spermine are aliphatic molecules with amine groups distributed along their structure and are fully protonated at physiological pH. It is described that multivalent cations with a charge of 3+ or greater induce the condensation and the aggregation of DNA in aqueous solution. With the aim to test if polyamines were able to protect DNA from digestion by endonucleases, we have incubated a commercial polydeoxyribonucleotide with different concentrations of spermidine. In order to evaluate if in our experimental conditions the binding of spermidine to DNA occurs, we have analyzed the binding of the polyamine by measuring the formation of DNA-aggregates and by analyzing the binding of ethidium bromide to DNA in the presence of spermidine.

Our results confirm the behaviour of DNA in the presence of spermidine described in the literature[1]: the polycation induces first the formation of aggregates that disappear after a further addition of spermidine.

In the course of our analysis we observed a strong pH enhancement in solutions containing spermidine at concentrations able to resolubilize the DNA aggregates. This alkalinization can produce a partial or complete deprotonation of spermidine that reduces its binding to DNA. On the other hand the adjustment of pH, by increasing the molarity of the buffer or by neutralizing the solutions with a strong acid, induces a rise of the ionic strength at values that can inhibit the formation of DNA aggregates.

We suggest that these observations should be take on account in the discussions about the mechanisms underlying the resolubilization of DNA aggregates induced by polycations.

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1.7

A REDOX SWITCH AND REGULATION OF ADENINE NUCLEOTIDE TRANSLOCASE PHOSPHORYLATION ARE IMPLICATED IN THE POLYAMINE EFFECTS ON MITOCHONDRIAL PERMEABILITY TRANSITION.

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Activation of protein kinases within signal transduction pathways and modulation of oxidative stress are physiological processes in which polyamines exhibit an essential role in mitochondria[1].

Polyamines are also able to prevent mitochondrial permeability transition (MPT), a phenomenon strictly related to intrinsic apoptosis. A number of evidences have demonstrated that MPT occurs in cultured cells and in vivo, however the molecular mechanism of polyamine action is not yet completely elucidated. Some hypothesis suggests that this protection, in particular spermine, involves the prevention of oxidative processes and/or the activation of protein phosphorylation at the level of key components of the transition pore (PTP). Spermine may act directly as a scavenger against the highly toxic hydroxyl radical or may indirectly cause an increase in reactive oxigen species when oxidized by polyamine oxidase. Indeed, polyamines are involved in signal transduction by activating tyrosine kinase and ERK 1/2.

Src family kinases (SFKs) are the major players in mitochondrial tyrosine phosphorylation and associated in signal transduction from plasma membrane to mitochondria. The presence of SFKs in mitochondria is associated with the proliferative status of the cells and an increased level of polyamines[2]. Proteomic studies reveal a number of phosphorylated proteins in mitochondria which have a primary role in regulating energy transduction (some subunits of ATP synthase, cytocrome C and cytocrome C oxidase) and MPT induction (adenine nucleotide translocase (ANT), voltage dependent anion channel (VDAC), creatine kinase and esokinase). We found two phosphorylated tyrosines located on ANT, Y190 and Y194, which protrude into the channel where nucleotides bind for translocation[3]. These observations are consistent with the hypothesis that ANT during its transformation in the PTP is regulated by tyrosine phosphorylation. In this regard it is to take into account that the PTP opening is the result of two critical cysteines oxidation always located on ANT. In conclusion the induction of MPT is controlled by tyrosine phosphorylation and a “redox switch”, even if a connection between them has not been evidenced. Our results indicate that the protective action of polyamines against agents inducing MPT involve their scavenging effect and tyrosine phosphorylation of ANT.

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2.1

ENDOCANNABINOIDS PROLONG PLATELET LIFE SPAN

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The ability of endocannabinoids (ECs), like 2-arachidonoylglycerol (2-AG) and anandamide (AEA), to modulate platelets functions represents an emerging field of interest. Indeed, ECs can act as (co-)agonist during platelet aggregation. It is well demonstrated that platelets are intrinsically programmed to die, as they express Bcl-xL and Bak, two proteins which constitute a molecular clock determining platelet life span.

Based on this background, we investigated if ECs are able to modulate the life/death balance in platelets.

In the first set of experiments, we treated platelets from healthy donors with AEA and Meta-AEA, its not-hydrolysable analogue, and performed viability assay and western blot analysis.

Platelets incubated at 37°C show decreased viability after 24 hours and more than 80% of MTT activity is lost at 48 hours. On the contrary, platelets stored at 22°C are stable after the same time tested.

10-100 nM Meta-AEA increase viability of platelets, while higher concentrations (10-100 μM) are not able to revert cell death occurring at 37°C. Platelets incubated in the presence of increasing concentrations of AEA show a pattern similar to that observed with Meta-AEA: the most efficacious dose appears to be 1 μM. Inhibition of intracellular AEA uptake (by the selective inhibitor OMDM1), as well as inhibition of AEA hydrolysis (by the use of the FAAH inhibitor URB597), enhance the protective effects of AEA, thus suggesting that it act extracellularly. This is further confirmed by the fact that arachidonic acid (AA), the major active product derived from AEA hydrolysis, exerts opposite effects, leading to cell death.

Specific cannabinoid (CB) receptor antagonists (SR141716 and SR144528 for CB1 and CB2 receptors, respectively) inhibit Meta-AEA-mediated survival, while an inhibitor (IRTX) of transient receptor potential vanilloid type 1 (TRPV1) is ineffective. Accordingly, platelets do not express TRPV1, while antibodies directed against CB1 and CB2 receptors recognize immunoreactive bands. We further confirmed the involvement of CB receptors by the use of specific agonists for CB1 and CB2 receptors, ACEA and JHW55, respectively.

Wortmannin, an inhibitor of PI3 kinase, abrogated Meta-AEA-mediated survival, thus suggesting an involvement of the PI3K/Akt pathway. Accordingly, Meta-AEA dose- and time-dependently increases phosphorylation and activation of the pro-survival Akt kinase, as well as expression of the anti-apoptotic Bcl-xL; on the contrary, protein levels of pro-apoptotic members of the Bcl-2 family (Bak and Bax) are unchanged. So, Meta-AEA changes the Bcl-xL/Bak ratio, increasing pro-survival factors.

2.2

P2X7 OVEREXPRESSION IS RESPONSIBLE FOR THE INCREASED INTRACELLULAR CALCIUM AND FUNCTIONAL DERANGEMENT IN SCHWANN CELLS FROM RATS WITH CHARCOT-MARIE-TOOTH TYPE 1A NEUROPATHY

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Charcot−Marie−Tooth (CMT) is the most frequent inherited neuromuscular disorder, affecting 1 person in 2500. CMT1A, the most common form of CMT, is usually caused by a duplication of chromosome 17p11.2, containing the peripheral myelin protein-22 (PMP22) gene: overexpression of PMP22 in Schwann cells (SC) is believed to cause demyelination, although the underlying pathogenetic mechanisms remain unclear. Here we report an abnormally high basal concentration of intracellular calcium ([Ca2+]i) in SC from CMT1A rats. By the use of specific pharmacological inhibitors and through down-regulation of expression by siRNA, we demonstrate that the high [Ca2+]i is caused by a PMP22-related overexpression of the P2X7 purinoceptor/channel leading to influx of extracellular Ca2+ into CMT1A SC. Correction of the altered [Ca2+]i in CMT1A SC restores the functional parameters of SC (migration, release of ciliary neurotrophic factor, myelination), which are typically defective in CMT1A SC.

These results establish a pathogenetic link between high [Ca2+]i and impaired SC function in CMT1A and identify overexpression of P2X7 as the molecular mechanism underlying both abnormalities. The development of P2X7-inhibitors is expected to provide a new therapeutic strategy for treatment of CMT1A neuropathy.

2.3

ABSCISIC ACID IS AN ENDOGENOUS STIMULATOR OF INSULIN RELEASE FROM HUMAN PANCREATIC ISLETS WITH CYCLIC ADP-RIBOSE AS SECOND MESSENGER

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Abscisic acid (ABA) is a plant stress hormone recently identified as an endogenous pro-inflammatory cytokine in human granulocytes[1]. Since paracrine signaling between pancreatic beta cells and inflammatory cells is increasingly recognized as a pathogenetic mechanism in the metabolic syndrome and type II diabetes, we investigated the effect of ABA on insulin secretion. Nanomolar ABA concentrations induce glucose-independent insulin release and increase glucose-stimulated insulin secretion from RIN-m cells and from human pancreatic islets. The signaling cascade triggered by ABA in both cell types sequentially involves a pertussis toxin-sensitive G protein, cAMP overproduction, protein kinase A-mediated activation of the CD38, cyclic ADP-ribose overproduction, eventually leading to a rise of the intracellular free Ca^{2+} concentration. ABA is rapidly produced and released from human islets and RIN-m cells stimulated with high glucose concentrations. In conclusion, ABA is an endogenous stimulator of insulin secretion in human beta cells. Autocrine production of ABA by pancreatic beta cells and its paracrine release by activated granulocytes and monocytes, suggest a possible role of ABA in the physiology as well as in the dysregulation of human insulin secretion.

1. Bruzzone S., Moreschi I., Usai C., Guida L., Damonte G., Salis A., Scarfì S., Millo E., De Flora A., and Zocchi E. (2007) Proc. Natl. Acad. Sci. USA. 104, 5759-5764.

2.4

DIACYLGLYCEROL KINASE ALPHA REGULATES CELL MIGRATION THROUGH PKCz, RhoGDI AND RAC.

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Diacylglycerol kinase enzymes (Dgks) convert diacylglycerol (DG) into phosphatidic acid (PA), thus acting as molecular switches between DG- and PA-mediated signalling. We previously showed that Dgka activation is required for VEGF-, HGF- and v-Src-induced cell migration of endothelial and epithelial cells, through a mechanism requiring Dgka phosphorylation on Tyr^{335} by Src itself (Cutrupi *et al*, 2000; Baldanzi *et al*, 2004; Baldanzi *et al*, 2007). More recently we showed that Dgka regulates HGF-induced membrane ruffling at the leading edge of migrating epithelial cells, by mediating activation and membrane targeting of Rac and Rac-dependent remodelling of actin cytoskeleton and focal contacts (Chianale *et al*, 2007).

We show inhere that the expression of myristoylated Dgka stimulates the formation of ruffle-like structures and is sufficient to recruit Rac at the outer plasma membrane of epithelial cell colonies. In addition, we show that Dgka is required for HGF-induced targeting at nascent ruffles of GTP-bound active RacV12 mutant. Together, these observations suggest that Dgka provides a signal promoting Rac targeting at the leading edge independently from its GTP loading.

Upon cell adhesion, Rac targeting to the plasma membrane is controlled by b1 integrin, through its dissociation from RhoGDI. Rac dissociation from RhoGDI occurs in presence of acidic lipids such as PA and PIP_2, and can be triggered by PKCz-induced threonine phosphorylation of RhoGDI; interestingly, PKCz is an atypical Protein Kinase C reported to be regulated by PA. Thus we investigated the hypothesis that Dgka may control Rac targeting through the regulation of PKCz and/or RhoGDI function

We show inhere that Dgka downregulation by specific siRNA impairs HGF-induced recruitment at the leading edge of both RhoGDI and PKCz, while active myr-Dgka is sufficient to promote Rac, RhoGDI and PKCz recruitment to plasma membrane, independently from b1 integrin activation. Cell treatment with a specific PKCz inhibitor impairs both HGF- and myr-Dgka-induced Rac and RhoGDI targeting to the plasma membrane. Moreover, the expression in epithelial cells of a constitutively active myristoylated PKCz induces *per se* the formation of long protrusions, completely unaffected by Dgka inhibition. These data strongly suggest that Dgka controls PKCz localization and function and, consequently, RhoGDI recruitment and dissociation from Rac. Indeed, Dgka inhibition by expression of a dominat-negative mutant impairs HGF-induced Rac dissociation from RhoGDI.

In conclusion, we envisage a model in which PA, generated by Dgka in a spatially restricted manner, regulates Rac function by driving membrane recruitment and activation of PKCz, which in turn regulates the dissociation of RhoGDI from Rac and finally Rac release and activation at the leading edge of migrating cells.

2.5

REACTIVE OXYGEN SPECIES HIF-1α INDUCED SURVIVAL IN METASTATIC MELANOMA

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Hypoxia, a common feature of solid tumors, is a major obstacle in the development of effective cancer chemotherapy often decreasing the efficacy of chemotherapic drugs in several solid tumors. The hypoxic environment is able to maintain an anti-apoptotic potential through the activation of critical genes associated with drug resistance and the overexpression of prosurvival proteins as Bcl-2. Hypoxia inducible factor1α (HIF1α) is the master regulator of hypoxic response and its overexpression and enhanced transcriptional activity are linked to tumour initiation and progression by inducing expression of genes mediating angiogenesis and motility, as well as tumor metabolism and survival. Increased level of HIF-1α are associated with resistance to therapy in head and neck, ovarian, esophageal and prostate cancer, thus suggesting that HIF-1α is a key contributor involved in drug resistance acquired by hypoxia. Besides inducing a glycolytic shift of tumour cells, hypoxia also paradoxically increases the intracellular level of reactive oxygen species (ROS) such as superoxide and hydrogen peroxide. Mitochondria appear to be their main source, although hypoxia may affect the intracellular redox state through NADPH oxidase engagement by autocrine growth factor production.

We observed that 1% hypoxia increases the resistance of human Hs29-4T metastatic melanoma to conventional chemotherapy with etoposide, mediating a signalling leading to tumor cells survival. The aim of our work is to identify the role of the redox component of this pro-survival spur in hypoxia signalling. In melanoma cells hypoxia leads to a strong and sustained increase of intracellular ROS, indicating mitochondria as the main source. Mitochondria-derived ROS are both necessary and sufficient to stabilize and activate HIF-1α both in normoxic and hypoxic conditions. In addition, inhibition of HIF-1α with specific siRNA, as well as inhibition of ROS production impairing mitochondrial source (rotenone) or NADPH oxidase (DPI), rescues the hypoxic protection from etoposide-induced apoptosis. Moreover VEGF and VEGF receptor neutralizing antibodies fully abrogate hypoxia-induced survival, thus suggesting a participation of VEGF signalling to sustain survival. Again, removal of mitochondria-derived ROS abrogates VEGF downstream signalling and survival. We therefore propose hypoxia-derived ROS as key modulators of the HIF-1α and VEGF-mediated resistance to chemotherapy of metastatic melanoma.

2.6

INCREASE OF INVASIVENESS IN HUMAN MELANOMA CELLS DURING HYPOXIA: ROLE OF REDOX SIGNALLING AND MET UPREGULATION

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Tumour hypoxia is linked to increased metastatic potential, but the molecular mechanisms coupling hypoxia to metastasis are poorly understood. The acquisition of a metastatic phenotype is often characterized by the activation of a transcription response, commonly called epithelium mesenchymal transition (EMT). Hypoxia has already been correlated with EMT in several cancers, mainly through the hypoxia inducible factor1-α (HIF1-α) transcription factor. HIF-1α, upon decrease of oxygen level, transcribes several proteins responsible for the phenotypic conversion of cancer cells from an epithelial to a mesenchymal, and more motile, phenotype. EMT leads to activation of motility signals, as autocrine motility factor, hepatocyte growth factor and its receptor Met, the stem-cell-derived factor1-α, as well the decrease of cell-cell constraints caused by decreased expression of E-cadherin. In addition, hypoxia has been correlated with oxidative stress, an other condition which has been causally linked to EMT and to malignant transformation.
The aim of our work is to understand the role of the redox component of hypoxia signalling in the EMT response of the Hs29-4T human metastatic melanoma cell line. 1% hypoxia induces a significant increase in cell spreading and cytoskeleton organization of melanoma cells. These cells respond to the hypoxic environment with an increase of their motility and invasiveness, likely undergoing to EMT, as indicated by their downregulation of E-cadherin and upregulation of Met. The Silencing the endogenous Met through an inducible lentiviral system severely inhibits motility and invasiveness in hypoxia. In addition, hypoxia is able to increase the natural tendency of melanoma cells to self-organize into tubular structures, a phenomenon commonly indicated as vasculature mimicry, and likely contributing to the metastatic spreading of these cancer cells. 1% hypoxia leads to a strong and sustained increase of intracellular reactive oxygen species (ROS), indicating mitochondria as the main source. Scavenging of these oxidative response using N-acetyl cysteine (NAC) or eliminating the mitochondrial source with rotenone, gives rise to a strong decrease of the motile and invasive response to hypoxia, regulation of expression of Met and E-cadherin, as well the ability of melanoma cells to self-organize into tubular structures. Collectively these data point on ROS as main regulators of the invasive response of metastatic melanoma cells towards a mesenchymal phenotype during hypoxia.

2.7

ABSCISIC ACID PRODUCED BY HUMAN MONOCYTE AND VASCULAR SMOOTH MUSCLE CELL RESPONSES INVOLVED IN THE DEVELOPMENT OF THE ATHEROSCLEROTIC LESION

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Abscisic acid (ABA) is a phytohormone regulating important physiological functions in plants. We recently demonstrated production and release of ABA by human granulocytes and functional activation of these cells by ABA[1]. Thus, ABA behaves as a new pro-inflammatory hormone in granulocytes. Monocytes orchestrate and execute inflammatory and immunological processes: they respond to cytokines and other inflammatory stimuli by expressing a wide range of genes and are known to play a pivotal role in atherosclerosis, together with platelets and vascular smooth muscle cells (VSMC). Exposure of human monocytes to ABA evokes an intracellular Ca^{2+} rise through the second messenger cyclic ADP-ribose, leading to NF-κB activation and consequent increase of ciclooxygenase-2 expression and prostaglandin-E_2 production, enhanced release of monocyte chemoattractant protein-1 (MCP-1) and of metalloprotease-9, all reportedly involved in the onset of atherosclerosis. Moreover, monocytes release ABA when exposed to thrombin-activated platelets, a condition occurring at the site of injured vascular endothelium: monocyte-derived ABA behaves as an autocrine and paracrine pro-inflammatory hormone stimulating monocyte migration and MCP-1 release and VSMC migration and proliferation. These results and detection of ABA in human arterial plaques identify ABA as a key molecule involved in the development of atherosclerosis and suggest a possible new target for anti-atherosclerotic therapy.

1. Bruzzone S., Moreschi I., Usai C., Guida L., Damonte G., Salis A., Scarfì S., Millo E., De Flora A., and Zocchi E. (2007) Proc. Natl. Acad. Sci. USA. 104, 5759-5764.

2.8

STROMAL MYOFIBROBLAST AND PROSTATE CANCER CELLS: AN ACTIVE PRO-INVASIVE BIDIRECTIONAL COMMUNICATION

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Prostate cancer is one of the main cause of male cancer death. However, the underlying molecular mechanisms contributing to prostate carcinoma progression and metastatic conversion are not completely clear. Recent studies have shown that tumor stroma is emerging as an active participant in tumor growth and progression. One of the cellular component of the reactive stroma are myofibroblasts, cells with *de-novo* acquired ability to synthesize large amount of extracellular matrix component, secrete angiogenic and pro-inflammatory factors and stimulate cell proliferation and invasion. Until now TGFβ is the only identified factor responsible for the fibroblast-to-myofibroblast transition. In this study our aim is to clarify the molecular events underlying this cellular transition as well as those accounting for the tumor invasion-promoting effect of myofibroblasts. Human fibroblasts (HFs) were transdifferentiated into myofibroblasts (MFs) by means of TGFβ− treatment. By incubating PC3 prostate cancer cells with conditioned medium (CM) from MFs or by directly co-colturing cancer cells with MFs, we observed a significant increase of cancer cell motility and invasiveness. The MF-CM induced a similar increase in PC3 cell motility when used as chemoattractant. Moreover, incubation of PC3 with MF-CM induced a strong increase in ROS intracellular level, in the expression and phosphorylation of c-Met and in the activation of the Src tyrosine kinase. Interestingly, we observed that PC3 cells incubated with MF-CM, or upon co-culturing with MFs, undergo epithelial-to-mesenchimal transition (EMT), as indicated not only by the activation of pro-invasive signals but also by the inhibition of cell-cell constraints caused by a downregulation of E-cadherin. Furthermore, we show evidence that PC3-CM is able to transdifferentiate HF in MFs, even if at a lesser extent with respect to what observed in response to TGFβ. Catalase treatment during HF incubation with PC3-CM abrogates their transition to MFs, as well as their ability to enhance cancer cell invasion. Collectively these data evidence a pro-invasive effect of MFs on prostate carcinoma cells, likely promoting an EMT, and suggest a role for oxidants as active player in PC3-promoted fibroblast transdifferentiation.

2.9

THE ROLE OF DNA-DAMAGE BINDING PROTEIN (DBB1), A COMPONENT OF E3 UBIQUITIN LIGASE COMPLEX IN $p27^{Kip1}$ TURNOVER

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DNA damage-binding protein (DDB), also referred to as UV-DDB, is a heterodimer made up of the 127 kDa DDB1 subunit and the 48 kDa DDB2 subunit. The DDB complex recognizes and binds some UV-damaged DNA lesions and is implicated in nucleotide excision repair (NER) of DNA damage[1].

Recent studies indicate an additional role of DDB1 as a fundamental protein in ubiquitination mechanism. In fact, this protein associated with Skp2 and Cul4A is part of the E3 ubiquitin ligase complex and induces proteolysis of $p27^{Kip1}$. The cyclin-dependent kinase inhibitory protein (CKI) $p27^{Kip1}$ has been shown to play a critical role in the regulation of cell cycle progression. Alteration of CKI levels and/or functions is implicated in cell transformation and in growth arrest after DNA damage. Levels of $p27^{Kip1}$, in fact, are decreased in many malignant cells and down regulation of $p27^{Kip1}$ in primary human cancers is mediated by accelerated proteasomal degradation of the protein CKI. Furthermore, there is an inverse correlation between the levels of $p27^{Kip1}$ and Skp2 in human tumors[2]. There is an extensive evidence that $p27^{Kip1}$ levels are decreased in human melanoma cells and tissues on the contrary, the role of DDB1-mediated ubiquitination and proteasomal degradation in down regulation of $p27^{Kip1}$ levels remains to be investigated. Therefore, the aim of this research is to investigate the role of DDB1 in the turnover of $p27^{Kip1}$ in human melanoma cells, in the cellular response to DNA damage and in senescent cells. In many cell types, in fact, severe DNA damage induces senescence and striking changes in gene expression, including overexpression of known cell-cycle cyclin-dependent kinase inhibitors $p21^{Cip1}$ and $p27^{Kip1}$.

We have previously demonstrated that DDB1 is expressed in senescent fibroblasts at levels comparable to those observed in exponentially growing cells and that its capacity of binding to UV-damage DNA is lost in senescent fibroblasts. Our results, therefore, indicate that DDB1 in senescent cells is not able to recognize some UV-damaged DNA lesions and to initiate the nucleotide excision repair (NER).

We used A2058, Colo 829, two melamoma cell lines, and BJ-hTERT cells, human foreskin fibroblasts.

Our results show that:

- in melamoma cells there is also an inverse correlation between the levels of $p27^{Kip1}$ and DDB1
- accumulation of $p27^{Kip1}$ could be triggered by reduced or absent DDB1-dependent response to UV damage
- the lack of DDB1 in ROC1/CUl4A/DDB1/Skp2 complex could affect $p27^{Kip1}$ turnover in senescent cells.

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2. Qing Li et al. (2004) J. Cutan Pathol. 31,0633-642

2.10

siRNA-MEDIATED DOWNREGULATION OF PKD1 AND PKHD1 GENES: DIFFERENT EFFECTS ON CALCIUM HOMEOSTASIS AND CELL PROLIFERATION

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Polycystic kidney disease (PKD) includes a large group of progressive renal disorders characterized by the development of renal cysts leading to end-stage renal disease. The most common PKDs are transmitted as autosomal dominant (ADPKD) and autosomal recessive (ARPKD) traits. The first is frequent (1:1000) and ~50% of patients develop renal failure by age 60[1], the last is a less frequent childhood disease (1:20000), with high mortality[2]. ADPKD is caused by loss of function of polycystin-1 (PC1), a large receptor protein, and of polycystin-2 (PC2), a Ca^{2+} permeable cation channel, interacting each other forming a receptor-channel complex. ARPKD is caused by mutations in PKHD1 encoding polyductin/fibrocystin-1 (FC1), a receptor-like protein with a still undefined function, but interacting with PC2. Consistently, studies of ADPKD and ARPKD converge on molecular mechanisms of cystogenesis, including intracellular Ca^{2+} deregulation, eventually leading to increased cell proliferation, apoptosis and dedifferentiation[3]

We have investigated the putative role of PC1 and FC1 on intracellular Ca^{2+} homeostasis by comparing evoked Ca^{2+} levels and Ca^{2+} oscillations in HEK293 embryonal and 4/5 tubular epithelial kidney cell lines with knockdown of PC1 and FC1 encoding genes (PKD1 and PKHD1) by RNA interference.

Either the PKD1 or PKHD1 downregulation affected Ca^{2+} homeostasis in kidney cells lines, but with different effects. In particular, in PC1-depleted [PC1(-)], and even more in FC1-depleted [FC1(-)] cells, ATP- or FBS-evoked Ca^{2+} entry was increased compared to control cells. On the other hand, only in PC1-depleted cells FBS-induced Ca^{2+} oscillation frequency was increased compared to control cells. Moreover, the increase in evoked Ca^{2+} in PC1(-) cells derived mainly from plasma membrane, while the increase in FC1(-) cell was associated to Ca^{2+} released from endoplasmic reticulum.

In conclusion, proteins belonging to different genetic forms of PKD share a common effect on cytoplasmic evoked-Ca^{2+} that occurs by modulating the opening of different channels; this is further supported by the observation that loss of FC1 does not cause a variation in Ca^{2+} oscillations, nor an increase in cell proliferation found in PC1(-) cells, but an increase in apoptosis. These data suggest that abnormal Ca^{2+} signaling is typical of both PC1(-) and FC1(-) cells, but is more relevant for the increase in cell proliferation in the pathogenesis of autosomal than in the recessive form of PKD pathologies.

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2.11

EPHA2 (RE)-EXPRESSION PROMPTS INVASION OF MELANOMA CELLS SHIFTING FROM MESENCHYMAL TO AMOEBOID-LIKE MOTILITY STYLE

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Eph tyrosine kinases instruct cell for a repulsive behaviour, regulating cell shape, adhesion and motility. Beside its role during embriogenesis, neurogenesis and angiogenesis, EphA2 kinase is frequently upregulated in tumor cells of a different hystotype, including prostate, breast, colon and lung carcinoma, as well as melanoma. Moreover, the highest degree of EphA2 expression and activation through phosphorylation is observed in metastatic lesions. Although a function in both tumour onset and metastasis has been proposed, the role played by EphA2 is still debated. Here, we demonstrated that EphA2 re-expression in B16 murine melanoma cells, that use a defined mesenchymal invasion strategy, converts their migration style from a mesenchymal to an amoeboid-like, conferring a plasticity in tumor cell invasiveness. Amoeboid motility, originally described for the amoeba *Dictyostelium discoidem,* has been reported for several eukaryotic cells needing rapid movements through flexible and weak adhesion sites, resulting in cell motion depending more on the rate of cytoplasmic contraction and protrusion. In addition, in response to particular environmental cues, cancer cells can *de novo* acquire an amoeboid-like motility, thus undergoing to what has been termed mesenchymal to amoeboid transition. Hence, our data propose that, in response to re-expression and activation of EphA2, melanoma cells activate a non-proteolytic invasive program which proceeds through the activation of cytoskeleton motility, the retraction of cell protrusions, a Rho-mediated rounding of the cell body, and squeezing among 3D matrix, giving rise to successful lung and peritoneal lymph node metastases. Our results suggest that, among the redundant mechanisms operating in tumour cells to penetrate the anatomical barriers of host tissues, EphA2 plays a pivotal role in the adaptive switch in migration pattern and mechanism, defining and distinguishing tumour cell invasion strategies. Thus, targeting EphA2 might represent a future approach for the therapy of cancer dissemination.

2.12

GLOBULAR ADIPONECTIN INDUCES GROWTH AND SURVIVAL OF MESANGIOBLASTS

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Adiponectin is a well-studied adipokine due to its insulin-mimetic and anti-aterogenic action. The hormone is present in the plasma in two distinct forms (full-length and globular) which can associate to form more complex aggregates. The hormone acts mainly in liver and skeletal muscle where it controls both glucidic and lipids metabolism. Besides the well-known metabolic effects of adiponectin in skeletal muscle, we recently observed that globular adiponectin acts as a pro-differentiative agent for C2C12 murine myoblasts, inducing their differentiation and fusion into mature myofibres. These findings suggested that globular adiponectin could have a more general role in skeletal muscle physiology, rising the possibility that the hormone could act on muscle cells progenitors. To verify this hypothesis we choose D16 murine mesangioblasts derived from dorsal aorta as a cellular model endowed with skeletal muscle stemness. We observed that globular adiponectin induces mesangioblast cell proliferation, together with activation of p42/p44 MAPKs. In addition, globular adiponectin acts as a pro-survival agent for mesangioblasts, by inhibiting the apoptotic cascade caused by growth factor withdrawal or extracellular matrix detachment. In particular, Annexin V assay, evaluation of mitochondrial potential and caspase-3 and-9 activities revealed a protection from apoptosis in globular adiponectin-treated mesangioblasts. Finally, we observed that the co-colture of rat L6 myoblats with globular adiponectin-treated mesangioblasts leads to an increased myogenesis as revealed by differentiation index and the interspecific fusion index (between L6 and D16 cells). These findings suggests that globular adiponectin plays a pleiotropic role in skeletal muscle development, acting both on muscle cell stem cells and on myoblasts differentiation.

2.13

HAPTOGLOBIN INFLUENCES APOA-I ACTIVITIES IN REVERSE CHOLESTEROL TRANSPORT

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Aims: Apolipoprotein A-I (ApoA-I), the major apolipoprotein of the high density lipoprotein (HDL), plays key roles in the removal of cholesterol excess from peripheral cells, in a recognized anti-atherosclerotic process called "reverse cholesterol transport" (RTC)[1]. In detail, it stimulates cholesterol (C) efflux and ApoE secretion from macrophages, and enhances Lecithin-Cholesterol Acyl-Transferase (LCAT) activity. The acute phase protein Haptoglobin (Hpt) was found to inhibit RCT, as binding ApoA-I and preventing its stimulation on LCAT. This study aimed to find out whether Hpt influences essential ApoA-I stimulated mechanisms in RCT, such as cholesterol efflux, LCAT activity, and ApoE secretion from macrophages.

Methods: Monocytes were isolated from human buffy coats, and kept for 6 days in culture for differentiation into macrophages. After treatment with ^{3}H-C for 24 h, the cells were incubated with ApoA-I (0.04 mg/mL), or ApoA-I plus increasing Hpt amounts (0.1-0.75 mg/mL). After 16 h of incubation, homologous plasma was added as LCAT source. After further incubation for 16 h, supernatants were aliquoted from culture medium to measure their radioactivity content, representing the released C. Supernatant lipids were extracted and separated by TLC to measure the LCAT activity as conversion of unesterified ^{3}H-C into C esters. Other macrophages, not treated with ^{3}H-C, were incubated with ApoA-I (20 µg/mL), or ApoA-I plus Hpt (200 µg/mL). The supernatants of these cultures were collected after 16 h, and analyzed by western blotting to detect the amount of secreted ApoE.

Results: Addition of Hpt to macrophages significantly ($P<0.01$) inhibited, with dose-dependent effect, the ApoA-I stimulation of C efflux (down to 27% of the control value, i.e. without Hpt), LCAT activity (down to 50% of the control value), and secretion of ApoE.

Conclusions: Macrophages play a key role in atherosclerosis onset and progression, as they differentiate to foam cell triggering the formation of lipid streaks and ateromatose plaques, when RCT is defective. Increase in Hpt concentration, as occurring during inflammatory process, might impair the efficiency of both cholesterol efflux and LCAT activity. Moreover, our results also suggest that Hpt might impair macrophage secretion of the anti-atherosclerotic protein ApoE. Therefore high levels of Hpt might contribute to adverse cardiovascular events.

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2.14

Sic1 PHOSPHORYLATION BY CK2 IS MODULATED BY CARBON SOURCE IN *Saccharomyces cerevisiae*

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CK2 is a highly conserved tetrameric kinase that phosphorylates a wide range of substrates[1]. In *Saccharomyces cerevisiae* CK2 is required for cell cycle progression both in G1 phase[2-3] and in mitosis[2], and it phosphorylates key cell cycle regulators, such as Cdc28[4], Cdc37[5], Cdc34[6-7-8] and Sic1[9-10]. Sic1 is a G1 inhibitor, active on the Clb5,6/Cdc28 complexes, whose activity is required for the onset of S phase[11]. Sic1 is a physiological relevant CK2 substrate[9-10], and mutants on the CK2 consensus site (Ser201Ala and Ser201Glu) alter cell growth-cell cycle coordination at the G1/S transition[9]. Furthermore, we previously showed that Sic1 level and localization are modulated by carbon source[12].

To deepen our knowledge on the role of CK2 phosphorylation at the G1/S transition, we are focusing on the analysis of the phosphorylation of Sic1, as a function of growth conditions and cell cycle phase. To this purpose, we developed a system to study the *in vivo* phosphorylation state of Sic1-Ser201, through immunopurification of Sic1 from yeast cells followed by LC-MS/MS analysis.

Here we report data indicating that Sic1-Ser201 phosphorylation is modulated by carbon source in G1 phase; in particular, Ser201 phosphorylation is higher in glucose grown cells than in ethanol grown cells. We further show that this difference is not due to a different level or localization of CK2 subunits (α, α', β, β') in the two nutritional conditions. Rather, we show that *in vitro* CK2 activity (using the synthetic peptide RRRADDSDDDDD as a substrate) is significantly higher in the cell-free extract of glucose-grown cells than in that of ethanol-grown cells, in keeping with our data on Sic1 phosphorylation.

This is the first evidence of nutritional regulation of CK2 activity in *S. cerevisiae*, suggesting an involvement of CK2 in the nutritional modulation of the G1/S transition.

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2.15

EFFECT OF ELLAGIC ACID ON PROSTATIC CANCER CELL LINES

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Prostate cancer is the most diagnosed cancer and the second leading cause of cancer deaths in men[1].

Oxidative stress was involved as a causative agent in a number of human diseases including prostate cancer and epidemiological data suggested that consumption of fruits and vegetables might be associated with a lower incidence of cancer. The fruit of the *Punica granatum* (pomegranate) contains hundreds of phytochemicals and its extracts have recently been shown to exhibit antioxidant properties, thought to be due to the action of ellagic acid (2,3,7,8-tetrahydroxy[1]-benzopyrano[5,4,3-cde][1]benzopyran-5,10-dione) the main polyphenol present in this fruit[2-4]. There is now increasing interest in the *in vivo* protective effects of natural antioxidants contained in dietary plants against oxidative damage caused by free radical species. In view of these considerations, this study investigated the effects of ellagic acid on LnCaP and DU145 prostatic cancer cell lines. BPH-1 cells were employed as non malignant cells. Cytodifferentiation of malignant/premalignant cells into more mature or normal-like cells and apoptosis during multistep carcinogenesis are theoretically suitable in cancer therapy. Thus, compounds capable of inducing differentiation or apoptosis are candidate chemopreventive and/or chemotherapeutic agents[5].The evaluation of chromogranin A levels was used as marker of neuroendocrine differentiation; since the low-affinity nerve growth factor receptor p75 (p75 NGFR), is less expressed in prostatic cancer cell lines than in normal cells, its expression was used as marker of cytodifferentiation. In order to elucidate mechanisms involved in anticancer activity of ellagic acid, we analyzed by western blotting analysis the expression of different proteins involved in cells growth and apoptosis. In addition, LDH release and ROS levels were evaluated. Our data evidenced, the ability of ellagic acid to reduce cell proliferation. In addition, our results provided morphological and biochemical evidence suggesting that ellagic acid is able to revert LnCap and DU145 cells from the neuroendocrine proliferating state to normal-like un-proliferating state. Then, our study furtherly support the growing body of data suggesting the bioactivities of natural compounds and their potential impact on cancer therapy and on human health.

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2.16

INTERACTION OF TUMOR CELLS WITH BRAIN VASCULAR ENDOTHELIAL CELLS: A SEARCH FOR CYTOSOLIC PLA_2-PKCα-ERK1/2 SIGNALING PATHWAY

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Cancer and metastatic cells communicate with endothelial cells (ECs) through an interactive network of cell-cell and cell-matrix signaling and could activate ECs by cell-cell contact or soluble factors. The largely undefined signal transduction mechanisms and cross-talk between human melanoma cell (HMC) lines and brain endothelial cells (ECs) involved in tumor cell interaction and adhesion has been here investigated. In immortalized rat brain EC cultures (GP8.3), conditioned media (CM, 48 h) prepared from SK-MEL28 and OCM-1 melanoma cells significantly enhanced EC proliferation, measured by BrdU ELISA test. Incubation with two kinase (PI3K/Akt and ERK1/2) inhibitors (wortmannin/ LY294002 mixture and PD98059, respectively), PLA_2 activity dual blocker ($AACOCF_3$) and COX-2 specific inhibitor (NS-398) caused a significant inhibition of cell proliferation. To clarify the role of calcium-dependent PLA_2, in addition to calcium-independent PLA_2, upon melanoma CMs, we evaluated the PLA_2 protein expression and activation by Western blot analyses. Results demonstrated that, in the presence of melanoma CM for 24 h, ECs significantly expressed $cPLA_2$ and $iPLA_2$ total protein and phosphorylated form of $cPLA_2$ at levels higher than controls. The increase of $cPLA_2$ protein synthesis and phosphorylation may support an increase in $cPLA_2$ activity[1]. To investigate the possible signaling mechanisms by which melanoma cell CMs may mediate phosphorylation of the endothelial $cPLA_2$, we next used the above mentioned inhibitors. Wortmannin/LY294002 significantly inhibit stimulated expression of phosphorylated $cPLA_2$ form to almost basal level. As the presence of melanoma CM in cultures increased $cPLA_2$ protein synthesis in ECs, we then assessed the $cPLA_2$ and $iPLA_2$ mRNA levels. Melanoma CM induced a significant increase in endothelial $cPLA_2$ and type VI-$iPLA_2$ mRNAs. By confocal microscopy, activation of $cPLA_2$, ERK1/2, PKCα and COX-2 in perinuclear and membrane regions of ECs grown in CM-stimulated cultures were clearly observed. Since PKCα-ERK1/2 signaling mediates CM induced proliferation via induction of expression of $cPLA_2$ activity, we electroporated exogenous $cPLA_2$ and ERK1/2 antibodies, binding to corresponding intracellular enzyme, into ECs: both of them abrogated CM-mediated cell proliferation. Thus MEK-PKCα-ERK1/2 and PI3K/Akt survival pathways are activated in EC cultures during the interaction with CM from both melanoma cell lines.

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2.17

CURCUMIN PROTECTS CARDIAC CELLS AGAINST ISCHEMIA-REPERFUSION INJURY: MECHANISM OF ACTION

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Ischemia-reperfusion (IR) injury, a condition that plays an important role in several circumstances of clinical interest, is multifactorial but it is generally accepted that an excessive production of reactive oxygen species (ROS) plays a prominent role in its development[1]. In heart muscle IR has been found to induce myocyte necrosis and apoptosis that appear to be the prevalent mode of death during, respectively, the ischemic period and the reperfusion. The intracellular signaling pathway leading to cardiomyocyte death in IR are poorly understood, however there is evidence that the nuclear factor kappa B (NF-kB), a pivotal transcription factor, is rapidly activated by ischemia in several tissues including myocardium[2].
Another pathway that might have a part in cardiomyocyte death during IR is that related to the c-Jun N-terminal kinase (JNK), which represents one of the "stress responsive" members of the mitogen activated protein kinase (MAPK) family[3].

Antioxidant treatments have a critical role in cell protection against IR injury and many substances have been proposed for this purpose. In the last years growing interest has been addressed to curcumin, a major active component of the food flavor turmeric, which is extracted from the powdered dry rhizome of Curcuma longa Linn (Zingiberacee), a perennial herb widely cultivated in tropical regions of Asia.

In this study we explored the effect of curcumin in cardiac cells subjected to a protocol simulating IR. 10 µM curcumin was administered prior ischemia (pre-treatment) or at the moment of reperfusion (post-treatment) and its effects were compared to those produced by a reference antioxidant (Trolox) used at an equal antioxidant capacity.

IR cardiac cells showed clear signs of oxidative stress, impaired mitochondrial activity and a marked development of both necrotic and apoptotic processes; at the same time, increases in NF-kB nuclear translocation and JNK phosphorylation were observed. Curcumin pre-treatment revealed to be the most effective in attenuating all the observed modifications and, in particular, in reducing the death of IR cells. This confirms that the protective effect of curcumin is not simply related to its antioxidant properties but involves other mechanisms, notably interactions in NF-kB and JNK pathways. These findings suggest that curcumin administration, in particular prior the hypoxic challenge, represents a promising approach to protect cardiac cells against IR injury.

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2.18

IDENTIFICATION OF ALTERNATIVELY SPLICED FORMS OF IL-6 GENE IN C2C12 CELLS.

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Interleukin-6 (IL-6) is a pleiotropic cytokine produced and secreted by various cells types. The human and mouse IL-6 gene contains four introns and five exons. Alternative splicing of this gene generates three mRNAs in human: IL-6δ2 (deletion of exon 2), IL-6δ4 (deletion of exon 4) and IL-6δ2δ4 (deletion of exons 2 and 4)[1]. On the contrary, only two mRNAs were found to be expressed in different tissues of mouse immunized with sheep erythrocytes. Furthermore, these splice vatiants, involved different exons: IL-6δ3 (deletion of exon 3) and IL-6δ5 (partial deletion of exon 5)[2]. The biological functions of these isoforms are poorly studied. However in vitro receptor mobility shift[1], reconstruction of hypothetical three-dimension structures and analysis of interactions with complex receptor[3] collectively suggest that these alternatively spliced forms could act as antagonists of IL-6 action.

In the present study we examined the expression of IL-6 isoforms in C2C12. In addition to full-length IL-6 (640 bp), we detected three different PCR bands both in myoblasts and myotubes. Sequencing of the corresponding PCR bands confirmed the presence of the two isoforms also saw *in vivo* in mouse: IL-6δ3 (526 bp) and IL-6δ5 (582 bp)[2]. Furthermore, this analysis, revealed a novel splice variant, IL-6δ3δ5 representing IL-6 lacking both exons 3 and the firsts 58 bp of exon 5 (468 bp). The amount of the various PCR products differed considerably. In particular the intensity of the full-length IL-6, IL-6δ5, IL-6δ3 and IL-6δ3δ5 bands was, respectively, hight, intermediate, low and very low intense. Analysis of the hypothetical structure and prediction of interactions with IL-6 receptors suggest that, all these isoforms, in different manner, could act as antagonists of IL-6 action. For example, the most expressed isoform IL-6δ5, retain the banding site for the second gp130 monomer but lost the E-helix and D-helix possibly losing the ability to interact with IL-6R and the first gp130 monomer. Therefore the formation of functional IL-6-receptor complex is likely to be impeded. The presence of splice variants of IL-6 gene in unstimulated myoblasts and myotubes suggest that the expression of these isoforms wasn't strictly related to inflammatory process, as reported in immunized mouse[2], and underline the importance to study what functional roles these variants play in muscle physiology.

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2.19

ROLE OF THE MECHANOTRANSDUCTION IN SKELETAL MUSCLE CELLS STIMULATED BY SPHINGOSINE 1-PHOSPHATE

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Stretch-activated channels (SACs) regulate trans-membrane currents providing cation and Ca^{2+} entry pathways, which have important regulatory roles in many cell processes[1]. Little is known on the physiological functions of SACs in skeletal muscle cells. In the present study we investigate whether a member of the TRP channel family (TRPC1) can be activated by mechanical stretch and whether it can be an effector of the pro-myogenic factor, sphingosine 1-phosphate (S1P) in C2C12 myoblasts.

Using short interfering RNA and whole-cell patch-clamp, we provided the first experimental evidence that TRPC1 can be activated by mechanical stretch in C2C12 myoblasts. Interestingly, by immunoprecipitation experiments we found that the physical interaction between actin-formed stress fiber and focal adhesion kinase as well as TRPC1 was enhanced by the bioactive lipid and significantly reduced by the Rho kinase inhibitor Y-27632, and the myosin II ATPase inhibitor blebbistatin. Moreover, TRPC1 expression was potentiated by the treatment with S1P and appeared to be essential for the skeletal muscle differentiation elicited by the bioactive lipid.

These findings demonstrate the existence of a structural coupling between actin filaments and plasma membrane and of a tensional force generated by S1P-induced SF contraction, that, in turn, contribute to sarcolemma stretching and SAC activation in myoblastic cells. Furthermore, beside to identify a novel effector in the signalling pathway of S1P leading to the phenotypic maturation of myoblasts, these data offer new possible targets for therapeutic intervention in those pathologies in which alterations of cation concentration appears to be crucially involved, such as muscle dystrophy.

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2.20

PHOSPHATIDYLSERINE SYNTHESIS DURING CHROMIUM (VI)-INDUCED APOPTOSIS IN HUMAN LYMPHOBLASTIC CELLS

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Various apoptotic stimuli increase PS synthesis[1-3] and it has been proposed that this event participates to PS exposure[1, 2]. However, the reduction of PS synthesis observed in macrophages induced to apoptosis by Group B Streptococcus (GBS)[4], seems in contrast with this possibility. Here we investigated on the correlation between apoptosis and PS synthesis in another experimental model, represented by MOLT-4 induced to apoptosis by Cr(VI)[5]. PS synthesis has been evaluated, as usually done, from the incorporation of [^{3}H]serine into PS. Apoptosis level and PS exposure were measured in parallel samples using propidium iodide and annexin V binding, respectively. Treatment of cells with Cr(VI) reduced PS radioactivity in a dose- and time-dependent manner that well correlated to the level of cell apoptosis. Cr(VI)-treatment reduced also conversion of newly synthesised PS to phosphatidylethanolamine (PE), a reaction catalysed by an enzyme located in mitochondria. The decrease of cellular [^{3}H]PS and [^{3}H]PE in Cr(VI)-treated cells appeared not due to a greater release of radioactive membrane vesicles in the medium. Thus, the response to the apoptotic stimulus was similar to that observed in GBS-infected macrophages[4]. In GBS-induced apoptosis, some pro-apoptotic proteins are released from the mitochondria[6]. Here, we evaluated the release of cytochrome c (cyt c) and of AIF from mitochondria after 6h treatment with 200 μM Cr(VI) (50% annexin V positive cells; 50% decrease of PS radioactivity). Western blotting analysis for cyt c and AIF in crude mitochondria fraction and in the post-mitochondrial supernatant[7] demonstrated that the two proteins were not released from mitochondria of apoptotic cells.

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2.21

TRAP1, A NOVEL ANTIAPOPTOTIC GENE RESPONSIBLE FOR MULTIDRUG RESISTANCE.

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Adaptation to oxidative stress can be considered a good tool to study molecular mechanisms involved in chemoresistance. In fact many anticancer agents, mainly platin derivatives, anthracyclines and other anti-neoplastics drugs induce cell death through generation of reactive oxygen species. However, cancer chemotherapy is often unsuccessful for the intrinsic or acquired cell drug resistance.

We demonstrated that expression of TRAP1, a mitochondrial chaperone, is significantly up-regulated in Saos-2 osteosarcoma cells chronically adapted to grow in mild oxidizing conditions, induced by diethylmaleate (DEM), a GSH depletor[1], and this correlates with a phenotype more resistant to H_2O_2-induced DNA damage and to apoptosis[2]. Furthermore TRAP1 is recently described as a component of a mitochondrial pathway selectively up-regulated in tumor cells, which antagonizes the proapoptotic activity of cyclophilin D and is responsible for maintenance of mitochondria integrity, thus favoring cell survival. Interestingly, novel antagonists of TRAP1/ ATPase activity causes sudden collapse of mitochondrial function and selective tumor cell death, suggesting that this pathway may represent a novel molecular target to improve anticancer therapy.

Since expression of inducible heat shock proteins correlates with increased resistance to apoptosis induced by a wide range of cytotoxic agents, we evaluated whether the up-regulation of TRAP1 expression may represent a mechanism responsible for resistance to chemotherapy in HT29 human colorectal carcinoma cells. A comparison of TRAP1 mRNA expression between DEM-adapted and chemoresistant cells demonstrates that TRAP1 mRNA levels are similarly induced in HT29 cell resistant to 5-fluorouracil and oxaliplatin[2]. Furthermore cell growth analysis by MTT dye assay, upon exposure of DEM-adapted Saos-2 cells and TRAP1 stable clones to 5-fluorouracil, demonstrated a direct correlation between TRAP1 expression and cell survival. Finally flow cytometric analysis of HT29 cells transiently overexpressing TRAP1, treated with 5-fluorouracil or oxaliplatin, demonstrated the TRAP1 protection from apoptosis. These results suggest that the increased expression of TRAP1 observed in human colorectal carcinomas could be part of a pro-survival pathway responsible for resistance to 5-fluorouracil and oxaliplatin-based chemotherapy. In addition a preliminary finding of increased TRAP1 expression in some colorectal cancer specimens suggests that TRAP1 may represent a novel molecular target in cancer theraphy.

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2.22

BIOENERGETIC STUDY OF K-RAS-TRANSFORMED FIBROBLASTS.

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Cancer cells are characterized by a high rate of glycolysis with production of lactate even in presence of oxygen, and by a reduced rate of aerobic respiration, the so-called Warburg effect (Warburg 1956). Nevertheless, the role of mitochondrial changes in cancer has not been unambiguously defined. We have used a system of K-ras-transformed fibroblasts (Chiaradonna et al 2005) in comparison with immortalized normal counterparts (NIH-3T3) to evaluate functional bioenergetic changes dependent upon ras oncogene activation. Cell viability was studied under different glycolytic conditions by changing daily the substrate-enrichment of the culture medium: 25 mM glucose and 5 mM galactose both in presence of 110 mg/l pyruvate were chosen. K-ras transformed cells have normal growth in high glucose, being higher the growing rate with respect to wild-type cells. At variance, decreasing the glucose content (and glycolysis rate) K-ras transformed cells grow much slower than the wild-type cells. Interestingly, ras-transformed fibroblasts grown in both glucose and glucose free medium have decreased ATP content and decreased expression of several mitochondrial genes related to oxidative phosphorylation, including genes encoding for cytochrome oxidase and ATP synthase subunits.

We have investigated bioenergetic parameters in digitonin-permeabilized cells grown in glucose medium. Contrary to succinate as substrate, glutamate/malate energized K-Ras transformed cells, resulted strongly depressed in both State 3 respiration and ATP synthesis rate. We have further measured the redox activity of Complex I (as rotenone-sensitive NADH dehydrogenase) in isolated mitochondria and a significant reduction of activity was shown in the transformed cells; the decrease of Complex I activity was in the range of NADH-dependent respiration decrease.

These data demonstrate that the decreased ATP synthesis and respiration rate are not the result of changes pertaining to either ATP synthase or cytochrome oxidase, but of alterations occurring at the level of respiratory Complex I. Since Complex I subunits had shown no change in the gene expression study of Chiaradonna et al (2006), we are currently investigating the content and supramolecular organization of OXPHOS complexes by polyacrylamide gel electrophoresis and western blotting.

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2.23

THE ROLE OF ERAD IN PLANT CELLS: A PRELIMINARY BIOCHEMICAL CHARACTERIZATION OF PLANT PROTEASOMES

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The secretory pathway of plants is a highly complex system concerned with the manufacture, quality control and transport of various polymers. Errors in transcription, translation and inefficient folding are among the many factors that can contribute to the accumulation of misfolded or unassembled polypeptides within the secretory pathway. These proteins are potentially very damaging and must therefore be eliminated. This was initially assumed to involve degradation in lytic vacuoles following targeting or autophagy. However, it was later demonstrated that the plant endoplasmic reticulum (ER) is endowed with a system that allows the retrotranslocation of terminally misfolded or orphan proteins to the cytosol where these selected substrates can then be degraded[1]. This quality control pathway known as ER-associated protein degradation (ERAD) is well described in mammalian cells, where it typically involves delivery of the selected proteins to dislocation machineries in the membrane, substrate recognition on the cytosolic plane of the membrane brought about by the ubiquitination machinery, extraction from the membrane and degradation by proteasomes. These events are tightly coupled such that it is normally very difficult to visualise a retrotranslocated substrate unless proteasomes are inhibited. Although several different ERAD substrates have now been described in plants, so far we are almost completely ignorant not only of the range of plant proteins that undergo ERAD, but of how many endogenous proteins are devoted to the disposal of ER proteins. Of note, over 1300 genes (5% of the Arabidopsis proteome) are devoted to the selective breakdown of proteins by the extensive ubiquitin/26S proteasome pathway in plant cells, but virtually nothing is known about these components in relation to ERAD, nor of the overall impact of this pathway relative to alternative disposal mechanisms. Addressing these important questions until now has been extremely difficult due to the limited information concerning both the biochemical properties of plant proteasomes and the activity of proteasome inhibitors in living plant cells. For this reason we characterized the cleavage specificities of proteasomes in protoplasts extracts of *Nicotiana tabacum* using fluorogenic substrates specific for the main peptidase activities of 26S particles. Furthermore, we evaluated the *in vitro* sensibility of these activities to several reversible and irreversible inhibitors widely used to inhibit mammalian and yeast proteasomes. Finally we measured the extent of proteasomal inhibition reached *in vivo* by administration of these inhibitors to protoplasts cultures. Based upon these results, we plan to develop more effective protocols of proteasomal *in vitro* and *in vivo* inhibition that will facilitate further study on ERAD in plants cells.

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2.24

SIGNAL TRANSDUCTION PATHWAY IN PrP^C-DEPENDENT NEURITOGENESIS

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Cellular prion protein (PrP^c) is a ubiquitous glycoprotein. It is localized at the cell surface via a glycosylphosphatidylinositol (GPI) anchor, and its physiological role is poorly characterized. It has been suggested that PrP^c participates in synaptic structure, neurite formation, copper metabolism, and signal transduction[1]. It also has a protective role in neurodegeneration due to oxidative stress[2]. Several cellular partners of PrP^c have been proposed, but no functional ligand of PrP^c has yet been identified clearly. Antibody cross-linking has been used to identify PrP^c induced signalling pathways, revealing a caveolin-dependent coupling of PrP^c to the tyrosine kinase Fyn[3]. In this study we detailed the intracellular events induced by PrP^c antibody-mediated cross-linking in PC12 cells. We found a Fyn-dependent activation of the Ras-Raf pathway, which leads to a rapid and transient phosphorylation of Erk1/2. In addition, this activation cascade relies on the engagement of integrins, and involves FAK activation. We demonstrated the tyrosine phosphorylation of caveolin-1 and paxillin as a consequence of PrP^c stimulation, and showed that phosphocaveolin-1 scaffolds and coordinates protein complexes involved in PrP^c-dependent signalling. Moreover, we found that caveolin-1 phosphorylation, like paxillin phosphorylation, is a mechanism for recruiting the C-terminal Srk kinase (Csk) to the plasma membrane and inactivating Fyn, so as to terminate cell signalling. Furthermore our data support a significant role for PrP^c as a response mediator in neuritogenesis. In fact cells grown in the presence of the anti-PrP^c antibody clearly show neurite extensions, which are not visible in control cells.

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2.25

RELATIONSHIP BETWEEN INTRACELLULAR OXIDATIVE STATUS AND INTERLEUKIN SYNTHESIS IN 18CO CELLS: INVOLVEMENT OF P38 AND ERK1/2 MAPKINASES

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Intestinal subepithelial myofibroblasts (ISEMF), present at the interface between enterocytes and the lamina propria mediate information flow between epithelium and the mesenchymal elements by secreting various cytokines, growth factor and inflammatory mediators. An excessive production of pro-inflammatory cytokines in the intestinal mucosa plays an important role in the pathogenesis and complications of inflammatory bowel disease (IBD). In fact, a deregulation of cytokine secretion occurs in intestine of these patients. The antioxidants affect the cytokine synthesis in response to appropriate stimuli with the involvement of MAP-kinases (MAPKs) and GSH levels decrease in the intestinal mucosa of IBD patients[1]. For this purpose we have evaluated the role of p38 and ERK1/2 MAPKs in the redox regulation of the production of IL-6 an IL-8, involved in the onset of acute inflammation, in a human intestinal myofibroblast cell line, 18Co. These cells were stimulated in different experimental conditions for 24 h with lipopolysaccaride (LPS), N-formyl-methionyl-leucyl-phenylalanine (fMLP) and tumor necrosis factor (TNFα), important mediators regulating IL production. The culture medium was used to assay IL-6 and IL-8 by ELISA Kits. GSH and GSSG levels were measured by HPLC method and phosphorylation of p38 and ERK1/2 MAPKs was performed by Western blot analysis.

A relationship between the cytokine synthesis and GSH/GSSG ratio has been demonstrated in 18Co cells. In fact, the decrease of this ratio, by inhibiting GSH synthesis with buthionine sulfoximine (BSO), induced an increase in the IL-8 and IL-6 production. The subsequent stimulation of BSO treated cells with LPS, TNFα and fMLP induced a further and significant increase of ILs production both respect to BSO treated cells and to untreated but stimulated cells. On the contrary, the N-acetylcysteine (NAC) addition to cells in which BSO was or not removed, by washing with PBS, restored both the GSH/GSSG ratio and the synthesis of interleukins to the normal values. BSO induced also a significant phosphorylation of p38 and ERK1/2 and this effect was abolished by NAC treatment. ILs production has been detected also in the presence of specific inhibitors of MAPKs to evaluate their physiological role in this process. Inhibition of MAPK pathways abrogated the increased release of interleukins in BSO treated and stimulated cells. These results show that the decreased GSH/GSSG ratio levels measured in the intestinal mucosa of IBD patients can be responsible of the increased cytokine production that occurs in these patients. This study shows also that this effect is mediated by activation of p38 and ERK1/2 MAPKs.

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2.26

REDOX REGULATION OF PDGF RECEPTOR TRANSACTIVATION INDUCED BY EXTRACELLULAR SPHINGOSINE 1-PHOSPHATE: ROLE OF G_i PROTEINS AND C-SRC-KINASE ACTIVITY.

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Introduction: Previous results have demonstrated that PDGF receptor (PDGFr) is redox regulated in NIH3T3 fibroblasts and that Sphingosine 1-phosphate (S1P) stimulation induces activation of NADPH oxidase and production of H_2O_2 [1,2]. S1P belongs to groups of platelet-derived lipid mediators regulating various biological processes. Extracellular S1P acts through specific G-protein coupled receptors (GPCRs) in various cells including NIH3T3 fibroblasts. In these cells, intracellular S1P is also related to PDGF mitogenic signalling as second messenger and to PDGF-induced cell motility by autocrin mechanisms of S1P receptors stimulation. S1P and some GPCRs ligands transactivate receptor tyrosine kinase (RTK) with important effects downstream the signalling pathways, even if S1P-stimulated transactivation of RTK has not been well studied. **Methods**: Cell culture, transfection experiments, western blot analysis were performed as reported in the reference[2]. **Results**: Previously, we demonstrated in NIH3T3 fibroblasts that S1P is able to activate tyrosine phosphorylation of PDGF receptor (PDGFr) and this effect is mediated by H_2O_2 production. Concentration- and time-dependent PDGFr tyrosine phosphorylation after S1P stimulation have been demonstrated and this effect was obtained in the presence of vanadate, specific inhibitor of tyrosine phosphatase that increased tyrosine phosphorylation. Similar results were obtained after cell stimulation by dihydro-S1P, S1P analogue that binds GPCRs and differently to S1P acts only as extracellular mediator. Phosphorylation of tyrosine 857 essential for the full activation and regulation of PDGFr kinase activity was also observed in S1P stimulated cells. Experiments performed by pertussis toxin (PTX), which specifically inhibits GPCRs, demonstrated that S1P-induced PDGFr transactivation occurred through GPCRs. Moreover, the specificity of this event was also determined. S1P-induced PDGFr transactivation was mediated by c-Src kinase activation with the involvement of H_2O_2 production. This has been demonstrated in cells treated with specific inhibitors of c-Src or PDGFr tyrosine kinase and with catalase. Effectively, c-Src kinase activation and H_2O_2 production induced by S1P stimulation occur upstream of PDGFr transactivation[2]. Finally, it has been demonstrated that PDGFr transactivation contributes to ERK1/2 activation and NIH3T3 fibroblasts proliferation due to S1P stimulation.

Conclusions: Taken together, these results show that H_2O_2 production and c-Srk kinase activation are involved in a possible redox regulation of PDGFr transactivation induced by S1P and this event can contribute to downstream activation of ERK1/2 and cell proliferation.

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2.27

CYTOCHROME OXIDASE, INHIBITING EFFECTORS AND THE CONTROL COEFFICIENT

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Respiration is controlled by NO[1] and by the electrochemical potential gradient at the level of cytochrome *c* oxidase. Two different mechanisms of inhibition by NO have been proved to be active *in vitro*, using the enzyme purified in detergent solution or in intact cells[2]. Similar cell lines (lymphoid cells) appeared more susceptible to one mechanism or the other, depending on the concentration of cytochrome *c* in the cell[3]

MATERIALS AND METHODS

NO- and O_2-amperometric measurements have been carried out in parallel and compared to the available rapid mixing spectroscopic results.

RESULTS & CONCLUSION

At high concentration of cytochrome c^{2+}, NO inhibits the enzyme in competition with O_2 by binding to heme a_3^{2+} (nitrosyl complex), whereas at low [cytochrome c^{2+}] NO is oxidized to nitrite, possibly by reacting with the Cu_B^{2+}. In both cases cytochrome oxidase is transiently inhibited; the inhibition is reversible, and the enzyme fully recovers activity in the presence of O_2 and reductants either by releasing NO or ejecting nitrite in the bulk phase.

We are presently studying, by Metabolic Control Analysis (MCA), the behaviour of a human liver carcinoma cell line (HepG2). The effect of the NO inhibition on the control coefficient of cytochrome *c* oxidase has been compared to that of the electrochemical potential gradient modulated by ionophores.

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2.28

EXPRESSION AND ACTIVITY OF GPR17, A NEW NUCLEOTIDE-CYSTEINYL-LEUKOTRIENE RECEPTOR, IN NEURONAL PC12 CELL MODEL.

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The GPR17 receptor, which is specifically activated by both uracil nucleotides and cystenil-leukotriene ligands and is mainly coupled to Gi-q protein [1], has been recently identified as a dualistic receptor with a phylogenetic position between P2Y and CysLT receptors. Purinergic signalling, having a important role in cell proliferation, migration and differentiation, has been recently involved in the control of mitogenic signalling activated by growth factor receptors (GFR) [2]. Vascular endothelial growth factor (VEGF) has neurotrophic and neuroprotective effects on neuronal and glial cells, thus stimulating the proliferation and survival of neural stem cells.

The aim of this work was to evaluate the GPR17 expression and involvement in neuronal differentiation-proliferation and its functional interaction with VEGFR in rat pheocromocytoma PC12 cells, as a neuronal model. Because of Nerve growth factor (NGF, 100 ng/ml) induces cell differentiation in sympathetic neurons, PC12 cells were grown in medium containing NGF (100 ng/ml) for different times (0-10 days).

The GPR17 expression levels were evaluated by RT-PCR and immunocytochemistry experiments. PC12 cells, at different NGF treatment days, were exposed to GPR17 agonists UDP-glucose and LTD4 for 24h, with or without VEGF (100ng/ml): then the MTS vitality assay was performed to assess cell proliferation. The levels of ERK ½ activation were also evaluated after cell exposure to GPR17 agonists and/or VEGF.

Experimental data showed that GPR17 receptor wasexpressed in PC12 cells in the early stages of differentiation: anyway its expression was highest after 10 days of neuronal differentiation (in serum-free conditions), in accordance to the dynamic expression of purinergic receptors during brain development.

The VEGF and both UDP-glucose and LTD4 significantly increased cell viability. UDP-glucose increases PC12 cell proliferation but had no synergic effects with VEGF. On the contrary, LTD4 increases PC12 cell proliferation and has a synergic effects with VEGF, at concentration within 5 nM. UDP-glucose and LTD4 effects on PC12 cell proliferation seem to be mediated by the activation of ERK1/2.

The results obtained show promising perspectives for the employment of GPR17 receptor as a new target for therapeutic approach in brain damage and repair.

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2.29

LOW OXIGEN TENSION AS A QUIESCENT FACTOR FOR NON-TRASFORMED CELLS.

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In the current bibliography “normoxia” is generally referred to an oxygen concentration of 20%, instead, physiological oxygen tension is highly variable in different tissues but nevertheless it’s very different from that of the atmospheric pressure. It has been calculated that oxygen tension is about 13% in the pulmonary alveoli and 7% in the peripheral capillaries. Hence, considering both tissue oxygen consumption and diffusion, an oxygen tension of about 1-3% is conceivable for most tissues.

In our working model we considered 1% oxygen tension as a physiological condition for NIH-3T3 cells, whereas we consider 20% an hyperoxic condition, to which a tissue could be exposed in particular conditions such as a wound.

Our data show that a low oxygen tension (1%) causes a reduced proliferation rate in NIH3T3 cells with respect to that grown at 20% oxygen tension, in response both to serum and to PDGF-BB stimulation. In addition we found that cells kept at low oxygen tension show a reduced migration capability with respect to NIH-3T3 in 20% oxygen atmosphere upon PDGF-BB stimulation.

We found that the exposition of NIH-3T3 cell to 1% oxygen tension for several days progressively reduce, through a post-transcriptional mechanism, the expression of PDGF receptor leading both to a weak cell proliferation response to high dose of PDGF-BB (30 ng/ml) and to a reduced cell migration at low PDGF-BB dose (2 ng/ml).

We have tested also the behavior of transformed cells, in particular melanoma cells from primary tumor (A375) and from metastasis (HS29), in the presence of 1% oxygen tension and we have found that they show an higher proliferation rate respect to that kept at 20% oxygen.

The opposite behavior of normal and transformed cells at low, physiological, oxygen tension may lead to the idea that, while for normal cells the low oxygen tension is a condition that contribute to the cellular quiescence of an adult tissue, for cancer cells the 1% oxygen tension gave instead a selective advantage both in terms of proliferation and migration. In this view cancers cells subvert what is normally a quiescent condition in a pro-proliferative one, acquiring a selective advantage over the other non transformed cells in the tissue. A logical consequence of this idea is that the changing in the.oxygen-dependence response is one of the step (together with loss of apoptotic control, loss in cell proliferation control, loss of contact inhibition, etc) on the long route to cellular transformation.

2.30

NEW SMALL MOLECULE ANTAGONISTS TO PROTEASE-ACTIVATED RECEPTOR-1 (PAR-1): INHIBITION OF RECEPTOR INDUCED CALCIUM RESPONSE IN A HUMAN MICROVASCULAR ENDOTHELIAL CELL LINE (HMEC-1).

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Protease-activated receptors (PARs) belonging to the large G protein-coupled receptors family (GPCRs) are activated by proteolytic cleavage of their amino-terminal extracellular domain, thus exposing a new amino-terminal sequence that functions as a tethered ligand to activate them. PAR-1, the first member of this unique GPCR subfamily to be cloned, responds to a selected group of serine proteases including thrombin, plasmin, factor Xa, activated protein C, and matrix metalloprotease-1. The cellular effects of thrombin, are largely, but not exclusively, mediated via the activation of PARs (PAR-1, PAR-3, and PAR-4) among which PAR-1 is a high-affinity receptor and the major effector of thrombin signaling in platelets and endothelial cells. Through activation of PAR-1, thrombin is the most potent stimulus for platelet activation and initiates inflammatory and tissue repair responses[1]. Thus, PAR-1 represents an important target for antagonist drugs which will be able to block the cellular effect of thrombin, including platelet activation, without inhibiting fibrin formation or platelet activation by other activators. To date, few PAR-1-targeting agents have been developed and tested in limited disease models such as thrombosis, and experimental liver fibrosis [2,3]. Recently, a panel of small molecules, which compete for binding at the carboxyl terminus of activated PAR-1 with a high affinity peptide mimicking the carboxyl terminal $G\alpha_q$, have been identified. One of these compound, Q94, inhibits thrombin receptor-activating peptide (TRAP) induced calcium current in a concentration dependent manner and blocks PAR-1-mediated CC-chemokine (CCL2) production in mouse lung fibroblasts[4].

Here, we examined the effects of four compounds which inhibit $G\alpha_q$ peptide binding to activated PAR-1 (similarly to Q94) on intracellular calcium increase induced by PAR-1 stimulation in HMEC-1. The purpose of this preliminary investigation was to identify potent allosteric antagonists which selectively interfere with G_q-mediated PAR-1 signalling.

All compounds caused either a modest or marked shift to the right of thrombin concentration-response curves and an evident reduction of thrombin maximal efficacy. Among the four compounds, compound PAR-1-0190 was the effective in reducing calcium transient with a complete block of the response at 10 μM. This compound has a molecular structure which is similar to that of Q94 while the other three compounds have quite different molecular structures. PAR-1-0190 and Q94 may represent leader molecules to design new selective allosteric antagonists of PAR-1.

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2.31

ANTITUMOR ACTIVITY OF NEW SUBSTITUTED 3-(5-IMIDAZO[2,1-b]THIAZOLYLMETHYLENE)-2-INDOLINONES

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In this work we assessed the antiproliferative activity of new synthesized imidazo-indolinone-derivatives in cancer cells. As preliminary screening the antitumor activity of the whole series of compounds was evaluated according to the protocols available at the National Cancer Institute of Bethesda. Compound **24** is the most potent of the series, particularly it is active against colon carcinoma cell lines. In order to investigate its mechanism of action, we first examined the effect on the cell cycle profile of HT-29 colon carcinoma cells. The cells were incubated for 24h in the presence of compound **24** at concentrations ranging from 0.5 to 2μM, afterward the analysis of DNA profiles was performed by flow cytometry. The results revealed that the molecule under test caused a dose-dependent accumulation of HT-29 cells in the G2/M phase. A similar effect was obtained with vincristine, used as a reference compound because in its structure it contains an indolic nucleus.

A cellular event strictly correlated with cell cycle progression is represented by the induction of ornithine decarboxylase (ODC), the limiting enzyme for the biosynthesis of polyamines, that are absolutely required for cell proliferation. It is known that ODC inhibitors can interfere with the cell cycle at G1-S and G2-M transitions. The ODC activity was measured after cell treatment and it was observed a dose-dependent inhibition of enzymatic activity.

Next we examined the effect of the molecule on cellular microtubule networks by using immunofluorescence techniques. The microtubules exhibit normal arrangement and organization in control HT-29. However, after treatment with compound **24**, an increase of cellular tubulin condensation was detected, indicating cytoskeleton reorganization, together with a great number of mitotic spindles. These observations suggested that compound **24** arrested the cells in the mitotic process similarly to vincristine. Morphological studies performed by using 4',6-diamidino-2-phenylindole (DAPI) staining revealed condensed chromatin along with fragmented nuclei, suggestive of activation of apoptosis. Following 48h of cycle arrest, the activity of caspase proteases acting on the amino acid sequence Asp-Glu-Val-Asp (DEVD) began to increase, confirming the onset of apoptosis.

We can conclude that the effect of the very active compound **24** in HT-29 cells, is associated with a block in cell cycle progression, together with inhibition of ODC. As expected, the arrest of the cell cycle is then followed by a late induction of apoptosis, with activation of caspase proteases. The results obtained show that compound **24** can interfere with proliferation of colon cancer cells by multiple mechanisms and that it could be a promising candidate as an antitumor drug toward colon carcinoma.

2.32

R)-9-HSA HAS A MORE REMARKABLE ANTIPROLIFERATIVE EFFECT COMPARE TO (S)-9-HSA.

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The endogenous lipoperoxidation product 9-hydroxystearic acid (9HSA) is an inhibitor of HDAC1 and its administration to HT29, a colon adenocarcinoma cell line, induces a proliferative arrest mediated by a direct activation of the p21 ^{WAF} 1 gene, bypassing p53 [1]. In a recent work the interaction of 9-HSA with the catalytic site of the 3D model of HDAC1 has been tested with a docking procedure. Noticeably, when interacting with the catalytic site, the (R)-9-enantiomer is more stable than the (S) one: in fact the energies of interaction are -8.45 kcal/mol and -1.97 kcal/mol for the (R) and (S) isomer, respectively, and the estimated free energies of binding are -6.31 kcal/mol and +4.98 kcal/mol for (R) and (S), respectively [2]. In this study (R) and (S)-9HSA were isolated and their biological activity tested in HT29. After 24 h of growth HT29 cells were treated with 50 μM of (R) or (S)-9HSA and then permeabilized by incubation with a solution of RNase and propidium iodide (PI). PI fluorescence was analyzed with a flow cytometer Epics Elite (Coulter), and cell cycle analyses were performed by M Cycle (Verity) and MODFIT 5.0 software.

A concentration of 50 μM (S)-9HSA induced a growth arrest in G0/ G1. At the same concentration (R)-9-HSA showed a more remarkable antiproliferative effect, resulting in a increase of cells in G0/ G1. To asses the effects of (R)- and (S)-9HSA on histone deacetylase 1 (HDAC1) HDAC2 and HDAC3 activity, HDACs were immunoprecipitated from HT29 cells and their catalytic activity were assayed by using [3H] acetate as the substrate. The amount of [3H] acetate released by deacetylase was expressed as cpm/mg protein. In presence of (R)-9HSA a drastic reduction of percentage of [3H] acetate released was observed, showing a stronger inhibitor effect on HDAC1, HDAC2 and HDAC3 activity than the S isomer.

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2.33

INTRACELLULAR ZINC INCREASE INHIBITS p53$^{-/-}$ PANCREATIC ADENOCARCINOMA CELL GROWTH BY ROS/AIF-MEDIATED APOPTOSIS

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Pancreatic cancer is characterized by high frequency mutations in *TP53*, *K-RAS*, *P16*, and *DPC4* genes. Despite advances in the understanding of the molecular biology, prognosis of this cancer remains dismal with a 5-year survival of less than 5% and the gold standard chemotherapy with gemcitabine is largely ineffective.
Zinc is an important modulator of various cellular activities with both catalytic and structural roles. It is the cofactor of over 300 enzymes and is involved in the stabilization of the three-dimensional structure of many proteins, including more than 100 transcription factors containing zinc finger domains. Owing to these critical roles, the cellular homeostasis of zinc ions is strictly controlled at different levels by cell membrane zinc transporters, metallothionein sequestration, and storing in intracellular compartments called "zincosomes". Although zinc is an essential element, its excess induces apoptosis in different cell lines involving mitochondrial injury and oxidative stress production.

In this work we show that treatment with non-toxic doses of zinc in association to the ionophore compound pyrrolidine dithiocarbamate (PDTC) inhibits p53$^{-/-}$ pancreatic cancer cell growth much more efficiently than gemcitabine, the gold standard chemotherapeutic agent for pancreatic cancer. Both the metal chelator *N,N,N',N'*-tetrakis(2-pyridylmethyl)ethylenediamine and the radical scavenger *N*-acetyl-$_L$-cysteine are able to recover cell growth inhibition by Zn/PDTC, demonstrating that this effect depends on the increased levels of intracellular zinc and of reactive oxygen species (ROS). Zn/PDTC treatment induces a strong apoptotic cell death that is associated to ROS-dependent nuclear translocation of the mitochondrial factor AIF, but not to the regulation of apoptotic genes and caspase activation. Primary fibroblasts are more resistant than pancreatic cancer cells to Zn/PDTC treatment and exhibit a lower basal and Zn/PDTC-induced enhancement of intracellular zinc. We show that Zn/PDTC induces p53 proteasomal degradation and that the proteasome inhibitor MG132 further increases fibroblast growth inhibition by Zn/PDTC, suggesting that p53 degradation plays an important role in fibroblast resistance to Zn/PDTC.

2.34

RhoA SILENCING REVERSES THE RESISTANCE TO DOXORUBICIN IN HUMAN COLON CANCER CELLS

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The efficacy of doxorubicin in the treatment of cancer is limited by its side effects and by the onset of drug resistance. Reversing such resistance could allow the decrease of the dose necessary to eradicate the tumor, thus diminishing the toxicity of the drug. We transfected doxorubicin-sensitive (HT29) and doxorubicin-resistant (HT29-dx) human colon cancer cells with RhoA small interfering RNA. The subsequent decrease of RhoA protein was associated with the increased sensitivity to doxorubicin in HT29 cells and the complete reversion of doxorubicin resistance in HT29-dx cells. RhoA silencing increased the activation of the nuclear factor-kappa B pathway, inducing the transcription and the activity of nitric oxide synthase. This led to the tyrosine nitration of the multidrug resistance protein 3 transporter (MRP3), and contributed to a reduced doxorubicin efflux. Moreover RhoA silencing decreased the ATPase activity of P-glycoprotein (Pgp) in HT29 and HT29-dx cells, as a consequence of the reduced expression of Pgp. RhoA silencing, by acting as an upstream controller of both MRP3 nitration and Pgp expression, was effective to reverse the toxicity and accumulation of doxorubicin in both HT29 and HT29-dx cells. Therefore we suggest that inactivating RhoA has potential clinical applications and might in the future become part of a gene therapy protocol.

2.35

CORRELATION BETWEEN THE PROTEIN COMPOSITION OF STAT3-DEPENDENT ENHANCEOSOME AND ITS ACTIVATION PATHWAYS IN CANCER CELLS

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In higher eukaryotes, transcriptional processes regulated by nucleoproteins complexes, called enhanceosomes, play a critical role in the integration of cellular signalling information. In this context the STAT3-dependent enhanceosome is particularly interesting. The signal transducer and activator of transcription-3, a cytoplasmatic latent transcription factor, is a point of convergence for numerous oncogenic signalling pathways. STAT3, constitutively activated in several cancer cells, up-regulates the expression of numerous genes, involved in promoting tumor cell proliferation, angiogenesis, metastasis and cell survival.

This research is focused on the influence of diverse extracellular signals on the composition of nuclear multi-protein complex STAT3 specific.

Melanoma cell (M14) is our cellular model of choice, because STAT3 is constitutively activated by src but is also responsive to stimulation by cytokine (IL-6 100ng/ml), and growth factors (EGF100ng/ml).

To characterize the STAT3-containing complexes we performed experiments *in vitro,* such as DNA affinity assay, and *in vivo* such as CoIP (Co-Immunoprecipitation); the proteins complexes so purified, were analyzed by SDS-PAGE followed either by Coomassie staining and mass-spectroscopy analysis, or by Western blotting with specific antibodies. The results obtained showed that the interaction between STAT3 and its co-activators is strongly dependent on the signalling pathways.

2.36

CORRELATION BETWEEN NEUROINFLAMMATION AND CHOLESTEROL METABOLISM IN GLIAL CELLS

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Some neurological disorders due to defective cholesterol metabolism and characterized by abnormal accumulation of unesterified cholesterol (Niemann Pick type C1, NPC1) or sterols (cerebrotendinous xantomathosis, CTX) are also characterized by glia activation and increased expression of inflammatory markers. Astrocytes and microglia possess a repertoire of sterol hydroxylases belonging to the superfamily of cytochrome P450s (CYPs) enzymes, which catalyze multiple hydroxylations of cholesterol. It has been shown recently that the metabolic conversion of cholesterol to hydroxylated derivatives that can be easily eliminated through the blood-brain barrier is critical to maintain cholesterol homeostasis and intact brain functions.

We have recently found that sterol hydroxylases are differently expressed in astrocytes and microglia, along with other genes involved in cholesterol homeostasis and their expression may be modulated at the level of gene transcription.

Aim of the present study was to better characterize the effects of an inflammatory stimulus (LPS) and cholesterol accumulation on the expression of genes involved in cholesterol metabolism and activation in glial cells.

To this end purified cultures of primary rat astrocytes and microglia were challenged with 5 ng/ml LPS for 4 h or loaded with 20 µg/ml cholesterol for 24 h. Gene expression was evaluated by real time RT PCR and activation was measured as release of biologically active Tnf-α.

We found that sterol 27-hydroxylase (Cyp27a1) is the only sterol hydroxylase to be up-regulated by LPS in both astrocytes and microglia. The induction of Cyp27a1 mRNA is maximal at 4 h and appears to be kinetically slower than the up-regulation of Tnf-α

The levels of Cyp27a1 protein do not change rapidly suggesting a dissociation between transcription and translation. The preliminary observation that biochemical inhibition of the p38 MAPK can partially prevent the action of LPS on Cyp27a1 suggest that p38 substrates are likely involved in the LPS-dependent transcriptional regulation of this gene.

On the other side cholesterol loading reduces mitochondrial functionality (assessed by means of MTT test) and increases the mRNA levels of Tnf-α in primary astrocytes. In the same conditions we observed reduced expression of some sterol hydroxylases (Cyp46a1 and Cyp7b1) but not of Cyp27a1.

Our results suggest that Cyp27a1 may be a crucial gene in the innate immunity response of glial cells and a possible link between cholesterol homeostasis and the inflammatory process.

2.37

STUDYING THE ERp57 FUNCTION IN LIVING CELLS

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ERp57/GRP58, a member of the protein disulfide isomerase (PDIs) family and also a glucose-regulated protein, is induced by a variety of cellular stress conditions. ERp57 is mainly located in the endoplasmic reticulum, but it has also been found in other cellular compartments[1], where its function is still unclear.

In order to identify a possible correlation between the stress-response and the nuclear location and function of ERp57, we identified ERp57-bound DNA fragments by means of ChIP experiments on HeLa cells[2].

The features of these DNA sequences, i.e. proximity of MAR regions and homology to the non-coding regions of orthologue genes of mouse or rat and DNAse hypersensitivity, are compatible with a gene expression regulatory function. Considering the nature of the genes concerned, one of which codes for a DNA repair protein (i.e. MSH6), we suggest that at least part of the mechanism of action of ERp57 takes place through the regulation of these, and possibly others still unidentified, stress-response genes.

Moreover, in M14 melanoma cells we found that the STAT3-ERp57 complex is also present in the nucleus, and that its nuclear import is strongly favoured by the activation of STAT3 induced by IL-6. Furthermore, ERp57 is associated with a number of STAT3-bound enhancers[3].

In order to understand the functions of ERp57 in the nucleus, we have exploited an RNAi strategy. The silencing of the protein ERp57 has an effect on the expression of genes identified by ChIP experiments (i.e. MSH6, TMEM, ETS1).

In addition, the inhibition of ERp57 expression is accompanied by the decrease of the expression of the STAT3-dependent genes. Evidence will be presented showing that the importance of ERp57 is not due to (or not limited to) the impairment of the nuclear import of STAT3.

Together these results suggest that this protein is involved in a variety of nuclear processes.

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2.38

THE SYNAPTIC PROTEIN NEUREXIN AND THE VASCULAR TYROSINE KINASE RECEPTOR TIE-2 PHYSICALLY ASSOCIATE IN BLOOD VESSELS

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The functional association between transmembrane receptors in order to finely regulate cell activities is well established.
In the past years, during a search for protein interactors of the vascular specific tyrosine kinase receptor Tie-2 we identified, among many candidates, another trasmembrane protein, Neurexin (NRXN). This protein had been studied exclusively in the nervous system where it localizes at the pre-synaptic side of chemical synapses. Based on the published data, NRXN and Tie-2 were expressed in different anatomical districts, and physical interaction between these two membrane-bound proteins was impossible to conceive. Since Vascular Biology is our main interest, we decided to investigate if Neurexin, as many other neuronal proteins, was expressed in the vascular system. We demonstrated that, among others, immature blood vessels of the chick embryo produce NRXN in the entire span of their developing wall (including the endothelial lining that expresses Tie-2).

At this stage we were in the right conditions to verify the initial hypothesis, i.e. that NRXN and Tie-2 can physically interact. As a first approach we overexpressed both proteins in a non-endothelial cell line and we verified that in these conditions NRXN and Tie-2 exist in stable preformed complexes. To improve the physiological significance of our data, we next moved to chirurgically excised arteries from chick embryos at day 18 of development, as a model to study the interaction. In these vessels Tie-2 is expressed preferentially by the endothelial layer while NRXN expression covers both the endothelial and the most luminal layers of vascular smooth muscle cells. In this setting we were also able to demonstrate that NRXN and Tie-2 form endogenous complexes. The best conditions to maintain this interaction were found after a set up phase of the extraction efficiency with buffers containing different detergents /cations. Moreover, we found that the optimal procedure to reveal the complex was to immunoprecipitate NRXN and immunoblot the resulting precipitate with the anti Tie-2 antibody. We believe that this could be explained by the fact that, in the arteries, Tie-2 is produced at least in a ten times greater amount than NRXN.

The findings here described strongly sustain the possibility that NRXN, a synaptic/vascular protein, exploits the Tie-2-initiated pathway to affect blood vessel biology. In fact, Tie-2 is one the few essential vascular players. At this stage we are investing to analyse the molecular/cellular mechanisms and the functions of this interaction in the vascular system. We are also planning to identify what part of Neurexin is needed for Tie2- binding by using mutational analysis.

2.39

MOLECULAR PROFILING OF DIFFERENTIATED THYROID NEOPLASM: DIAGNOSTIC AND CLINICAL IMPLICATIONS.

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The differential diagnosis of the thyroid nodule includes numerous entities, non-neoplastic and neoplastic, benign and malignant and not infrequently pose diagnostic and treatment difficulties.

The use of fine needle aspiration cytology (FNAC) has significantly improved the ability to identify specific high-risk disorders and to facilitate their management in an expeditious and cost-effective manner but the follicular-derived neoplasms (adenoma, carcinoma and the follicular variant of papillary carcinoma) manifest overlapping cytomorphologic features.

The purpose of this research was to identify novel genes that can be targeted as diagnostic and clinical markers of indifferentiated thyroid tumours.

Human thyroid carcinoma cell lines utilized in this study were obtained from primary thyroid neoplasms through biopsy and from FNAC, with the informed consent of the patients (Centro Telesforo), and cultivated at the Department of Pathology and Experimental Medicine and Clinic, University of Udine.

Analysis were performed using western blotting and real-time PCR. PCR-based methods offer a simpler and practical approach to quantify gene expression also with limited amounts of material.

Moreover, data reported so far demonstrated that H_2O_2 is crucial for the generation of thyroid hormones but is a potential source of reactive oxygen intermediates. These can lead to DNA damage if antioxidant defences are inadequate. Therefore, in this study we examined the effect of H_2O_2 on ARO, WRO and NPAP cells with the aim to investigate changes in the level of expression of selected genes (by real-time PCR) and a different cellular distribution of selected protein targets (by western blotting).

2.40

INHIBITION OF NF-KB ACTIVATION PREVENTS UPREGULATION OF SKP2 AND CKS1B BUT NOT OF THEIR mRNA FOLLOWING STIMULATION OF T-LYMPHOCYTES WITH ANTI TCR/CD3 AND ANTI CD28 IMMOBILIZED ANTIBODIES.

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Stimulation of primary T cells by engagement of the TCR-CD3 complex along with the coreceptor CD28 initiates signal transduction cascades that lead to the activation of NF-AT, NF-kB and AP1 transcription factors required for cytokine production and cell proliferation. This event is associated with the degradation of the cyclin-dependent kinase inhibitor $p27^{kip1}$ by the 26S proteasome following its ubiquitination by the ubiquitin ligase $SCF^{skp2\text{-}cks1}$ [(1)]. SCF complexes are RING type ubiquitin ligases that consist of Cul1, Rbx, Skp1 and a member of the F-box protein family. The RING domain containing protein Rbx1 together with Cul1 form a catalytic core complex that recruits a ubiquitin conjugating enzyme; the variable F-box protein binds the substrate and Skp1 serves as an adapter that links the F-box protein to Cul1. Skp2 and its cofactor Cks1B are the substrate binding subunits of the $SCF^{skp2\text{-}cks1}$ ubiquitin ligase [(2)].

In the aim to differentiate between NF-κB-dependent and NF-κB-independent events occurring during T-lymphocytes activation, the effects of the inhibition of NF-kB pathway on the stability of $p27^{kip1}$ and the expression of Skp2 and Cks1B following activation of resting T-lymphocytes were evaluated.

Resting T -lymphocytes were isolated from buffy coats of healthy donors by density gradient cetrifugation on Lymphoprep followed by immunopurification. Cells were maintained at 37°C in a 5% CO_2 humidified atmosphere in a medium supplemented with 10 mM HEPES, 1mM glutamine, 1 mM sodium pyruvate, 50 UI/ml penicillin, 50 µg/ml streptomycin and amphotericin A T-lymphocytes were activated by incubation in the presence of immobilized anti-CD3 + anti-CD28 antibodies in the absence and in the presence of inhibitors of NF-κB activation. At the end of incubation cells were lysed and mRNA and protein expression was evaluated by qRT-PCR and western immunoblotting, respectively.

Activation of resting T-lymphocytes with immobilized antibodies against CD3 + CD28 caused a significant number of cells entering cell cycle. Following a 48h stimulation, around 23% of cells were S phase. Cell cycle entry was accompanied by decreased levels of $p27^{kip1}$. Analysis of Skp2 and Cks1B expression revealed increased levels of Skp2 and Cks1B both at the mRNA and protein levels. Activation of resting T-lymphocytes in the presence of inhibitors of NF-κB pathway determined a decrease in the number of cells entering cell cycle which was accompanied by a decrease of Skp2 and Cks1 at the protein but not at the mRNA level. In these conditions the down regulation of $p27^{kip1}$ was instead unaltered.

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2.41

BIOLOGICAL EFFECTS OF A NEW CLASS I HDAC SELECTIVE INHIBITOR IN HT29 COLON CANCER CELLS.

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Chromatin structure is mainly regulated by histone post-translational modifications. In particular, histone lysine acetylation is modulated by the opposing activity of two enzyme classes, namely histone deacetylases (HDACs) and histone acetyl-transferases (HATs).

Aberrant levels of HDACs have been reported in most human malignancies, leading to the identification of HDACs as one of the promising targets for cancer treatment. In this regard, many HDAC inhibitors have been developed in the last few years, some of them being in late-phase clinical trials as new antineoplastic drugs. Current studies are aimed to improve inhibitor selectivity on HDAC isoenzymes, resulting in more restricted therapeutic targets and lower cell toxicity.

In this study, MC1855, a new class I HDAC selective inhibitor, has been tested in HT29 human colon cancer cells. MTT assay was performed to determine MC1855 IC_{50}, and the effects on HT29 proliferation were analysed by flow cytometry. MC1855 negatively affects HT29 growth with a 24h IC_{50} in the range 1.2 - 5.7 µM. The treatment with 5 µM MC1855 induced a strong growth arrest characterized by an increase of cells in the G2/M phase of cell cycle.

This antiproliferative effect is mediated by the induction of the $p21^{WAF1}$ gene, analysed by semi-quantitative PCR. In addition 24 hours administration of MC1855 resulted in the induction of the proapoptotic protein Bax, as revealed by confocal microscopy. In addition, histones extracted from HT29 cells treated with this inhibitor were characterized by liquid–chromatography coupled to mass spectrometry[1] in order to determine genome-wide histone post-translational modifications. The observed molecular events were found to be associated to a diffuse histone hyperacetylation status, covering histone classes H2-A, H2-B, H3 and H4.

These data indicate MC1855 as a new potential HDAC inhibitor, its administration causing an interesting antiproliferative effect in colon cancer cells, and the growth arrest seems to be associated to a broad histone hyperacetylation.

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2.42

CYTOTOXICITY EVALUATION OF A POLYAMINE-QUINONE LIBRARY CONJUGATES IN HUMAN COLON CARCINOMA CELLS

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Naphtoquinones and anthraquinones have a wide spectrum of anticancer activity: they covalently bind to and intercalate into DNA, inhibit DNA replication and RNA transcription, act as DNA topoisomerase II poisons, produce oxidative stress, induce DNA breakage and chromosomal aberrations. Moreover quinones, such as emodin and shikonin, are able to modulate receptor tyrosine kinase (RTK) activity, an innovative molecular target for successful mechanism-based cancer therapies[1,2]. RTKs are mediators of cellular proliferation and their mutations are often associated with hyperplasia and tumor development. Targeted inhibition of RTKs has thereby become an attractive therapeutic strategy in the treatment of cancer, and has resulted in several small-molecule RTK inhibitors recently approved for clinical use worldwide. Polyamines have long been associated with cell growth and cancer: their important roles in angiogenesis and invasion have recently been identified, and now polyamine analogues are being developed as anticancer drugs to target polyamine metabolic enzymes and inhibit polyamine biosynthesis. Besides by modulating cell growth, some analogues also play an important role in cell apoptosis by regulating gene expression. Therefore, following the concept that a library built on the basis of selected natural products should yield better hits at a higher rate than classical compound libraries, we planned to design a small library combining aromatic quinone scaffolds with polyamines, varying from diamines to triamines and tetramine, as potential new chemical entities against cancer. We recently reported preliminary biological results showing that such a polyaminoquinone derivative designed following this rationale, FR18, shows cytotoxic activity in a human colon carcinoma cell line by activating apoptosis. A library of 24 derivatives designed by combining two natural products-derived fragments was prepared and tested to determine their anticancer potential in HT29 colon cancer cells. All library members inhibit cell proliferation as measured by MTT mitochondrial functional assay, with IC_{50} values in the 1-100 μM range. We recently reported preliminary biological results showing that such a polyaminoquinone derivative designed following this rationale, FR18, shows cytotoxic activity in a human colon carcinoma cell line by activating apoptosis. This finding further encouraged us to perform SAR studies, synthesizing a focused polyaminoquinone library. Herein, to our knowledge, we report the first parallel synthesis with a polymer assisted purification of a polyamine-quinone conjugates library together with their antiproliferative properties.

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2.43

REGULATION OF HEPATIC LIPID AND CARBOHYDRATE METABOLISM BY THE LIVER X RECEPTOR

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Nuclear receptors are ligand-activated transcription factors that modulate gene expression in response to endocrine and environmental signals. Members of the family that work as heterodimers with the Retinoid X Receptor (RXR) have emerged as sensors of various dietary components, including lipids, fatty acids, retinoids, vitamins, cholesterol, bile acids, and xenobiotics.

The Liver X Receptors (LXR alpha and beta) are nuclear receptors that are activated by oxidized forms of cholesterol (oxysterols). They serve as sensors of intracellular accumulation of pathogenic cholesterol, activating a program of gene expression aimed at removing harmful levels of cholesterol. In addition to their role in regulation of cholesterol homeostasis, recent studies have demonstrated that the LXRs also coordinate carbohydrate and lipid metabolism in the liver and that they modulate expression of key genes in glucose metabolism. The liver plays a central role in glucose homeostasis, as it has the distinct ability to produce and consume glucose. Upon feeding, glucose influx triggers gene expression changes in hepatocytes to suppress endogenous glucose production and convert excess glucose into glycogen, or fatty acids to be stored in adipose tissue. This process is controlled by insulin. In addition to stimulating pancreatic insulin release, glucose also regulates the activity of Carbohydrate Responsive Element Binding Protein (ChREBP), a transcription factor that modulates lipogenesis.

We recently described another mechanism whereby glucose determines its fate: we showed that glucose binds and stimulates the transcriptional activity of the Liver X Receptors. D-glucose binds and activates both LXRs at physiological concentrations expected in the liver, inducing expression of LXR target genes with similar efficacy to oxysterols. Cholesterol homeostasis genes that require LXR for expression are upregulated in liver and intestine of fasted mice re-fed with a glucose diet, suggesting that glucose is an endogenous LXR ligand. Our results suggest that LXR acts as a transcriptional switch that integrates hepatic glucose metabolism and fatty acid synthesis: excess glucose is sensed by the same transcription factor responsible for control of fatty acid synthesis.

2.44

CATHEPSIN S AND D EXPRESSION AS A BIOCHEMICAL CHECKPOINT FOR THE GENERATION OF TOLEROGENIC DENDRITIC CELLS FROM HSC-CD34$^+$

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Thrombopoietin (TPO) and IL-16, combined with a conventional cocktail composed of Flt3L, GM-CSF, IL-4 and TNF-alpha, direct the differentiation of human CD34$^+$hematopoietic stem cells toward the development of tolerogenic dendritic cells. However, the molecular mechanisms by which dendritic cells are shifted away from an immune stimulatory phenotype are largely undefined.

Here, we report that TPO+IL-16-mediated signaling leads to STAT3 phosphorylation and drives specific down-regulation of cathepsins S and D, two members of the pool of proteases involved in antigen presentation. Expression of these enzymes is finely time-controlled at both transcriptional and post-transcriptional levels, and apparently linked to a concerted modulation of the endogenous cathepsin inhibitor cystatin C. Notably, CD34$^+$cells induced to differentiate toward the alloreactive pathway by the cytokine cocktail lacking TPO+IL-16 switched the course of dendritic cell differentiation from immunity into tolerance when either cathepsin gene was silenced via RNA interference. Knockdown cells displayed markedly reduced levels of MHC-II, decreased efficacy in antigen uptake and impairment of stimulatory activity toward autologous CD4$^+$T cells comparable to those resulting from TPO+IL-16 treatment.

Thus, cathepsins S and D enforce a biochemical checkpoint within the immunosuppressive pathway elicited by TPO+IL-16, suggesting that either enzyme may be targeted for cell manipulation aimed at immunotherapy.

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2.45

PROTEOMIC PROFILING OF VESICLES RELEASED BY 8701-BC CELLS.

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Tumor cells were shown to release, *both in vitro and in vivo*, "vesicles" that vehicle several proteins involved in cell-cell and cell-matrix interactions. An interesting challenge is the characterization of the protein content of such vesicles that may increase the understanding of their potential roles *in vivo*.

8701-BC, a continuous line of breast carcinoma cells, was shown to release "membrane vesicles" with a diameter ranging from 100 to 1000 nm and playing a role in tumor progression mechanisms. On the other hand, production of "exosomes", smaller vesicles known to be involved in immune response activation, had not been revealed.

The first goal of this study was to separate different vesicle populations from 8701-BC cell conditioned medium. To this aim, after two low speed centrifugations performed to remove cells and cell debris, the medium was differentially centrifuged. Western analysis, carried out using specific antibodies, revealed that the 15,000xg pelletted fraction contains β1-integrin, a protein which had been shown to be clustered in membrane vesicles shed by 8701-BC cells[1], but not Hsc70, a protein found in exosomes[2, 3]. On the contrary, Hsc70 is detectable while β1-integrin is not present in the fraction obtained by a further centrifugation at 100,000xg of 15,000xg supernatant. Moreover, the absence of Cytochrom C in both fractions excludes the contamination with apoptotic vesicles. These results suggested that 8701-BC cells release both membrane vesicles and exosomes and that their separation can be achieved by differential centrifugation.

Then, to analyze the whole protein content of the vesicle preparations, a proteomic approach was chosen. Protein 2D-PAGE analysis of the different fractions was performed and the resulting gel images were analyzed *in silico*, using ImageMaster 2D Platinum software.

The preliminary comparative proteomic analysis revealed a set of protein spots differently abundant in the vesicle fractions. These data strongly encourage for further investigation using 2D-PAGE coupled with MS-MALDI-TOF analysis which could help to elucidate physiological roles of the two different kinds of vesicles.

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2.46

EFFECT OF AN INDOLE-DERIVATIVE IN HUMAN OVARIAN CARCINOMA CELLS.

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Ovarian cancer remains one of the most difficult gynaecologic cancers to treat, owing to its aggressive biology and high relapse rate, as well as the toxic side effects of available chemotherapeutic agents used against this recurrent disease. Therefore, it is important to develop new antiproliferative molecules with fewer side effects and superior pharmacological properties.

Among anticancer agents, those that target microtubules constitute one of the most effective classes of chemotherapeutics employed for survival prolongation in the advanced state of the disease. Most of them, such as *Vinca* alkaloid (Vincristine, Vinblastine) or the semi-synthetic ones (Vinolrebine), contain in their structure an indolic nucleus, responsible for the therapeutic activity. These drugs are used extensively for the treatment of a wide variety of human cancers, including ovarian, prostate, NSCLC, and breast cancers.

In a previous work we investigated the antitumor activity of a new class of indole-derivatives towards 60 different human cancer cell lines[1]. In particular we noted that compound **3l**, (3*E*)-3-{[2-chloro-1-(4-chlorophenyl)-5-methoxy-6-methyl-1*H* -indol-3-yl]methylene}-5-hydroxy-6-methyl-1,3-dihydro-2*H* -indol-2-one, was able to induce a strong and irreversible cytostatic effect without showing any cytotoxicity in human ovarian carcinoma cells, IGROV-1 [1].

The aim of this work is to characterize the biochemical pathway by which the new indole-derivative produces growth inhibition in IGROV-1 cells.

Firstly to assess whether **3l** interferes with the cell cycle progression we performed flow cytometric studies: treated cells showed a marked increase in the G0/G1 peak with respect to controls (89% vs 66%).

In order to identify the molecular target of the compound we followed its cellular internalization, by monitoring the concentration in the IGROV-1 nuclear fractions for 48 hrs. A method based on LC-ESI-MS was developed for the quantitative determination of the molecule under test in the cellular samples. The analysis revealed that drug localizes in the nucleus starting from 6 hrs after treatment , where it remains for 48 hrs.

These preliminary results indicate a correlation between the cell cycle arrest and accumulation of **3l** in the nuclear compartment for 48 hrs. Further studies are in progress to investigate whether the compound brings about posttranslational modifications, such as phosphorylation and acetylation, of nuclear protein involved in the control of the cell cycle.

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2.47

HISTONE POST-TRANSLATIONAL MODIFICATIONS BY HPLC-ESI-MS AFTER HT29 CELL TREATMENT WITH HDACS INHIBITORS.

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Human malignancies of all kinds show alterations in the epigenetic features, indicating that all cancer patients could potentially benefit from epigenetic therapy. In particular, an high activity of histone deacetylases (HDACs) causes epigenetic alterations associated with a genome-wide histone hypoacetylation. Consequently, HDAC non selective inhibitors have entered late-phase clinical trials as new antineoplastic drugs. However they can cause contradictory effects by inhibiting different isoforms at the same time. Selective HDAC inhibitors could be in principle able to avoid these situations and also result less toxic.

The goal of the present work has been the study of a possible correlation between the degree of histone post-translational modification and the effects caused by treatment with HDAC I selective (MS275, MC1855), Class II HDAC selective (MC 1568)[1] and non selective (SAHA) HDACs inhibitors. This correlation along with the whole analysis of the histones isoforms, could afford a mean to better understand the mechanisms of action of new, more potent, and selective HDAC inhibitors directly on the cells.

Following our previous studies[2], liquid – chromatography coupled to mass spectrometry was applied to characterize histones and their post-translational modifications in HT29 cells after time and concentration dependent treatment with HDAC inhibitors.

The results were correlated to the power of HDAC inhibition, the effects on the cell cycle, and protein expression. The most important results were that MC 1568 produced only a modest H4 hyperacetylation even at high doses (50 μM), was ineffective on the other histones, and resulted non cytotoxic. In order to have cytotoxic effects therefore HDACs inhibitors must inhibit HDAC class I. In fact we observed that, besides H4, SAHA (non selective) and MC1855 (class I HDAC selective) induced a generalized histone hyperacetylation when administered at 50 μM, and were found to be cytotoxic. Levels of intermediate inhibition of H4, H3, H2A, and H2B as those obtained after treatment with MS275 produced only less marked effects on the cell cycle by inducing a G0/G1 arrest. It can be concluded that the sole H4 hyperacetylation does not cause cell death.

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2.48

ANTI-APOPTOTIC EFFECT OF ROS PRODUCED BY NAD(P)H OXIDASE IN HUMAN LEUKAEMIA CELLS

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A growing body of evidence indicates that growth factors stimulate ROS production in a variety of cell types through receptor-transducing pathways, although the detailed mechanism is poorly understood [1]. Growth factor-induced ROS production seems to be necessary for optimal propagation of mitogenic signals in neoplastic proliferation and for glucose transport activation. Malignant cells are known to have accelerated metabolism, high glucose requirement and thus increased glucose uptake rate.

In this study we show that in the human acute myeloid leukaemia (AML) cell line M07e the growth factor IL-3 induced ROS formation and stimulated the synthesis of glucose transporter 1 and of the cytosolic NAD(P)H oxidase subunit p47phox. In fact, both intracellular ROS content and glucose transport activity were decreased by inhibitors of plasma membrane ROS sources such as diphenylene iodonium and apocynin. Among the catalytic subunits implicated in NAD(P)H oxidase activity, M07e cells express the phagocytic gp91phox protein (Nox2). The inhibition of ROS generation with NAD(P)H oxidase inhibitors stimulated apoptosis in leukaemia cells as confirmed by typical internucleosomal DNA fragmentation and effector caspase activation. Similar effects on ROS content, glucose transport modulation and apoptosis induction were also shown by the incubation with receptor tyrosine kinase inhibitors, such as anti-leukaemic drugs blocking the stem cell factor receptor c-kit. In fact, IL-3 seems to transmodulate c-kit phosphorylation. The receptor tyrosine kinase c-kit is essential for the development of normal hematopoietic cells and has been proposed to play a functional role in acute myeloid leukemia. Binding of the c-kit ligand SCF initiates a signal transduction cascade including receptor autophosphorylation and subsequent phosphorylation of intracellular substrates. Therefore, we could speculate that a receptor transmodulation or receptor trans-phosphorylation occurs in the context of leukaemia cell growth responses to individual cytokines. Otherwise, an autocrine SCF production could occur, as shown in most AML cells.

The results suggest that ROS production induced by IL-3 via NAD(P)H oxidase protects leukaemia cells from apoptosis. Opposing to this mechanism may play an important role in acute myeloid leukaemia treatment, thus representing a novel therapeutic strategy.

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2.49

GENOMIC APPROACHES TO THE DISCOVERY OF MOLECULAR TARGETS OF LOW MOLECULAR WEIGHT PHOSPHOTYROSIN PROTEIN PHOSPHATASE (LMW-PTP) IN TUMORIGENESIS.

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Protein tyrosine phosphorylation plays a key role in the generation of signals necessary for different cellular events such as growth, migration and invasion of normal and malignant cells. Recent studies have assessed the role of low molecular weight protein tyrosine phosphatase (LMW-PTP) in cell transformation and tumour onset and progression, observing a significant increase in LMW-PTP mRNA and protein expression in different human tumors and in particular in breast and colon cancer[1,2 and 3]. Moreover, its enhanced expression is generally prognostic of a more aggressive cancer. Negative modulation of LMW-PTP expression leads to profound changes in cell motility, possibly due also to LMW-PTP-dependent Src Tyr-phosphorylation. LMW-PTP modulation seems also to influence apoptosis. Finally, we believe that the analysis of the pattern of expression of a very large repertoire of genes will contribute to clarify the role of LMW-PTP in tumorigenesis. Our aim is to evaluate how LMW-PTP could play a role in modulating cell transformation, trying to understand the cause-effect relationship between expression level of LMW-PTP and tumorigenesis, using whole genome expression approach, such as microarray techniques, in combination with ectopic LMW-PTP modulation. Moreover, we are also analyzing the phenotypic changes in cell adhesion and motility induced by LMW-PTP modulation. Whole genome expression analysis are currently performed with microarray techniques in MCF10A cells where expression of LMW-PTP has been modulated by either transient ectopic expression or with RNAi-induced silencing, to increase or decrease LMW-PTP expression in the cell. Results must be yet statistically analyzed and evaluations will be available soon. In the same experimental conditions it was possible to observe that LMW-PTP silencing leads to a strong increase in cell motility, observed already after 12 hours, using a on-plate wound-ealing assay. A crucial parameter such as phosphorylation of the activatorial Tyr416 of Src protein was also evaluated: Src specific phosphorylation shows a 2-fold increase, possibly due to a direct action of LMW-PTP. Moreover, MMC2 metallo-protease expression is markedly induced (about 5-fold) by LMW-PTP negative modulation. Evidences suggests that in these conditions there is also a partial protection from anoikis induced by detachment of the cells from extra-cellular matrix.

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2.50

ENZYMATIC Methylation OF DEAMIDATED PROTEINS PREVENTS THE apoptosis INDUCED BY OXIDATIVE STRESS OF endothelial cells

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BACKGROUND AND AIM. Proteins undergo *in vivo* spontaneous post-biosynthetic deamidation/isomerization of asparagines, generating L-isoaspartyl residues. Such alterations slowly occur during aging processes and are dramatically increased by physical-chemical stresses brought to living cells. Deamidated-isomerized proteins are often structurally altered and functionally impaired. The isospartyl protein carboxyl-*O*-methyltransferase (PCMT; EC 2.1.1.77) initiates the repair of the isopeptide bond at the isoaspartyl site. In line with the role of PCMT in the maintenance of protein structural stability upon cell stress, preliminary results speak in favor of the involvement of this enzyme in the apoptosis processes.
Aim of this project is to shed light on the role of PCMT in apoptosis and to clarify the relevant mechanism(s) involved.

METHODS. Endothelial cells from porcine aorta (PAE) were transfected with pcDNA3.1 expression plasmid, carrying human wild type *PCMT* or its antisense or various mutants. Cells were then subject to oxidative stress induced by H_2O_2 concentrations (0.1 - 0.5 mM range). Apoptosis was monitored by: A) DNA fragmentation assay; B) Western blotting, using anti-PARP and anti-caspase3 antibodies; C) FACS analysis (propidium iodide staining). PCMT substrates were purified through proteomics, using human recombinant PCMT as a byte coupled to SulfoSbed, followed by mass spectrometry.

RESULTS. 1) PAE cells overexpressing the*"wild type"* human PCMT are resistant to apoptosis. 2) This is specifically due to the methyltransferase activity, since negative dominant mutants are devoid of any antiapoptotic effect. 3) Transfection with antisense *PCMT* induces high sensitivity to apoptosis even at low H_2O_2 concentrations. 4) Cells transfected with either antisense *PCMT* or mutants accumulate unrepaired highly deamidated-isomerized proteins, upon oxidative stress. 5) The mechanism for apoptosis is mediated by activation of the caspase3/PARP common pathway. 6) Proteomics allowed the identification of Hsp70, Hsp90 and Bcl-xL as PCMT substrates involved in apoptosis. In particular Bcl-xL is an antiapoptotic protein which has been previously described to undergo deamidation *in vivo.*

CONCLUSIONS. PCMT is involved in the regulation of cell response to proapoptotic stimuli such as oxidative stress. The mechanism of this protection involves methyl esterification of specific isoaspartyl residues derived from asparaginyl deamidation. We propose that PCMT repairs the deamidated forms of key anti-apoptotic proteins thus preserving their structural stability and biological function.

2.51

QUALITATIVE AND QUANTITATIVE ANALYSIS of carboxylic acids of CHEMICAL-CLINICAL INTEREST THROUGH THE USE OF coupled Techniques as HPLC-UV, HPLC-FID e HPLC-ESI-MS/MS.

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Neuroblastoma is the most common extra cranial solid cancer in childhood. The difficulty with neuroblastoma lies in early diagnosis. Unfortunately, it's normally diagnosed when the disease had metastasized to other parts of the body (bones, lymph nodes, skin and liver). If the neuroblastoma is found in time, treatment is often very successful. The prognosis is not so good in the later stages, however. In about 90% of neuroblastomas, elevated levels of catecholamines or their metabolites are found in the urine or blood.

Vanilmandelic acid (VMA, 4-hydroxy-3-methoxymandelic) and homovanillic acid (4-hydroxy-3-methoxy- phenylacetic, HVA) are the main acidic catabolites of norepinephrine and dopamine, respectively, and are excreted in urine. The concentrations of these metabolites increase significantly in neurological disorders so that HVA and VMA are exploited as biological markers of neuroblastoma.

The main goal of the present work was to develop a method for the rapid quantitative analysis of VMA, HVA in urine samples using multi-detection techniques as HPLC–ESI- MS/MS, HPLC-UV and HPLC-FLD.

The high selectivity and sensitivity of tandem MS and fluorescence make these techniques suitable for determination of low concentrations of metabolites in complex matrices, such as urine. The developed methods use mandelic acid as internal standard (IS), both for the evaluation of recovery and assessment of ionization efficiency towards these metabolites. In parallel a qualitative analysis of VMA was also pursued. In fact, it is very interesting to observe that in the catabolic pathways of L-noradrenaline and L-adrenaline, the stereocenter configuration of some metabolites is not specified, particularly for VMA. For this reason, an attempt to identify if this molecule is present as unique enantiomer, racemic mixture or enantiomeric excess "in vivo" in the human organism was also made. To resolve the VMA standard racemic mixture the indirect resolution strategy was used. This method is based upon diastereomers formation by reaction with a chiral tagging reagent. This way their HPLC separation using an achiral stationary phase is possible, exploiting the different physicochemical properties of the diastereomers.

2.52

OSTEOBLAST RESPONSE TO MECHANICAL STRESSES IN VITRO IN THE PRESENCE OF ESTRADIOL

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Bone remodelling is the result of different factors, such as piezoelectric responses, prostaglandin production, intra- and extra-cellular biochemical factors stimulated by natural or applied mechanical forces, whose efficiency is a very important topic in orthodontics. It is well known that tooth movement is due to both strain and compression forces, whose dimension and duration are crucial to obtain good and quick clinical results. Sexual hormones are also active on the bone cycle: estrogen deficiency, for instance, leads to increased bone resorption due to inhibition of osteoclast apoptosis and to decreased bone production[1].

In this study, we attempted to evaluate the effect of strain forces on osteoblast-like SaOS-2 cell line in the presence or absence of estradiol, to evaluate the action of these two different variables taken together.

We subjected SaOS-2 cells to mechanical strains caused by two types of continuous forces (1: slow, consisting in 2 hours of stress and 2 hours of rest, 2: very slow, consisting in 24 hours of stress followed by 24 hours of rest) or by interrupted forces (consisting in 20 seconds of stress, 20 seconds of rest for 1 hour) in the presence and in the absence of estradiol. The method to stress the cells is a modification of that of Hasegawa[2]. Cells were seeded in six well plates, in transwells which are provided with transparent, permeable and reversibly deformable bottom. To stress the cells, a template with a convex surface was placed under the transwells. ALP activity was assayed as a marker of osteoblast differentiation by colorimetric method. In the estradiol absence, ALP activity decreased following stress though not significantly, demonstrating a weak stop signal in cell differentiation. The activity didn't change after the rest period in type 1 stress, while it showed a significant increase of 52% in type 2 stress, thus suggesting that long stress is a stimulus to osteoblast differentiation.

Incubation of cells in the presence of estradiol caused a 45% increase of ALP activity with respect to control cells; such increase, even if in different amount, is always present in all the experimental conditions. ALP activity increased significantly in interrupted stress after the rest (58% with respect to non stressed cells). No significant variations are shown after both types of continuous stresses. Continuous long time stress seems thus to be the optimal condition for bone remodelling, nevertheless interrupted stress applied to osteoblasts in the presence of estradiol, caused the highest ALP activation.

This different behaviour shown in the presence or absence of hormones suggests dentists that this factor must be taken into account when using orthodontic appliance in subjects with different hormonal conditions.

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2.53

microRNA EXPRESSION DURING THE INDUCTION OF CELLULAR SENESCENCE

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Cellular senescence is a special form of permanent growth arrest that is considered a tumor suppressor mechanism and an alternative to apoptosis, as well as a possible contributor to tissue aging. Various stimuli, including dysfunctional telomers, sublethal oxidative stress, DNA damage and activation of certain oncogenes, trigger cellular senescence that is usually controlled by p53 and p16-retinoblastoma (p16-Rb) tumor suppressor pathways.

Accumulation of senescent cells has been viewed for a long time as potential cause of aging and recent evidences support the hypothesis that this phenomenon may contribute to aging not only by accumulation *in vivo* of senescent cells in different tissues, but also by reducing the regenerative potential of the tissues, as a consequence of the stem cell senescence. The cellular senescence is characterized not only by the appearance of several markers, but also by modifications of gene expression pattern.

We previously reported that young human embryo fibroblasts (IMR90) exposed for few days to low doses of the GSH-depleting agent DEM, acquire the senescent phenotype[1].

A recently identified class of short (20-23 nucleotides) non-coding RNAs, that act as "regulators" at post-transcriptional level, are the microRNAs (miRNAs). miRNAs suppress gene expression via imperfect base pairing to the 3' untranslated region of target mRNAs, leading to repression of protein production or, in some cases, to mRNA degradation. Since recent studies indicate that miRNAs affect a broad spectrum of biological activities including development, proliferation, apoptosis and cancer, we have investigated whether the miRNAs have a role in the induction of cellular senescence provoked by mild and chronic oxidative stress. We performed a microarray analysis for 380 known human miRNAs, by using as probes RNAs from normal and DEM-treated young IMR90. Preliminary results show that 4 miRNAs are up-regulated in IMR90 exposed to DEM; validation of these results by real time PCR will be presented.

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3.1

INCREASED ALANINE CONCENTRATION AS METABOLIC BIOMARKER OF EXPOSURE TO ORGANOPHOSPHATES IN INVERTEBRATES.

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Biochemical biomarkers are of particular value as they represent sublethal changes resulting from individual exposure to pesticides. Organophosphorous insecticides (OPs) bind covalently to the active site of acetylcholinesterase (AChE) and measurement of AChE activity represents an obvious biochemical biomarker to detect OP exposure. On the other hand, exposure to insecticides is expected to produce a series of biochemical changes, including not only decreased level of target enzymes, but also an increased level of enzymes involved in the biotransformation pathway and a disturbance of the normal physiology and energetic metabolism of the animal[1]. To our knowledge no studies have been performed on the role of metabolic intermediates and/or end-products as biochemical biomarkers.

In this study we measured the metabolites concentration in freshwater macroinvertebrates exposed to OPs under laboratory conditions. Then we examined these metabolites in natural communities exposed to OPs and other pesticides. Laboratory studies were performed on midge larvae (*Chironomus riparius*), a well known sentinel organism for freshwater ecosystems. During a survey of metabolite concentrations in *C. riparius* larvae exposed to insecticides we found unusual high concentration of alanine in animals treated with fenitrothion (FT) which increased up to 7-fold the basal level. This phenomenon was accompanied by decreased trehalose, pyruvate and glutamate and increase of transaminase activity. These effects were not detected when animal were exposed to carbamate insecticides, suggesting that metabolic response elicited by FT could by related to oxidative stress induced by this compound as documented in literature.

Metabolite analysis was then applied to a field research which included biochemical analysis of several freshwater macroinvertebrates collected from Meolo River, a site exposed to agricultural pollution (in particular OPs, herbicides and fungicides), and Upper Livenza River, a low pollution reference site. Animals collected from Meolo River displayed lower AChE activities and higher alanine concentration compared to Livenza and a metabolic pattern compatible with exposure to OPs similar to that described from laboratory studies. Alanine determination is confirmed as a valuable biochemical biomarker to OPs exposure.

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3.2

THE EFFECTS OF DIFFERENT ANAESTHETICS ON PLASMA SEROTONIN IN *DICENTRARCHUS LABRAX*

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Serotonin (5-HT) has not been detected in all trombocytes of fishes but in teleost it has been shown to regulate the hypothalamic-pituitary-interrenal activity and the secretion of plasma cortisol in response to different stressors[1]. European sea bass *Dicentrarchus labrax* is subjected to different rearing conditions, that may affect production, product quality, but mainly fish physiological functions and welfare. In order to investigate the effects of different anaesthetics in reducing the stress related to handling and in affecting fish metabolism, plasma 5-HT with tryptophan (Try) and cortisol levels were measured in *D. labrax*, without anaesthesia and with anaesthesia using tricaine methanesulphonate (MS-222), 2-phenoxyethanol (2-PE) and cloves oil. Fish (n. 5, mean weight ± D.S. 82 ± 15 g) were caught from off-shore cages and transferred to a tank of water (150 l). Sampling was done without anaesthetic (Group I, control) after 31 days acclimation in aquarium to 21,0 °C, pH 7,35. Fish (n. 15, mean weight ± D.S. 61 ± 11 g), reared in aquarium, were anaesthetized before sampling with MS 222 (50 mg/l) (Group II), 2-PE (0,4 ml/l) (Group III), cloves oil (0,04 ml/l) (Group IV). All fishes were exposed to anaesthetic for a medium time of 7-8 min. Blood, in heparin, was taken from caudal vessel. PCV, plasma glucose, TPP, TG, TCho (spectrophotometer) were analysed. Cortisol levels were determined in duplicate by EIA kit (Radim). Plasma samples were added to standard N-Methylserotonin and protein precipitation reagent, vortex-mixed, incubated at 4°C and centrifugated. The surnatant was analysed by HPLC and electrochemical detector. In Group I plasma 5-HT leves are very low, comparable to those reported by Rubio[2]. Blood variables are similar in Group II,III,IV; Glucose, PCV, TPP levels are comparable to control, suggesting that the anaesthetics did not affect the biochemical status of fishes, while TG and TCho concentrations are significatively lower owing to maintenance diet. Increased cortisol levels owing to all anaesthetics are directly related to time exposure. As in other species subjected to handling[3] cloves oil is less effective than MS-222 in reducing cortisol responses. 5-HT and Try values show similar trend. Plasma 5-HT levels are strongly increasing only in Group II. Try levels in Group III and IV are comparable to control values while in Group II are significatively higher vs. Group I and IV ($P<0,01$) and Group III ($P<0,05$). The results obtained support the hypothesis that the anaesthetic MS 222 probably interact with brain serotoninergic system, thus inducing an increase of plasma 5-HT and Try levels.

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3.3

ANALYSIS OF THE CATHELICIDIN PROTEIN FAMILY IN SALMONIDS

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The cathelicidin family of host defence peptides is regarded as an important component of the host innate immune system. Its members are synthesized as precursors with a characteristic N-terminal cathelin-like domain, well conserved also in evolutionary distant vertebrates, and with cationic C-terminal regions, carrying the active antimicrobial peptide, with considerable sequence diversity. Cathelicidin-related proteins were found in mammals, birds, primitive vertebrate Atlantic hagfish and most recently also in ray-finned fish such as rainbow trout and atlantic salmon[1,2].

By using genomic PCR amplifications, RT-PCR tissue analyses, and peptide chemical synthesis, we have here investigated and characterized the cathelicidin family in different salmonid ans osmerid species such as brown trout *(Salmo trutta fario),* brook trout *(Salvelinus fontinalis)*, grayling *(Thymallus thymallus)* and rainbow smelt (*Osmerus mordax*).

With few exceptions, two different genes, CATH-1 and CATH-2, were found in each species, with almost identical cathelin-like domains and largely varied cationic C-terminal domains. Unique hallmarks were found in all these peptides: the presence of a high number of Gly and Ser residues, which collocate them in a novel distinctive group of antimicrobial peptides, and the presence of a six-amino-acid repeated sequence, detected in a variable copy number among the different cathelicidins, which reflects the existence of a genetically unstable region similarly to that found in some mammalian cathelicidin genes. RT-PCR assays indicate that salmonid cathelicidins are expressed in a wide range of tissues in healthy fish, including head and trunk kidney, spleen, gills, skin, stomach and brain. Some of the C-terminal peptides have been chemically synthesized and used to investigate their spectrum of antibacterial activity against a number of bacterial fish pathogens.

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3.4

STRUCTURAL CHARACTERIZATION OF SILICATEIN FROM THE MARINE SPONGE *PETROSIA FICIFORMIS*

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Silicateins, members of the cathepsin L family, are enzymes that have been shown to be involved in the biosynthesis/condensation of biosilica in spicules from Demospongiae (phylum Porifera). Axial filaments from *P. ficiformis* spicules have been extensively analyzed by mass spectrometry. Preliminary MALDI-MS experiments on the intact protein samples showed a complex group of signals in the 22.9-24.2 KDa range, clearly indicating an high degree of heterogeneity in the silicateins family. Further bottom-up analysis with tandem mass sequencing were thus performed on both the whole sample and on the 1D electrophoresis bands. Peptides deriving from proteolytic digestions where separated by reversed-phase chromatography and analyzed by Nanospray-MS/MS. The previously published sequence of silicatein beta deduced from *P. ficiformis* cDNA has been completely confirmed except for a single point mutation (a valine instead of a leucine). Functional silicatein sequence begins with H_2N-LPETVD, but strong evidences of a significant degree of methylation of the N-terminus were collected in tandem mass sequencing experiments. Extensive use of manual interpretation of tandem mass data of smaller chimotryptic peptides proved to be a crucial breakthrough in silicatein analysis. Several post translational modifications were detected in our experiments. Three phosphorylation sites Tyr 97, Ser 213 and Ser 66 were found. Experiments have been planned in order to assess the correct grade of phosphorylation. Preliminary results show that phosphorylated/non phosphorylated ratio roughly ranges from 5 (S213) to 20% (Y97) . It should nevertheless be noted that the reported phosporylations are those surviving the HF extraction procedure and might not correctly represent the correct amount of phosphorylation of silicatein in its natural conditions inside spicules. Other relevant modifications were an extensively oxidized histidine at H15 and the modified thyonyl group of Cys 157 to cisteic acid. The elucidation of details on silicatein structure and its post-translational modifications are essential to better understand the mechanism of biosilica precipitation and its nanoscale organisation and it will allow a future employ of these peculiar proteins as biotechnological devices for the realisation of shapes and dimensions controlled silica particles suitable for microelectronics and nanotechnology.

3.5

FUNCTIONAL METAPROTEOME ANALYSIS OF MICROBIAL POPULATIONS IN ENRICHMENT CULTURES ON POLYCYCLIC AROMATIC HYDROCARBONS

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A better understanding of microbial ecology would lead to clarify the essential processes mediated by microrganisms such as bioremediation of polluted sites. A variety of tools, such as nucleic acid-based methods, have been used in the past to gain insights into genetic structure and diversity of environmental microbial populations (metagenome). However, knowledge of genetic structure of microbial community does not help to acquire useful information on functional pattern such as metabolic capacity, population dynamics, and physiological responses to changeable environmental conditions. To overcome this limit, the large-scale study of whole community expressed proteins (metaproteome) has the potential to yield insights into the functional role of the microbial components in ecosystems.

In this study we performed a parallel analysis of the metagenome and the metaproteome of enrichment cultures established using an historically contaminated soil as inoculum and naphthalene or phenantrene as sole carbon source. Denaturing gradient gel electrophoresis (DGGE) fingerprinting of 16S rRNA genes indicated that enrichment cultures presented a lower microbial biodiversity than the soil used as inoculum but, surprisingly, the bacterial communities in the polycyclic aromatic hydrocarbons (PAH)-amended cultures were highly similar to the control cultures. On the contrary, Real-Time PCR quantification of PAH catabolic genes, namely the upper-pathway naphthalene dioxygenase gene (nahAc) and the lower-pathway catechol 2,3-dioxygenase gene (C23O), indicated that strains harboring PAH catabolic genes, and thus potentially capable of PAH degradation, were enriched in the cultures treated with PAHs, and in particular with naphthalene. Entire proteome of cultured bacterial communities was separated by two-dimensional gel electrophoresis using the 3-10 pH range. Spot analysis, performed using specific software, highlighted quantitative and qualitative differences in the protein expression pattern of PAH-treated cultures with respect to the control. PAH treatment induced a strong increase of the total number of expressed proteins, 505 and 452 spots in naphthalene and phenantrene cultures, respectively, compared to 361 spots in control cultures. Interestingly, naphthalene and phenantrene treated cultures shared 88 treatment-specifc proteins. Moreover, the number of spots that showed no significant quantitative differences were higher in phenantrene respected to naphthalene cultures, confirming Real-Time PCR results. In conclusion we can emphasize as metagenome and metaproteome characterization of complex microbial communities can provide useful information to link their genetic structures with their functions in the environment.

3.6

MURINE PULMONARY INFLAMMATORY RESPONSE FOLLOWING INSTILLATION OF SIZE-FRACTIONATED TYRE PARTICLES (TP)

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Epidemiological studies have demonstrated associations between acute exposure to elevated levels of particulate matter (PM) and increased risk of cardiopulmonary disease. PM is composed of an inert carbonaceous core covered by layers of adsorbed pollutant molecules. Attention has been focused on one component of the respirable fraction, the tyre derived particles (TP which represent 8-10% of PM10 and 2.5 % of PM2.5). In a recent *in-vitro* study we demonstrated that organic extract of TP causes disassembling of the alveolar plasma membrane at discrete points and causes deregulation of caveolae/rafts expression (Beretta et al, 2007), while the lung inflammatory response evoked by TP *in-vivo,* is still unknown.

Therefore, we evaluated the pro-inflammatory potential effects induced by size-fractionated TP (10 μm) intratracheally instilled. TP morphology and characterization were performed by transmission electron microscopy. Male BALB/c mice (7-8 week-old) were intratracheally instilled with microsprayer syringe (Penn Century) with 10 (TP1), 100 (TP2), 200 μg (TP3) of TD10 in 100 μl of saline 0.01% Tween 20, or 100 μl of saline 0.01% Tween 20 (Control). After 24 h, 10 mice from each group were euthanized, the trachea was cannulated and the lungs were lavaged 3 times with 0.6 ml of saline. The resulting lavage (BAL) was centrifuged 1500 g, 15 min, 4 °C. Total cells were counted using Trypan blue; cell aliquots were smeared on slides using Cytospin and stained with Diff-Quik for differential count. The supernatant was assayed for total proteins, LDH, MIP2, TNFα β-Glucoronidase, Alkaline Phosphatase, SOD and total GSH.

TP present the typical shape of tyre debris: the 10μm TP are mainly distributed under the dimension of 10μm (more than 90%).Biochemical analyses, cell number and cell type of BAL didn't show significant differences in comparison with control. Conversely, TP2 dose induced significant increase in the number of total cells and in particular in polymorphonuclear (PMNs) percentage (70% compared to 29% in control). Biochemical analysis revealed an increase in alkaline phosphatase activity (correlated to increase of PMNs and marker of alveolar cells type II damage)and MIP2 level, but no change in LDH, β-Glucoronidase, SOD, GSH and TNFα. The highest dose TP3 produced a marked increase in PMNs percentage (up to 80 %), β-glucoronidase and LDH. Among all the tested parameters, the most sensitive end-point appeared to be the PMNs number in the BAL, which was significantly elevated in a dose-dependent way. The other parameters monitored, such as anti-oxidant enzymes and proinflammatory cytokines appeared to be less indicator systems, almost at the end time analyzed

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4.1

NUTRITIONAL MODULATION OF IL-23 RECEPTOR AS POTENTIAL REGULATOR OF BOWEL INFLAMMATION

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Recent immunologic advances have established that T cells and inflammatory cytokines play a pivotal role in inflammatory bowel disease (IBD)[1]. Particularly, Crohn's disease is associated with high levels of interleukin-23 (IL-23) secretion[2], mucosal interleukin-17 (IL-17) expression and serum IL-17[3].

IL-23 is a newly discovered member of the interleukin-12-related cytokine family. IL-23 is primarily involved in the differentiation of pathogenic T cells, which are characterized by the production of the pro-inflammatory IL-17.

Controlling the expression/production of IL-23 and IL-17 is an approach that would allow the development of novel therapeutic and nutritional tools in human IBDs. In fact, diverse micronutrients are believed to exert their effects through modulation of the expression of genes related to inflammation.

In humans, the functional IL-23 receptor complex is predominantly expressed on activated/memory T cells, T-cell clones, and natural killer cell lines[4].

In this study we established whether Kit 225 cells might be a suitable model system for investigating nutritional modulation of IL-23 pathway. Kit 225 are interleukin-2-dependent human T cells derived from chronic lymphocytic leukemia. The expression of IL-23 receptor in this cells was evaluated with quantitative real time PCR and Caco-2 cell line was used as comparison. Once established IL-23 receptor complex expression in our model system, we evaluated its effect on the expression and release of some cytokines, in the presence or absence of IL-23 stimuli.

Future experiments will elucidate whether specific micronutrients can modulate IL-23 receptor activity, therefore reducing IBDs incidence or symptoms. This nutrigenomic approach appear essential for the developing of immunonutrition as optimal new therapy.

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4.2

PRO-APOPTOTIC EFFECTS OF *ORIGANUM VULGARE* IN HUMAN COLON CANCER CACO2 CELLS

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Oregano spice is widely used in Mediterranean diet, which is associated with a low risk for colon cancer. Although the medicinal benefits of oregano, such as the antioxidant, anti-inflammatory and antimicrobial activities, are well known, nonetheless only few data are available on its effect in cancer prevention, especially concerning the mechanisms of action. Here, we investigated the effects of *Origanum vulgare* ethanolic extract on cell proliferation, redox balance and apoptosis of the human colon cancer Caco2 cell line and compared them with normal human primary fibroblasts.

We found that 500 µg/ml oregano extract led to growth arrest and cell death of Caco2 cells, without harming normal cells, through a mechanism involving the expression of biochemical hallmarks of apoptosis and alteration of redox state. Oregano led to activation of pro-caspase-3, as indicated by the increase in the expression of 17 kDa cleavage product, which in turn promoted cleavage of full-length PARP to its 89 kDa fragment. To gain insight into the mechanism of action, we analyzed the activation of caspases and regulators, involved either in the death receptor or mitochondrial pathways. Treatment of Caco2 cells with oregano induced processing of pro-caspase 8: the cleaved, active fragment p18 was significantly increased (4 fold) after treatment with oregano , thus suggesting an involvement of the death receptor-dependent signalling. Oregano also activated the intrinsic apoptotic pathway: after a 24 hours incubation, the expression of APAF-1 increased of 1.6 fold, while the expression of pro-caspase-9 decreased of 0.48 fold. Expression of pro- or anti-apoptotic members of the Bcl-2 family also appeared to be modulated: oregano increased protein levels of pro-apoptotic Bak, while down-regulating the expression of anti-apoptotic Bcl-xL. Furthermore, oregano promoted a perturbation in the mitochondrial membrane potential, as measured by uptake of the fluorescent dye JC-1, thus corroborating its influence on the death mitochondrial pathway. Despite its antioxidant capacity (2.4 mmol of Trolox equivalent/g of oregano extract dry weight) oregano lowered total glutathione levels and increased the content of its oxidised form, when added to cultures.

In conclusion, our data suggest a scientific basis for oregano spice involvement in prevention of cancer. Recently, the pro-oxidant action of phenolic compounds present in spices seems to explain their preferential cytotoxicity towards cancer cells. The amounts of extract used in our experiments were derived from about 2.5 mg of dried leaves, quantities far less than those normally found in the diet. Therefore, the anti-tumoral effects described in this study are easy to reach in a Mediterranean diet.

4.3

POLYUNSATURATED FATTY ACIDS IN THE CONTROL OF CHOLESTEROL AND TRYGLICERIDE SYNTHESIS: ROLE OF SREBP

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The plasma triglyceride and cholesterol-lowering effect is considered an important one among other possible contributing factors for the protecting activity of polyunsaturated fatty acids (PUFAs) in cardiovascular diseases. Both cholesterol and fatty acids biosynthetic processes are controlled by a common family of transcription factors indicated as sterol regulatory element binding proteins (SREBPs). SREBPs are intrinsically bound to endoplasmic reticulum membranes and must be escorted to the Golgi apparatus, where proteases liberate mature forms of SREBPs. Once released, mature SREBPs translocate to the nucleus to bind to the SRE (sterol response element) in the promoter regions of key genes of lipid metabolism. The proteolytic processing of SREBPs is regulated by free cholesterol intracellular concentration. When sterol concetration is high SREBP is confined into the endoplasmatic reticulum, when it is low cellular SREBP moves to activation in the Golgi, and the mature and active form of SREBP is released.

In this study, we evaluated the effect of the supplementation of different fatty acids to the culture medium of HepG2 cells on SREBP-mediated gene regulation. HepG2 cells were transfected with the SRE-luciferase (SRE-luc) gene, and SRE-luc activity determined after supplementation with scalar concentrations of n-6 and n-3 PUFAs. Modification of cell fatty acid composition was also determined by gascromatography. Furthermore, to evidence whether an increase of free cholesterol intracellular concentration could participate to the proteolytic regulation of SREBP activity, the distribution of free cholesterol within cell after PUFAs supplementation was assessed by fluorescence microscopy, in the absence/presence of U18666A, a pharmacological molecule that blocks cholesterol translocation.

All supplemented PUFAs caused a significant reduction of SRE-luc activity, let us hypothesize a consequent reduction in the expression of genes related to fatty acids and cholesterol biosynthesis. PUFAs added to the culture medium were not only remarkably incorporated into cell lipids, but also converted into their longer and more unsaturated metabolites. Filipin staining showed free cholesterol distribution in both plasma membrane and cytosol. Free cholesterol transport was clearly blocked by the addition of U18666A, but when the pharmacological molecule was added to EPA supplemented cells, a free cholesterol translocation was still present. This result suggest an EPA-induced cholesterol release from plasma membrane to cytosol.

Our data may improve knowledge of the mechanisms by which fatty acids control fatty acid and cholesterol synthesis, and provide insight into the development of new therapeutic strategies for a better management of lipid metabolism.

4.4

SUPPLEMENTATION WITH ISOFLAVONES: METABOLIC EFFECTS IN YOUNG HEALTHY WOMEN

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Consumption of soy products has been correlated to a lower incidence of different diseases such as CVD, hormone-related cancers and osteoporosis, that has been partially attributed to isoflavone intake[1-3]. The aim of this study was to investigate the metabolic effects of isoflavone (IF) supplementation in healthy women. A dietary intervention with isoflavone supplements was performed in 8 healthy women under a free diet (age 29 ± 8, BMI 22 ± 3), selected by a specific selection criteria. Volunteers were supplemented with 80 mg/d of total isoflavones (42 mg genistein, 30 mg daidzein and 8 mg glycitein) for 6 months followed by 2 months of suspension.

At the beginning (T=0), after 2 (T=1), 4 (T=2) and 6 (T=3) months of treatment and after the suspension (T=4) samples of blood were collected. Plasma concentrations of isoflavones (genistein and daidzein), variables of antioxidant status like total antioxidant capacity (TEAC, Lag-time), plasma vitamin status (α-tocopherol, retinol, ascorbic acid); markers of oxidative stress like erythrocytes membrane fluidity (fluorescence anisotropy) and susceptibility of DNA against induced oxidative stress (comet assay); serum lipid profile (concentration of triglyceride, total cholesterol, HDL cholesterol and LDL cholesterol) were analysed. Statistical analysis were performed by ANOVA test for repeated measure.

Plasma concentrations of isoflavones significantly increased according to dietary intervention suggesting a good compliance of volunteers to the protocol. Markers of plasma antioxidant capacity show a trend (not significant) toward an increase at T=2 and T=3 and a return to the basal level at T=4. Plasma concentrations of vitamins show a variability possibly related to different dietary habit within the experimental period (ascorbic acid). Regarding membrane status we observed a significant increase of fluidity at T=2 T=3 with respect to the basal level and final point ($p<0.05$). DNA susceptibility against induced oxidative stress (H_2O_2) was significantly decreased due to dietary intervention ($p<0.05$), showing a significant inverse correlation between DNA damage and plasma IF concentration ($p<0.01$). Finally, the analyses of serum lipid profile pointed out a positive effect of IF on LDL cholesterol (significantly decreased at T=3, $p<0.05$), while other variables were not affected.

The obtained results suggest a positive effect of IF supplementation in healthy young women against oxidative stress evaluated by measurement of membrane fluidity and DNA oxidative damage. These results support that the healthy effect ascribed to soy consumption could be at least partially related to the antioxidant potential of isoflavones.

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4.5

KINETICS OF THE LIPOPEROXYL RADICAL-SCAVENGING REACTION OF BETANIN AND BETANIDIN IN SOLUTION.

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ROS-mediated damage to membranes and lipoproteins is involved in the initiation and/or progress of a number of diseases including atherosclerosis, coronary hearth disease and cancer. Betacyanins, water-soluble nitrogenous pigments formed by condensation of betalamic acid with cyclo-DOPA or cyclo-DOPA derivatives, are dietary bioavailable phytochemicals whose antioxidant effects have been recently reported in LDL and RBCs both *in vitro* and ex *vivo* experiments[1,2].

In this work we studied the reaction between betanin, the main dietary betacyanin, and its aglycone, betanidin, with lipoperoxyl radicals generated by azoinitiator-induced oxidation of methyl linoleate in methanol, and identified the oxidation products of the pigments to clarify the anti-radical reaction mechanism.

Betanin, in the range 1-50 μM, behaved as a retarder of the lipid peroxidation causing a concentration-dependent decrement of the propagation rate and generating betalamic acid. Consumption rate of betanin was linearly related to its amount (r^2=0.98), suggesting adverse autoxidation reactions.

Betanidin behaved as a chain-terminating lipoperoxyl radical scavenger as effective as α-tocopherol, with a calculated inhibition constant of 1.23 x 10^5 $M^{-1}s^{-1}$ and a stoichiometric factor of 1.99. Its oxidation product was identified as betanidin *o*-quinone.

Betanin is characterised by a monophenol structure whereas betanidin is an *o*-diphenol. A mechanism can be proposed in which phenoxyl radicals generated from betanin following reaction with peroxyl radicals cause autooxidation and cleavage of the molecule. On the other hand, the two orthohydroxyls in the phenol moiety of betanidin, while remarkably increasing their reactivity, affect betacyanin transformation. The aryloxyl radical formed following reaction with lipoperoxyl radicals can suffer a dismutation to produce betanidin quinone and re-generate betanidin.

Our results contribute to elucidate the chemical basis of the known antioxidant action of the betacyanin pigments.

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4.6

ASCORBIC ACID ANTIOXIDANT ACTIVITY IN PRESENCE OF STABILIZATION AGENTS.

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Ascorbic acid has a fundamental role for preservation and correct maintenance of living organism. Close to its well known antioxidant properties, it is an unreplaceable cofactors of enzymatic reaction. Many organisms biosynthesize L-ascorbic acid, but other have the need to introduce this fundamental vitamin with the vegetables. One of the main problems involving ascorbic acid assimilation is correlated to its instability and degradation into biologically inactive compounds. As reported in Barreca et al.[1] trehalose preserves ascorbic acid against thermal degradation. In this work we analyses the protective influences of the sugar on antioxidant properties of ascorbic acid. 500 μM ascorbic acid solution was incubated at 25°C and at 60°C in presence or absence of 1.0 M trehalose for 20 minute. After incubation, the vitamin solution were analyzed by UV-Visible spectroscopy. The free radical scavenging effect of compounds was assessed by using the stable free radical 2,2-diphenyl-1-picrylhydrazyl. Ascorbic acid alone or plus trehalose, at a concentration ranging from l to 100 μM, in a final volume of 3.0 ml, was mixed with 80 μM DPPH in methanol. The changes in absorbance at 517 nm was monitored over 30 min. DPPH concentration in the cuvette has been chose to give absorbance values less than 1.0. UV-Visible spectroscopy did not reveal significant changes in major absorbance band of ascorbic acid, in presence of trehalose. Otherwise a remarkable decrease in the same band intensity was observed for solution incubated at 60°C without sugar. The antioxidant assay showed that absorbance of DPPH alone did not change over all the period of the experiments. The results obtained with ascorbic acid alone or plus trehalose 1.0 M, after incubation for 20 min a 25°C, are the same for both solution, inducing a rapid decrease of absorbance at 517 nm. The efficient concentration (EC_{50}) obtained was the same for both solution (6.55 μM). After incubation for 20 min at 60°C, the results obtained in presence of 1.0 M trehalose are superimposable with the curves at 25°C, while ascorbic acid alone showed very little antioxidant potency. The antioxidant potentials of pure vitamin C and vitamin C plus trehalose were the same irrespective of the sugar concentration, indicating that vitamin solution could play an effective role as radical scavenger. The same test shows also that at 60°C the presence of trehalose preserves ascorbic acid scavenger potency, presenting a biological activity equivalent to the pure L-ascorbic acid. Otherwise at 60°C ascorbic acid solution looses nearly completely this capacity.

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4.7

FIRST EVIDENCE OF POTENTIAL ANTIOXIDANT ACTIVITY OF *ALLIUM SATIVUM* PROTEIN EXTRACT.

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Abstract: *Allium sativum* L., commonly known as garlic, belong to Alliaceae family. It grows wild in areas where it has become naturalised; it probably descended from the species *Allium longicuspis*, which grows wild in south-western Asia. Garlic has been used as both food and medicine in many cultures for thousands of years, and represents a popular herbal remedies. It's claimed to help prevent heart disease, reduces cholesterol and cancer risk by neutralizing dietary mutagens and free radicals, significantly inhibits abnormal platelet aggregation and oxidation of LDL and VLDL cholesterol, helps to remove iron and other toxic heavy metals from the body, protects neurons from aging damage, boosts immune function and lower blood pressure in some people[1]. *Allium sativum* may also possess cancer-fighting properties due to the presence of allylic sulfur compounds such as diallyl disulfide, believed to be an anticarcinogen and allicin, a powerful antibiotic and anti-fungal compound. It is major component include: alliin, ajoene, vitamins, minerals and flavonoids. In our work we focused attention on protein fraction of garlic extract. We examined protein content and its potential antioxidant activity and capacity to inhibit tyrosynase activity. Garlic bulb were homogenate into powder in a chilly mortar. Protein were precipitated by salts and suspended with buffer. Protein content were estimated by Bradford assay. Radical scavenging activity were analyzed, in function of protein concentration, against the free stable radical 2,2-diphenyl-1-picrylhydrazyl (DPPH). The measurement of the reductive ability was evaluated by ferric reducing/antioxidant power.

Antioxidant assay shows the potentiality of protein extract precipitated by salts, to eliminated DPPH radical species. Useful information arise also by salts precipitation at different salts ratio (20, 30, 40, 60, 80 and 100%). Ferric reducing/antioxidant power assay reveals that about 40% of total garlic extract reducing activity is attributable to protein fraction. This last shows also tyrosynase inhibitory that is function of its activity. Results underline the importance of garlic protein fraction not only for feeding but also for commercial preservation utility.

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4.8

ANTIOXIDANT ACTIVITY IS INCREASED IN *XENOPUS* EMBRYOS DEVELOPED IN SIMULATED MICROGRAVITY

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The operation of the space station and the human exploitation of the space will require long duration missions, characterized by two condition that can negatively impact on human health: microgravity and space radiations .

In the human organism, solar radiation or low wavelength electromagnetic radiations (such as gamma rays) from the Earth or space environment can split water to generate the hydroxyl radical, which can initiate chain reactions leading to lipid peroxidation. These reactive free radicals (ROS) can react with the non-radical molecules, leading to oxidative damage of lipids, proteins and DNA, involved in various diseases, such as cancer, cell degeneration, and inflammation.

In this view radiation constitutes the most important hazard for humans during long-term space flights. Radiation protection is therefore mandatory to safeguard the well-being of astronauts or jet crew and to prevent the occurrence of future damage.

The aim of this experiment was to determine if microgravity might influence endogenous antioxidant systems so modifying the ability of living organisms to counteract to space radiation.

Xenopus laevis embryos of different ages were exposed to simulated microgravity using a Random Positioning Machine (RPM). Changes of liquid were performed daily. Morphology and enzymatic activities were measured.

As previously described embryos exposed to microgravity have axial malformations. For what biochemical assays concern it can be definitively conclude that: µg (RPM) did not cause an increase of mortality compared with controls. The exposure of embryos to RPM for 3 days caused an activation of HSP-60 and HSP-70; longer periods of incubation did not cause the same effect. When the embryos developed in simulated µg it was possible to observe an increase of activity of glutathione (GSH) related enzymes. Also GSH content wwas increased after exposure to RPM. These might result in a higher resistance to stress, such as anoxia or perhaps radiation induced oxidative stress.

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4.9

LONG CHAIN PUFAS OMEGA-3 INCORPORATION IN CELL MEMBRANE MICRODOMAINS OF BREAST CANCER CELLS

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Long chain PUFAs are important molecules for membrane order and function; they can also modify inflammation-inducible cytokines production, regulation of eicosanoid production, plasma TAG synthesis, blood pressure, and gene-expression.

Furthermore omega-3 have been hypothesized to influence colorectal carcinogenesis through many mechanisms (e.g. inhibiting COX2, increasing apoptosis, reducing angiogenesis). In breast cancer, supplementation with DHA synergistically enhances taxane cytotoxicity, down regulate HER-2/neu (c-erbB-2) oncogene expression, modifies the production of the heparansulfate syndecan1, suggesting a gene-nutrient interaction of critical importance for mammary carcinogenesis and supporting the hypothesis that omega-3 can be used as modulators of cancer cell chemo-sensitivity.

Aim of the study was to evaluate the effects of supplementation of AA, EPA and DHA in two lines of human breast cancer cells characterized by different expression of ER receptor. The fatty acid are all incorporated in cell membrane phospholipids with different specificity.

Moreover after treatments fatty acids are partially metabolized from both cell lines. In particular EPA is promptly converted to DPA,; DHA is partially re-converted in EPA while AA is integrated without being further metabolized.

However both omega-3 fatty acids induced cell apoptosis, with different degree and sensitivity, while AA increased cell proliferation in both cell lines.

Further studies will investigate the role of membrane changes induced by omega-3 fatty acid in micro domain function and signal transduction related to cancer cell proliferation.

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4.10

EFFECT OF POTASSIUM METABISULFITE IN HUMAN FIBROBLASTS

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Potassium metabisulfite (E224) is used as an antimicrobial substance in many kinds of foods. Potassium metabisulfite (PMB) is a sulfite that is chemically very similar to sodium metabisulfite (SMB), and is sometimes used interchangeably [The Ministry of Agriculture, 2004]. Both PMB and SMB are converted in aqueous solution to bisulfate and sulfur dioxide. Sulfites are often used as preservatives in wines to prevent spoilage and oxidation, dried fruits, dried potato products, biscuits and chocolate, jam, and sausage and salami. Since potassium metabisulfite is a widely used preservative in food industry, it may be of interest to investigate the effects of this substance on cultured animal cells. On this regard, it has been recently demonstrated that PMB had genotoxic and cytotoxic effects on human peripheral lymphocytes[1].

In this study Human Dermal Fibroblasts (HDF) were treated with different concentrations (150, 300 and 600 mg/ml) of potassium metabisulfite and the effects on cellular growth and protein expression pattern were analyzed at various intervals of time. Our results clearly indicated that the highest dose of PMB caused a dramatic cell death from one day of incubation. Nevertheless, a significant decrease on cell growth was observed even at low concentrations of the sulfite. SDS-PAGE of cell extracts revealed a substantial increase on the amount of a 70 KD protein in all samples. 2D electrophoresis analysis reveal extensive changes on protein expression pattern of treated cells compared to the control and will be used to identify target proteins by mass spectrometry (MS). Overhall results demonstrate that PMB has dramatic effect on human cultured cells and its use as food preservative should be carefully assessed.

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4.11

AUTOCATALYTIC AND MITOCHONDRIA-ASSISTED FLAVINYLATION OF RECOMBINANT RAT DIMETHYLGLYCINE DEHYDROGENASE.

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The mitochondrial matrix located dimethylglycine dehydrogenase (Me_2GlyDH, EC 1.5.99.2) is a key enzyme of folate one-carbon metabolism and choline catabolism, which covalently binds FAD via a histidyl(N3)-(8α)FAD linkage. Like most mitochondrial proteins, Me_2GlyDH is synthesized in the cytosol as a higher molecular weight precursor protein containing an N-terminal mitochondrial targeting peptide, which is removed on protein import inside the organelle by the mitochondrial processing peptidase (MPP).

Both the mature (mMe_2GlyDH) and the precursor (pMe_2GlyDH) forms of rat Me_2GlyDH have been produced in *Escherichia coli*, purified in both their apo- and holo-forms and identified by ESI-MS/MS[1, 2].

Purified recombinant holo-mMe_2GlyDH and holo-pMe_2GlyDH show typical flavoprotein absorbance spectra, with a main peak at 277 nm and two minor peaks at about 349 and 457 nm. They exhibit an enzymatic specific activity (240 nmol/min/ mg protein at 30 °C , pH 7.0) similar to that of the native enzyme and are in a folded state, as demonstrated by circular dichroism (CD) analysis. Underflavinylated forms of Me_2GlyDH perform a 70-80% lower specific activity. Trypsin-resistance experiments and CD analysis indicate that apo-mMe_2GlyDH is mostly in an unfolded state.

In vitro flavinylation experiments demonstrate that: i) covalent attachment of FAD to recombinant apoenzyme can slowly proceed autocatalytically, without third reactants; ii) the removal of mitochondrial presequence from apo-pMe_2GlyDH by MPP is not required for covalent autoflavinylation; iii) addition of proteins from mitochondrial matrix to apo-Me_2GlyDH strongly accelerates holoenzyme formation, allowing for a total recovery of a fully flavinylated enzymatically active holoenzyme.

These results allow to rule out the existence of a mitochondrial holoenzyme synthetase. A proposal for an alternative flavinylation pathway involving mitochondrial proteins will be presented in this communication.

This work was supported by Grant from MIUR (FIRB 2003, project RBNE03B8KK) to MB.

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4.12

BODY FAT DISTRIBUTION MAY INFLUENCE BOTH LIPIDEMIC AND OXIDATIVE PROFILES IN WOMEN. RESULTS OF CROSS-SECTIONAL STUDY

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Visceral-abdominal adiposity, a widely recognized cardiovascular disease (CVD) risk factor, is frequently associated with atherogenic dyslipidemia[1]. The Reactive Oxygen Species (ROS)-mediated oxidative modification of LDL cholesterol is implicated in the etiopathogenesis of several forms of CVD.

We evaluated the interrelationships among Body Fat Distribution assessed by Dual Energy X-Ray absorptiometry (DXA), oxidant (hydroperoxides) and antioxidant (total antioxidants, Uric Acid and Thiols) markers and lipidic profile (Total, LDL and HDL cholesterol, Tryglicerides): This cross-sectional study was based on a sample of 132 healthy women.

We found significant correlations between abdominal fat and LDL cholesterol ($r = 0.300$, $p<0.05$), LDL/HDL ratio ($r = 0.339$, $p<0.001$), tryglicerides ($r = 0.267$, $p<0.001$) and HDL cholesterol ($r = -0.199$, $p<0.05$). Total and abdominal fat, were significantly correlated with total antioxidants ($r = 0.257$, $p<0.01$ and 0.259, $p<0.01$ respectively) due to the essential contribute of Uric Acid, the highest concentrated endogen antioxidants of human organism, which is mostly associated with fat stored in abdomen ($r= 0.371$, $p<0.001$). Hydroperoxides only showed a positive correlation with fat localized on legs ($r = 0.226$, $p<0.01$). All association persisted after adjusting for age values. Finally, no significant associations between any components of lipid profile and antioxidant and oxidant markers were discovered.

Our results are consistent with a possible influence of body fat distribution on two well known CVD risk factors as dyslipidemia and Oxidative Stress. It is confirmed that the fat stored in abdomen is mostly linked to an enhanced LDL/HDL ratio. This relationship has been explained as one of the principal reason why abdominal-visceral fat is considered a key player in the development of type 2 diabetes and atherogenesis. Interestingly legs fat mass, widely considered much less harmful than abdominal, shows to be positively correlated with systemic Oxidative Stress. This last result may open new perspectives in the comprehension of mechanisms involved in the generation of ROS.

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4.13

POSSIBLE ROLE OF OXIDATIVE STRESS IN THE PATHOGENESIS OF CHRONIC VENOUS INSUFFICIENCY. PRELIMINARY RESULTS OF A POPULATION STUDY

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BACKGROUND: It is now appreciated that Oxidative Stress (OS) is involved in the pathogenesis of the lesions in certain chronic arterial diseases (as in atherosclerosis)[1]. In contrast, little information is available on the implication of oxidant species in pathologies affecting the venous system.
OBJECTIVES: The aim of our population study was to evaluate whether OS could have role in the onset of chronic venous insufficiency (CVI), one of the most spread and severe venous diseases.
METHODS: For this study we have selected 2 samples of women from a total of 350 patients attending a Vascular Surgery Clinics: the first group included 71 women devoid of any type of pharmacologic intervention; the second consisted of 65 subjects who either intensively or occasionally used medicals. Both samples were subdivided in 3 groups: healthy; patients with varicose veins; and patients with diagnosed CVI. Sera from all subjects were employed for colorimetric assessment of Oxidant Power (PO) given by hydroperoxides concentration measured by D-Roms test and total antioxidant power (TAP), determined through a ferric reduction (FRAP) technique.
RESULTS: In the first sample a slight, but not significant ($p>0.05$), increase in Oxidant and Antioxidant power is observed in ill with respect to healthy subjects. The average values of both parameters are higher in patients with CVI than those with varixes. The use of medications appears to indistinctly increase PO ($p<0.001$) in healthy, varixes and CVI groups while the increment in TAP is only significant in CVI patients.
DISCUSSION: Our preliminary results are not consistent with an implication of OS in pathogenesis of CVI. Indeed, the progression of venous disease, from the mild varicous veins to the severe CVI is not accompanied by an increase of OS. Interestingly, the use of medicals for the cure of this venous disease seems to improve the antioxidant defense.

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4.14

STRUCTURE-FUNCTION RELATIONSHIP EVALUATION OF SYNTHETIC VITAMIN E ANALOGUES IN C6 MURINE GLIOMA CELLS AND PERIPHERAL BLOOD LEUKOCYTES.

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A growing body of evidence is accumulating on the possible use of vitamin E analogues as anti-cancer compounds. The succinyl ester of α-tocopherol (α-T) tocopheryl succinate (α-TS) and the ether-linked acetic acid analogue, namely α-TEA, are considered the prototypes of the anti-cancer analogues of vitamin E. Actually they are potent pro-apoptotic agents for human cancer cells in vivo and in vitro in that stimulate the mitochondrial pathway of apoptosis and ROS generation. Only recently amide derivatives of α-T (TNH) have been produced and biological evaluations of these analogues are awaited. In this study we used a systematic in vitro drug screening approach to investigate a series of synthetic vitamin E analogues that include esters and ethers based on α-TS and α-TEA structures, and a series of TNHs that are derived from the basic structure of amino-tocopherol. The analogues were based on the condensation of the hydroxyl group in position 6' of the chroman ring with proper cyclic anhydrides and carboxylic acids providing succinic, maleic, fumaric and benzoic acids. Di and poly-halogenated amide derivatives of these acids were also prepared. Cell viability was investigated using MTT assay and cell death was assessed by cytofluorimetry. The test compounds were assessed for anti-cancer activity in the concentration range 0.1 – 10 μM using C6 murine glioma cells; peripheral blood mononuclear cells (PBMC) from healthy donors were used a non-cancerous cells to test the specificity of the anti-cancer activity. The four natural forms of vitamin E (namely α–, β–, γ–, δ–Toc) served as reference compounds. The main evidence provided by this study was that α-TS and α-TEA were confirmed to behave as strong pro-apoptotic analogues with IC50 values for the cell growth inhibition < 0.1 μM, but both of them showed low specificity. As far as it concerns cell viability, α-TEA produced a higher IC50 value than α-TS and a better apoptotic/necrotic ratio. The same findings were obtained for γ-TEA. In comparison with TS and TEA, TNHs and the other ether and ester derivatives investigated in this study were less effective in reducing C6 cell viability, but were also effective pro-apoptotic agents and showed higher specificity of activity. Intriguingly, TNH2 was one of the most active compounds. The different types of dicarboxylic acid used to functionalize the chroman group of α-T do not seem to influence significantly the activity of the test compounds, apart from the presence of a phenol group that significantly increased activity and specificity of TEA analogues. In conclusion, when tested in C6 glioma cells, α-TS and α- or γ-TEA are strong, but unspecific, in vitro anticancer agents. Other synthetic analogues such as TNHs and ether-linked benzoic acid analogues show a higher specificity to activity ratio, and thus are good candidates for the use in further pre-clinical evaluations.

4.15

MATERNAL SUPPLEMENTATION WITH HIGH DOSES OF α-TOCOPHEROL INFLUENCES HIPPOCAMPAL PKC ACTIVITY OF RAT OFFSPRING.

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Vitamin E is considered important to sustain foetal development and thus it is commonly recommended as a supplement during pregnancy. The net placental transfer of vitamin E, however, appears to be low and this has stimulated to use relatively high doses to maximize the delivery to foetal tissues. The effects of vitamin E supplementation on gene expression and signaling pathways of foetal tissues remain unexplored. Dietary loads of vitamin E as α-tocopherol (α-Toc) have been reported to affect neuronal plasticity and protein kinase C (PKC) signalling in adult rat hippocampus, and PKC is involved in a variety of developmental processes of CNS.

Based on this background, we investigated in rats the effects that the maternal supplementation with high doses of α-Toc may exert on PKC activity of offspring developing hippocampus. Moreover, we assessed the bioavailability of the supplemental vitamin E in the mothers and offspring measuring α-Toc levels in liver and brain tissues.

The results showed that PKC inhibition was highest at bird, when PKC activity was virtually abolished (-95% respect to controls). Thus, p-PKC levels remained significantly reduced during the span of hippocampal maturation, with significant differences between supplemented and control offspring. These differences in offspring PKC activity decreased with age (-75% until 14 days, and then -50% at 21 days of age) and disappeared in adulthood (i.e. after 60 days). These changes showed a correlation with the changes of liver vitamin E in the mother and offspring, but this correlation was much less evident when brain vitamin E was assessed.

This study shows that maternal exposure to high doses of α-Toc during pregnancy and lactation in rat causes a massive even if transient inhibition of PKC activity in offspring developing hippocampus.

4.16

DISTRIBUTION OF HOMOCYSTEINE AND CYSTEINE IN PLASMA LIPOPROTEIN FRACTIONS

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S-thiolation of plasma proteins have been detected in healthy humans, in patients with cardiovascular diseases and it is a recurrent phenomenon in oxidative stress elicited by reactive oxygen species. The low-molecular-weight aminothiols homocysteine (Hcy) and cysteine (Cys) can exist in the plasma either free or bound to thiol-combining groups, many of which are present in proteins (Pb), particularly albumin.

Recent studies have demonstrated that also plasma lipoproteins (LP) are susceptible to form disulfide-linked products with Hcy and Cys and that the N-homocysteinylation is accompanied by structural and functional alteration and could increase the atherogenicity of LDL. However, the mechanism through which Hcy contributes to these abnormalities remains however still undefined.

In order to ascertain the link between physiologic thiols and lipoproteins (LP), we determined simultaneously the distribution of Hcy and Cys, in vivo, bound to different plasma protein fractions by disulfide linkage. Human VLDL, LDL, HDL and LPDS (lipoprotein-free protein fraction) were obtained from plasma of normolipemic subjects volunteers (University workers) prepared by differential ultracentrifugation. The purity of the isolated subfractions were controlled by electrophoresis on agarose gel and no significant albumin content was found in the isolated lipoprotein fractions. The Hcy and Cys bound to single plasma protein fraction was calculated as difference between total and free aminothiols. Hcy and Cys in plasma and in lipoprotein fractions were analyzed by the HPLC method[1].

Protein-bound Hcy and Cys (Pb-Hcy and Pb-Cys) (9.10 and 196.79 μmol/L, respectively) represents about 76% and 79% of total plasma Hcy and Cys (11.98 and 249.33 μmol/L, respectively). The absolute Pb-Hcy and Pb-Cys distribution between the single LP fractions was as follows (μmol/L): VLDL=0.59 (6.4%) and 36.78 (18.7%) respectively; LDL=0.77 (8.5%) and 9.13 (4.6%) respectively; HDL=1.44 (15.8%) and 6.63 (3.4%) respectively; LPDS=6.30 (69.3%) and 144.24 (73.3) respectively. The Hcy and Cys/protein ratios in each single LP fraction (nmol/mg of protein) suggest a higher binding capacity for Hcy and Cys by VLDL and LDL probably due to their content in Apo B.

The detection of all aminothiols (Hcy, Cys, Cys-Gly, GSH) linked to Apo B may be important to understand the mechanisms of thiol-disulfide exchange reactions and to evaluate if this binding may have a complex role in promoting lipoprotein oxidation or structural and/or functional modifications in lipoprotein thiolation both in vivo and in vitro.

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4.17

SULFORAPHANE MODULATES NRF2/ARE PATHWAY IN CARDIAC CELLS

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Sulforaphane (SF), a naturally occurring isothiocyanate abundant in *Cruciferous* vegetables, has gained attention as a potential chemopreventive compound[1] thanks to its ability to induce several classes of genes implicated in reactive oxygen species (ROS) and electrophiles detoxification. Antioxidant responsive element (ARE)-mediated gene induction is a pivotal mechanism of cellular defence against the toxicity of electrophiles and ROS. The transcription factor NF-E2-related factor-2 (Nrf2), is essential for the up-regulation of these genes. Nrf2 phosphorylation by protein kinases such as Akt kinase, extracellular-signal-regulated kinase (ERK), c-Jun NH2-terminal kinase (JNK) and protein kinase C (PKC) influence the activation of the Nrf2/ARE pathway[2, 3].

Recently, we have demonstrated that SF elicits cardioprotective activity by up-regulating a battery of antioxidants and phase II enzymes[4]. The aim of this study was to verify SF mechanism of action leading to cardioprotection by investigating the effect of SF treatment on Nrf2 activation in primary cultures of neonatal rat cardiomyocytes. In particular we focused our attention on the ability of SF to activate different mitogen–activated protein kinases (MAPK) related to Nrf2 translocation to the nucleus.

Cultured rat cardiomyocytes, prepared and grown as previously reported[5], were supplemented with 5 μM SF for different times. Western blot analyses of Nrf2, phsopho-p38, phospho-JNK, phospho-Akt, phospho-ERK1/2 were performed using specific antibodies and following the manufacturer's recommended protocols. Nrf2 translocation to the nucleus was evaluated by immunoblotting of different cellular fractions. The expression of Nrf2 was determined by RT-PCR.
SF was able to modulate MAPK phosphorylation and Nrf2 translocation to the nucleus. This translocation was blocked by cell treatment with specific MAPK inhibitors demonstrating that Nrf2 activation is mediated by these pathways. SF was also able to up-regulate Nrf2 expression. So, the cardioprotective activity of SF could be ascribed to its ability to modulate both MAPK signaling pathways and Nrf2 expression.

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4.18

IS DNA DOUBLE STRAND BREAKS RECOGNITION RELATED TO LONGEVITY ?

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In mammals, species lifespan can vary by more than 100 fold (shrew 2 years, bowhead whale 211 years). Despite considerable research, the cellular mechanisms that make this variation possible remain unclear. In regard to these mechanisms, several predictions can be made. First, they must impact fundamental biochemical processes. Second, they would be expected to be related to structural differences between species at the cellular level. Furthermore, the goal would be to find significant correlation between cellular differences and the life span magnitude. As a tool to investigate these mechanisms, we have developed a series of skin fibroblast cell lines derived from mammalian species with a wide variation in lifespan (man, cow, bat, dog, mouse etc.). Using these lines, we have previously shown that the reported dependence of replicative capacity on longevity[1] is most likely due to the dependence of replicative capacity on body mass, which is itself correlated with longevity[2]. Therefore, comparative studies of longevity must address the influence of body mass.

The fact that DNA-PKcs and Ku 80 ablation in mice reduces average lifespan approximately 25% and 50% respectively and that Ku 80 null mice display symptoms of premature aging supports the potential role of these nuclear proteins in the aging process. DNA-PKcs and Ku are key proteins in double strand damage recognition. So we tested the capacity of skin fibroblast nuclear extracts from different mammalian species to bind DNA double strand breaks using an electrophoresis super-shift method that we have previously developed and that is now widely used in the field of DNA damage/repair[3]. Our results indicate that Ku-dependant DNA double strand break recognition increases exponentially with longevity and suggest that an enhanced ability to detect critical DNA damage may be a key requirement for longevity.

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4.19

SULFORAPHANE TREATMENT PREVENTS ACUTE EXHAUSTIVE EXERCISE INDUCED MUSCLE DAMAGE IN RATS.

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Acute, exhaustive exercises lead to a burst of reactive oxygen species (ROS) generation that increases the GSSG/GSH ratio in plasma[1], and causes structural damage to muscle cells as evidenced by an increase in plasma activity of cytosolic enzymes such as lactate dehydrogenase (LDH) and creatine kinase. Acute exhaustive exercise can be considered an interesting model of oxidative stress and muscle damage. To prevent and counteract ROS generation and oxidative stress during exercise, many studies have been focused on the use of natural compounds with ROS scavenging properties acting as direct antioxidants, but the overall results are still inconclusive. In this study we have investigated the possibility to counteract exhaustive exercise induced oxidative stress and muscle damage in rats by treating animals with sulforaphane (SF). SF is a naturally occurring isothiocyanate present in the human diet and originating from the ingestion of Cruciferous vegetables. SF is known to induce phase 2 enzymes with antioxidant properties in many tissues, but no data are still available on skeletal muscle tissues. In our study male Wistar rats (age 4 months, weight 230±20g) were treated every 24 hours with SF (25 mg/kg bw i.p.) for three days before undergoing an acute exhaustive exercise protocol. The exercise protocol consisted in running on a treadmill at 24 m/min and 7% gradient. Exhaustion was defined as the point at which the animals failed to get off the shock grid and thus had to be manually repositioned to the front of the treadmill on three consecutive occasions. Animals were sacrificed and LDH activity was determined on plasma samples. The activities of phase 2 enzymes such as NAD(P)H:quinone oxidoreductase (NQO1), glutathione-S-transferase (GST), glutathione reductase (GR), glutathione peroxidase (GPx), thioredoxin reductase (TR) and superoxide dismutase (SOD) and catalase (CAT) were evaluated in homogenates from freshly excised vastus lateralis skeletal muscle. SF treatment significantly induced the activity of NQO1, GST and GR in skeletal muscle tissues, with no effect on GPx, TR, SOD and CAT activities. Upregulation of phase 2 enzymes correlated with a decrease in oxidative damage in muscles, as evidenced by a significant decrease in LDH release in plasma after SF treatment. Our data, for the first time, demonstrate that SF could play a critical role in the modulation of muscle redox environment leading to the prevention of exhaustive exercise induced muscle damage. These results suggest that SF could become an interesting natural compound in the development of a dietary intervention that promotes oxidant scavenging through phase 2 protein induction and in the development of new dietary supplements for physical active people.

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4.20

MODULATION OF THE MMP-9 EXPRESSION BY OLIVE OIL PHENOLS

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Although the beneficial effects of olive oil consumption on human health are widely recognized, the functions and the mechanisms through which specific components of olive oil exert their effects still need to be fully deciphered. Although *in vivo* studies suggest that these compounds are responsible for the anti-inflammatory and anti-atherosclerotic actions of olive oil, less is known on the direct effects and mechanisms of action of these molecules in circulating cells. Monocytes participate in the early stages of atherosclerosis and, by expressing several molecules, including metalloproteinases (MMPs), contribute to amplify the inflammatory response.

With the aim of elucidating the mechanisms through which olive oil-derived phenols are beneficial on markers related to cardiovascular diseases, we investigated the effects of an olive oil phenolic extract (PE) and the main individual phenolic compounds (PCs, namely oleuropein aglycone, apigenin, luteolin, tyrosol, and hydroxytyrosol) on the modulation of MMP-9 in monocytes.

We found that PE significantly counteracts the effect of TNF-α on the expression and secretion of MMP-9. Oleuropein aglycone resulted to be active at the concentrations found in PE although other compounds probably contribute to the activity exhibited by the extract. We demonstrated that the PE acts at the transcriptional level preventing the stimulation of MMP-9 promoter activity. Finally, we found that the effect of PE on gene expression is ascribable to impairment of NF-kB signalling.

We assayed individual compounds present in PE that could contribute to the biological activity. Our findings regarding the ability of both flavonoids to down-regulate the MMP-9 promoter and to attenuate the NF-kB-driven transcription are much higher than those found in the PE used in our experiments thus excluding a role of these compounds in the effects exerted by the PE.

In the present study we clearly demonstrated that PE inhibits MMP-9 expression thus supporting the hypothesis that inhibition of proteolytic activity by PCs could be, at least in part, responsible for the reduction of invasiveness of tumour cells.

In this context our study elucidates some of the molecular mechanisms through which olive oil, a phenolic rich source, can be beneficial to human health, as widely demonstrated by *in vivo* studies.

4.21

CHAMOMILE INFUSIONS INHIBIT PROTEASES INVOLVED IN GASTRIC INFLAMMATION.

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Chamomile, prepared with dried flowers from *Matricaria recutita* L., is one of the most commonly consumed herbal tea. The drug is used for the treatment of gastrointestinal complaints (minor spasms, epigastric distensions, gastritis and gastric inflammation). Several classes of bio-active compounds have been identified in the extracts of chamomile including phenolic acids, coumarins and flavonoids such as the glycosides of apigenin, quercetin, patuletin, luteolin and several derivatives[1]. Several studies showed that chamomile infusions possess a protective effect on gastritis and gastric ulcer[2-3], but the mechanisms involved in this effect are not well established. Matrix metalloproteases (MMPs) and neutrophils elastase (NE) are proteases that degrade extracellular matrix in physiological and pathological conditions. Since MMPs and NE are involved in gastric inflammation, the aim of this work was the evaluation of the effect of chamomile infusions of dried capitula (CFI) and sifted (SFI) flowers on MMP-9 and NE, and the identification of the compounds responsible for the observed effect. Each infusion was analyzed by LC-MS/MS in order to verify whether compositional differences affected biological activity.

Analysis of CFI and SFI by LC-MS/MS showed a complex profile. The compounds unequivocally identified were the flavonoids apigenin-7-O-glucoside (api7glu), luteolin-7-O-glucoside (lut7glu), patuletin-7-O-glucoside (pat7glu) and hyperoside (hyp). Api7glu was more abundant in CFI than in SFI, whereas the opposite was for lut7glu. Pat7glu was the most abundant in both the infusions, whereas hyp was the lowest.

CFI and SFI inhibited enzymatic activity of MMP-9 catalytic domain in a concentration-dependent manner. At 1500 µg/ml the inhibition was 28 % and 55 % for CFI and SFI, respectively. Api7glu and lut7glu (10 µM) showed an inhibitory activity of 40 % and 30 %, respectively, demonstrating their contribute to the effect of the infusions. The inhibitory effect of CFI and SFI was confirmed on MMP-9 released by human adenocarcinoma cells (AGS cells). CFI was able to inhibit MMP-9 secretion from AGS cells (85 % at 1500 µg/ml). The inhibitory effect of the infusions on NE was also tested. Concentration-response curves were performed and IC_{50} of CFI and SFI on NE were 369,2 µg/ml and 536,7 µg/ml, respectively. The individual compounds that showed an inhibitory effect on NE activity were api7glu (IC_{50} 74,3 µM), lut7glu (IC_{50} 8,6 µM), pat7glu (IC_{50} 10,4 µM), and chlorogenic acid (IC_{50} 31,3 µM).

In conclusion, the present study shows some biochemical mechanism of action for the effect of chamomile infusions and supports the use of chamomile in the treatment of gastrointestinal inflammation.

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4.22

HOMOCYSTEINE-INDUCED ENDOTHELIAL DYSFUNCTION: PROTECTIVE EFFECTS OF OLIVE OIL POLYPHENOLS.

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Elevated serum homocysteine (Hcy) levels represent an independent cardiovascular risk factor and may contribute to the pathogenesis of atherosclerosis by altering endothelial functions.[1].

The mechanism of Hcy-induced cell adhesion has been investigated[2]. In particular, we have recently demonstrated that the lowering of intracellular adenosine concentration is responsible for Hcy-induced increased adhesiveness of EA.hy 926 cells. In hyperhomocysteinemia, indeed, the Hcy-induced reversal of S-adenosylhomocysteine (AdoHcy) hydrolase reaction causes the increase of the intracellular formation of AdoHcy at the expense of free intracellular adenosine. This event results in a lowering of extracellular adenosine, and likely in an impairment of the adenosine-related signal transduction.

Olive oil polyphenols, because of their powerful antioxidant activities, modulate several cardiovascular risk factors and contribute to the low incidence of cardiovascular diseases in the Mediterranean Area[3]. To examine the effect olive oil polyphenols on Hcy-induced endothelial dysfunction, and to elucidate if their protective effects are strictly related to their scavenging activities, structurally related phenolic compounds, endowed with different scavenging activities, have been selected and assayed for their ability in reducing the Hcy-induced monocyte adhesion as well as cell surface expression of intercellular adhesion molecule-1 (ICAM-1), in EA.hy 926 cells. The tested compounds include hydroxytyrosol (Dopet) and its *in vivo* metabolite homovanillyl alcohol (Mopet), sharing the same antioxidant properties. Tyrosol, the Dopet monophenolic analogue, devoid of antioxidant activity, has also been assayed. Our data indicate that Dopet and Mopet significantly and equally reduce cell adhesion and ICAM-1 expression, starting from a concentration as low as 0.75 µM. Also tyrosol appears protective, although to a less extent. Similar results have been obtained testing hydroxycinnamic acid derivatives, including caffeic, ferulic and p-coumaric acid. Taken together, our results indicate that in our model system the tested olive oil phenolic compounds are able to affect the specific Hcy-activated signalling, which results in ICAM-1 expression, through redox-independent mechanisms that remain to be elucidated.

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4.23

ANTIOXIDANT ACTIVITY IN SOLUTION AND BIOLOGICAL MEMBRANES OF SEVEN CULTIVARS OF SICILIAN PEACH (*PRUNUS PERSICA,* L. MILL.)

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Consumption of fruit and vegetables has been recognized to be of great importance in the Mediterranean diet, because of health benefits against inflammation and various chronic diseases. Healthy properties are due to the presence of some vitamins, minerals, fiber and phytochemicals. Among the latter, polyphenols, widespread in the plant kingdom, are known for their redox and antioxidant properties[1].

Peaches are important as table fruit, as well as ingredient largely utilized in the confectionery industry. Though a few studies reported about the antioxidant potential of some peach varieties in solution, the antioxidant activity in biological environments has not been investigated yet.

The antioxidant capacities of methanolic extracts of peel and flesh of seven sicilian peach cultivars in solution, have been assessed using TEAC (Trolox Equivalent Antioxidant Capacity), and TAA (Total Antioxidant Activity) assay. Metal-dependent lipid membrane oxidation of bovine liver microsomes, measured as TBARS, was assessed only for the flesh. The total phenols were determined by a spectrophotometric enzymatic method.

The TEAC values ranged 1.2 – 9.5 and 2 – 6.4 (µmol Trolox / g fresh weight), and the TAA values ranged 12.9 – 98.3 and 33.4 – 60.3 (mg GAE / 100 g fresh weight) for flesh and peel, respectively. Guglielmina and Daniela cultivars showed the highest values of TEAC and TAA.

A strong correlation was verified between total phenolics and antioxidant capacities expressed as either TEAC or TAA of flesh (vs TEAC r^2 = 0.98; vs TAA r^2 = 0.96) and peel (vs TEAC r^2 = 0.92; vs TAA r^2 = 0.96).

Flesh extracts from all cultivars , at a concentration 1mg fresh weight/ mL assay mixture, inhibited TBARS formation in microsomes in the range of 10 – 90 %. Only Fair Line cultivar needed 10 mg fresh weight/ mL assay mixture to show an inhibition of 25%.

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4.24

MITOCHONDRIAL LOCALISATION OF THE HUMAN FAD SYNTHETASE ISOFORM 1

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Mitochondria are the cellular site at which many flavoproteins are located, where they act as dehydrogenases and oxidases in co-operation with the riboflavin-derived redox cofactors FMN and FAD. We demonstrated the existence of a mitochondrial FAD synthesising pathway starting from imported riboflavin both in rat[1] and yeast[2] and proposed that, besides working as traditional cofactors, riboflavin/riboflavin metabolites may play a direct role in transcriptional/translational regulation of apo-flavoprotein expression, both in humans[3] and in yeasts[4]. In the aim to elucidate the mode by which mitochondria maintain the proper level of FAD, we overproduced in *E. coli*, identified and purified two human isoforms of FAD synthetase (EC 2.7.7.2), the enzyme which adenylates FMN to FAD, produced from two different transcript variants of FLAD1 gene[5, 6]. Transcript variant 1 encodes a 587-amino acid protein with a predicted molecular mass of 65.3 kDa (hFADS1), and the second variant encodes a 490-amino acid protein with a predicted molecular mass of 54.2 kDa (hFADS2), which lacks an N-terminal region of 97 amino acids present in hFADS1. Analysis using protein prediction programs showed that hFADS1 contains an N-terminal mitochondrial targeting sequence (17 residues, molecular mass 2.1 KDa). Here we report mitochondrial import assays aimed to directly prove the mitochondrial localisation of hFADS1p. For this purpose, the cDNA encoding hFADS1 was cloned into the pCMVTNT™ vector and used as template in the coupled transcription/translation rabbit reticulocyte lysate system. One main [^{35}S]-Met labelled product migrating at about 63 kDa on SDS-PAGE was generated and processed to an about 2 kDa faster band upon incubation with freshly isolated rat liver mitochondria. The import of the protein into mitochondria was demonstrated by the protection from digestion by externally added trypsin. hFADS1p processing was inhibited by the uncoupler carbonylcyanide p-trifluoromethoxyphenylhydrazone (FCCP), as expected for a process depending on mitochondrial membrane potential. The same experiments were carried out with the *in vitro* translated hFADS2 protein, starting from pCMVTNT™–hFADS2 plasmid. hFADS2p was neither processed by nor imported into isolated mitochondria. These results represent the first direct demonstration that hFADS1 transcript encodes for the mitochondrial FAD synthetase.

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5.1

PRECISION AND ACCURACY IN NON-OPTIMAL REAL-TIME PCR: ADVANTAGES OF A NEW DATA ANALYSIS METHOD

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Quantitative real-time PCR addresses the evident requirement for quantitative data analysis in molecular medicine, biotechnology, microbiology and diagnostics[1]. Although, the cycle-threshold (*Ct*) method is the present "gold standard", it is far from being a standard assay. Uniform reaction efficiency among samples is the most important assumption of this method. Nevertheless, some authors have reported that it may not be correct and a slight PCR efficiency decrease of about 4% could result in an error of up to 400% using the *Ct* method[2]. This reaction efficiency decrease may be caused by inhibiting agents used during nucleic acid extraction or copurified from the biological sample. We propose a new method (Cy_0) that does not require the assumption of equal reaction efficiency between unknowns and standard curve.

The Cy_0 method is based on the fit of Richards' equation to real-time PCR data by nonlinear regression in order to obtain the best fit estimators of reaction parameters. Subsequently, these parameters were used to calculate the Cy_0 value that minimizes the dependence of its value on PCR kinetic.

The *Ct*, second derivative (*Cp*), sigmoidal curve fitting method (*SCF*) and Cy_0 methods were compared using two criteria: precision and accuracy. Our results demonstrated that, in presence of optimal amplification conditions, the Cy_0 method showed the same effectiveness respect to the *Ct* and *Cp* methods. However, when PCR efficiency was slightly decreased, lowering amplification mix quantity or adding a biological inhibitor such as IgG, the *Ct, Cp* and *SCF* methods were markedly impaired while the Cy_0 method gave significantly more accurate and precise results.

These data demonstrate that Cy_0 represents a valid alternative to the standard methods for obtaining a reliable and precise nucleic acid quantification even in sub-optimal amplification conditions overcoming quantitative inaccuracy due to slight amplification inhibition.

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5.2

XYLab: AN INTERACTIVE PLOTTING TOOL FOR MIXED MULTIVARIATE DATA OBSERVATION AND INTERPRETATION.

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The correct display of data is often a key point for interpreting the results of experimental procedures. Multivariate data sets suffer from the problem of representation, since a dimensionality above 3 is beyond the capability of plotting programs. Moreover, non numerical variables such as protein annotations are usually fundamental for a full comprehension of biological data. Here we present a novel interactive XY plotter (XYLab) designed to take the full control of large datasets containing mixed-type variables, provided with an intuitive data management, a powerful labelling system and other features aimed at facilitating data interpretation and sub-setting[1].

The XYLab loads data from simple column-based tables to build up an XY scatter plot with per-point pop-up labels. The plot area is controlled by three easily accessible selectors, named "X", "Y" and "Lab" since columns containing numerical variables are automatically detected and used to feed the first two selectors, while the latter may also contain non-plottable variables such as text-based ones.

XYLab is aimed at offering to the user an easy-handling, fast and full control of what to plot and which labels to show: a simple change in the variable choice from the selectors makes an update of the plot with automatic rescaling and optimization. This allows to visually explore a number of data trends and interrelations in minutes.

XYLab takes full control over its labels. In fact, we implemented a "search-in-plot" procedure: we introduced a text box that is read before plotting the points and that may contain a query directed against the variable selected in the "Lab" selector. Such query can be verbose, acting on text-based labels, or numerical (e.g. greater/lesser than), acting on numerical labels. All the positive matches are scored directly in the plot by changing the point appearance, without affecting their position in the Cartesian space.

Another peculiar aspect of the XYLab is a sub-setting mechanism that we called "select-and-paste". Being the plot an interactive area, the user can draw a rectangle in a region containing interesting points and all the associated features are automatically visualized in a dedicated program area, ready to be exported. Thus, the plot itself guides the data selection and avoids the tedious task to look at the full data table to trace-back the desired information.

In conclusion, the XYLab offers a simple and intuitive plotting interface aimed at the rapid interpretation of large multivariate datasets in which text and numbers have a comparable importance.

The program, along with its manual and a test dataset, is available at www4.unifi.it/scibio/bioinfo/XYLab.html

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6.1

STRESS BY AROMATIC COMPOUNDS IN SULFOLOBUS SOLFATARICUS: DETOXIFICATION, REGULATION AND BIOMONITORING.

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Most of the archaeal microbial cells, the third domain of life, are adapted to grow in extreme environments not only regarding temperature but also pH, ionic strength and the presence of high concentrations of detergents and organic solvents. As all living cells, they possess a wide variety of finely regulated biochemical systems to defend from environmental stress and, in fact, they own in their genomes regulative sequences responsive to different stress agents[1]. Generally, the response can be initiated by binding of transcription factors to particular ligands, such as environmental signals.

The thermophilic archaeon *S. solfataricus* responds to stress by aromatic compounds increasing the expression of a MarR-like operon and of an alcohol dehydrogenase gene (*Sso2536adh*)[2]. The system involves the MarR family transcription factor BldR, which binds to its own promoter inducing auto-activation and increasing the coexpressed drug export permease level. BldR also binds to the *Sso2536* promoter stimulating the gene transcription, the accumulation of the ADH enzyme, and hence the enzyme-catalyzed conversion of the aldehydes to the less toxic alcohols[3]. A homologue of Bldr, Bldr2, has been recently characterised. DNA binding assays demonstrated that this protein is indeed a transcription factor. Biochemical characterization, as well as transcriptional analyses, suggested that Bldr2 could be also involved in the detoxification/catabolism of aromatic aldehydes.

Knowledge of the molecular mechanisms underlying this stress response has revealed crucial to set up a microbial sensing device for the measurement of water-dissolved aromatic aldehydes. The biosensor is an *E coli* strain expressing the GFP under the control of the responsive *Sso2536* promoter, and the gene for the sensor protein BldR.

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6.2

TOLUIDINE BLUE-MEDIATED PHOTODYNAMIC EFFECTS ON STAPHYLOCOCCAL BIOFILMS

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Staphylococci are important causes of nosocomial and medical-device-related infections. They often form biofilms, sessile communities of microcolonies encased in an extracellular matrix that adhere to biomedical implants or damaged tissue. Infections associated with biofilms are difficult to treat; sessile bacteria in biofilms are more resistant to antibiotics than their planktonic counterparts. Photodynamic treatment (PDT) has been proposed as an alternative approach for the inactivation of bacteria in biofilms.

In this study, we have investigated the effect of the photodynamic action of toluidine blue O (TBO) on the viability and structure of biofilms of *Staphylococcus epidermidis* and of a methicillin-resistant *Staphylococcus aureus* strain.

Significant inactivation of cells was observed when staphylococcal biofilms were exposed to TBO and laser simultaneously. The effect was found to be light dose dependent. Confocal Laser Scanning Microscopic study suggested damage to bacterial cell membranes in photodynamically treated biofilms. In addition, Scanning Electron Microscopy provided direct evidence for the disruption of biofilm structure and a decrease in cell numbers in photodynamically treated biofilms. Furthermore, the treatment of biofilms with tetrasodium EDTA followed by PDT enhanced the photodynamic efficacy of TBO in *S. epidermidis*, but not in *S. aureus*, biofilm. The results suggest that photodynamic treatment may be a useful approach for the inactivation of staphylococcal biofilms adhering to solid surfaces of medical implants.

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6.3

DESIGN, HETEROLOGOUS EXPRESSION AND CHARACTERIZATION OF A CHIMERIC MOLECULE COMPOSED OF TWO PLANT DEFENSE PROTEINS

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Plants have their own networks of defence against pathogens that include a vast array of proteins and other organic molecules produced during pathogen attack: pathogenesis-related proteins (PR proteins), defensins, ribosome-inactivating proteins (RIP), lipid-transfer proteins (LTP), killer proteins (KP), protease inhibitors (PI), etc. (1). Recombinant DNA technology is currently being used in agriculture to create genetically modified plants which result more resistant to pathogen infections. In order to provide a more effective strategy in plant disease control, we designed a bifunctional chimeric molecule containing two different protein modules: PD-L4, type 1 ribosome-inactivating protein (RIP) and a subtilisin/chymotrypsin inhibitor (WSCI). PD-L4 is a monomeric protein (Mr about 30k), isolated from *Phytolacca dioica* L. leaves that hydrolyzes the N-glycosidic bond of a specific adenosine in the major rRNA and acts as toxic or antiviral agent (2). WSCI is a small protein (Mr 8,126.3k), isolated from *Triticum aestivum,* capable to inhibit bacterial subtilisin and pancreatic chymotrypsin as well as to interfere with proteolytic activities present in the midgut of some phytophagous insect larvae (3). *pd-l4-cDNA* and *wsci-*cDNA have been isolated and cloned in *E. coli* expression vectors (2, 4). Here we report on: i) the strategy used to clone a genetic cassette, containing the cDNAs of PD-L4 and WSCI separated by a double strand oligonucleotide linker, in the pGEX-2T expression vector; ii) the expression of the recombinant chimeric protein into *E. coli* BL21-DE3 strain; iii) the characterization of the recombinant product.

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6.4

CONJUGATES BETWEEN FOOD PROTEINS AND MAGNETIC NANOPARTICLES: PRODUCTION AND PROPERTIES

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Advances in processes for producing nanostructured materials have made possible the production of magnetic nanosupports with potential applications in the biochemical and biotechnological fields. This kind of support is generally synthesized by encapsulating magnetic materials within a polymer layer. The interest for magnetic nanosupports is not only limited to the obvious ease of their separation under micro- and nanofluidic conditions. Most relevant is the fact that nanostructures can be conjugated to biologically active molecules, including hormones, antibodies, drugs, and various peptides, taken up by cells, and circulated among tissues expressing their cognate receptors. Given their intrinsic magnetism, magnetic nanoparticles may be used as tracers in NMR and MRI experiments, and are easily detectable in standard transmission electron microscopy.

This work is aimed at producing and characterizing conjugates between magnetic nanoparticles and bioactive proteins, and at assessing their use as biological tracers (for instance in monitoring the intracellular and/or intra-tissutal path of bioactive molecules of food origin), and their suitability for controlling enzyme activity in a number of applications.

Dextran-coated iron oxide nanoparticles were modified in order to obtain an activated coating which allowed the covalent binding of different bioactive proteins through simple chemical procedures. Proteins considered in our studies included food allergens, enzymes, and antibodies to food proteins. Dot blotting with specific antibodies, followed by immunoenzymatic detection of the bound antibodies, demonstrated the actual presence of these allergenic proteins on the conjugated nanoparticles. As a further test of protein immobilization, immunoprecipitation experiments were performed to demonstrate the coupling of the proteins to the functionalized dextran-coated nanoparticles. Both immunological approaches confirmed the suitability of our immobilization strategy.

We also prepared conjugates between analytical-grade trypsin and dextran-coated nanoparticles, and assessed the immobilized enzyme activity on synthetic substrates. These experiments led us to estimate the coupling yield of our coupling approach, which compares with those reported in the literature for equivalent procedures[1].
Preliminary tests aimed at assessing the citotoxicity of unmodified dextran-coated nanoparticles showed that the viability of differentiated HT-29 cells, from a human adenocarcinoma cell line, after various times of incubation with the nanoparticles was close to that of control untreated cells, suggesting full biocompatibility of the unmodified particles with this peculiar cell line.

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6.5

GENETIC VARIABILITY ANALYSIS OF APULIA SHEEP BREEDS BY USING MICROSATELLITES.

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The dramatic contraction of autochthonous sheep breeds increased the need for native genetic resource conservation. Infact, the local sheep breeds, characterized by a limited size, strictly depend on the maintenance of genetic differences. Conservation of genetic diversity and resistance to disease are of great importance in animal science and the analysis of breed genotype can supply the basis for conservation programs. The drastic decrease in the Moscia Leccese breed, even more in the Altamurana breed (<500 animals), among the Apulia native genotypes (provinces of Bari and Lecce), focused attention to the possibility of genetics protection of these races.

The genetic variability and genetic distance of sheep populations Altamurana (AL), Moscia Leccese (ML), Delle Langhe (DL), Sarda (S), Massese (M) and Bergamasca (B) were analyzed. In this study, 8 microsatellite molecular markers (OarCP49, FCB11, OarAE129, FCB304, INRA063, MAF214, CSRD247 e HSC) were used. These microsatellites were amplified by multiplex-PCR and analyzed for genetic variability and genetic distance among individuals having a scrapie ARR/ARR genotype.

161 individuals were analyzed: 49 AL (46 of them with a ARR/ARR scrapie resistent genotype), 17 ML, 34 S, 18 DL, 28 M e 15 B. ML, S, DL, M e B individuals were not consanguineous and belong to different sheep herds. Allele frequencies, the average allele number per population, observed (H_{obs}) and expected average heterozygosity (H_{exp}), genetic distances and Hardy-Weinberg equilibrium were analyzed with GENETIX 4.05 and FSTAT softwares. Genetic relationships among the breeds were analyzed by the factorial correspondence analysis (AFC). Genetic distance among individuals within populations, based on common alleles, was analyzed with MICROSAT software.

A total of 130 alleles was detected across the 8 loci. The average number of alleles per locus observed was: AL=8.8, DL= 6.5, M=9.0, S=9.6, ML=6.9, B=7.0.

Average values of expected (H exp) and observed (H obs) heterozygosity were respectively: AL=0.747± 0.10 and AL=0.701± 0.07; DL=0.671± 0.15 and DL=0.619± 0.18; M=0.749 ± 0.08 and M=0.695 ± 0.09; S=0.719 ± 0.10 and S=0.665 ± 0.14, ML=0.743 ± 0.07 and ML=0.694 ± 0.11; B=0.755 ± 0.06 and B=0.808 ± 0.09.

The reduction of intrapopulation heterozigosity *Fis* was 0.0741±0.01 and revealed an excess of homozigotes with respect to Hardy-Weinberg equilibrium condition. The total heterozigosity reduction *Fit* was 0.134±0.01. The reduction of interpopulation heterozigosity was 0.065±0.01 and, on the basis of Wright classification, it denotes a low differentiation among the studied populations. On the basis of the AFC analysis, DL e AL populations were the most primitive with respect to the other populations and they have a probably common origin, whereas S e M populations have a recent origin.

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6.6

FUNGAL LIGNINOLYTIC ENZYMES IN TEXTILE INDUSTRIAL WASTE DEPURATION

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Synthetic dyes find application in different industrial divisions including textile. These compounds result to be recalcitrant to degradation and toxic to higher animals becoming of environmental concern in case of release in aquifers. Physico-chemical treatment methods (e.g. electrolysis, sonication or ozonization) which bring to the complete destruction of dye molecules are somewhat unsuccessful, very expensive and producing large amounts of toxic wastes which are difficult to dispose of.
Many representatives of different classes of synthetic dyes, have been subjected to discoloration by fungi, in particular, the ligninolytic basidiomycetes have received extensive attention due to their powerful lignin-degradating extracellular enzymatic systems[1]: laccases and peroxidases.

Laccase (Lc) is a blue oxidase capable of oxiding phenols and aromatic amines. Lignin peroxidase (LiP) is characterized by its ability to oxidize high-redox-potential aromatic compounds and manganese peroxidase (MnP) by Mn^{2+} oxidation, Mn^{3+} chelates acting as diffusing oxidizers. Versatile peroxidase combines the catalytic properties of the two above peroxidases. These enzymes are also able to decolorized several industrial dyes. The ligninolytic basidiomycetes are edible and their industrial treatment produces a significant amount of spent mushroom substrate (SMS), still characterized by high levels of residual enzymatic activity.

We have demonstrated that the SMS and the extracellular culture fluid derived from the spent mushroom substrate of the lignolitic basidiomycete *Pleurotus Ostreatus* were able to decolorize the synthetic diazo-dye reactive Black DM 5594, trade name “Lanasol” black, and the synthetic dye acid blue “Follone” and “Follone” complete recipe. The enzymatic assays on the extracellular culture fluid of *Pleurotus Ostreatus*, revealed that laccases are predominant on the manganese and versatile peroxidase, while no lignine peroxidase activity was found. The optimum pH was 4 with ABTS as substrate. The laccase stability to pH indicated that this enzyme was stable in a range of pH 4-9. The laccase stability to temperature indicated that the enzyme is stable until 60 ºC. To shed light on the potential toxicity of commercial azo dyes, we have used a human epithelial cell line (WISH) for the cytotoxicity tests and feeds of some common plants for the germinability tests. The azo reactive dyes Lanasol red, black, blue and yellow are toxic for cells and feed plants. On the other hand these azo reactive dyes pre treated with SMS, have lost their toxic effect on the cells and for the feed plants. The azo acid dyes, blue, red and yellow Follone and a real waste water deriving from a local textile manufacturer are no toxic for cells and feeds as observed for the azo reactive dyes.

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6.7

BINDING EQUILIBRIUM ASSESSMENT BETWEEN MUCIN AND ARABINOGALACTAN, A NO-VISCOUS POLYMER USEFUL IN THE TREATMENT OF DRY EYE

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Mucins are the major glycoprotein components of the mucous that coats the surfaces of cells lining the respiratory, digestive, and urogenital tracts. Their function is to protect epithelial cells from infection, dehydration, and physical or chemical injury. In the eye, corneal and conjunctival epithelia produce hydrophilic mucins that hold tears onto the ocular surface. Eye pathologies, as dry eye syndrome can be associated to mucin alterations. Dry eye is associated with tear film deficiency, owing to insufficient supply or excessive loss, and anomalous tear composition. One of the essential functions of the tear film is to lubricate the ocular surface during blinking and eye movement. The eyes are constantly in motion, and the ocular surface is delicate; without adequate lubrication, ocular surface damage occurs and symptoms of discomfort develop or increase. Ocular lubrication is also essential for contact lens wearers, which utilize artificial tears to increase both lubrication and retention of the tear film. A general feature of artificial tears is their high viscosity which should increase the residence of the substance on the ocular surface. However, the high viscosity may lead up to inconveniences such as sticky feeling and solidification. In this regard, a strategy to antagonize the discomfort of dry eye is the use of low viscosity polysaccharidic molecules that may endure on the ocular surface without unpleasant disadvantages. Arabinogalactan (AG), a natural polysaccharide present in conifers of the genus Larix (Larch), was recently shown to exert a corneal protective action.

This study is devoted to assess mucoadhesive properties of purified AG (supplied by Opocrin) by evaluating its ability to interact with mucins through gel filtration chromatography. The methodological approach is based on the shift possibly occurring in the elution profile of a ligand when subjected to gel filtration chromatography in the presence of the target and on the measurement of the ligand bound to the target while emerging from a chromatographic column equilibrated with different ligand concentrations.

Mucin (MUC1) from bovine submaxillary glands and AG display, when chromatographed separately on a Sephacryl S300 column, well distinct elution peaks. A significant change in the elution profile of AG, compatible with a transient coelution of the two molecular species, is observed when the polysaccharide is chromatographed together with mucin. On the contrary, no effect is exerted on the elution profile of AG by different proteins and glycoproteins with molecular mass comparable with mucin. Frontal gel chromatographic approach, performed with MUC1 (1 mg of protein/ml) and AG at concentrations ranging from 0.06 to 0,23 mg/ml allowed to assess the effectiveness of the interaction process.

6.8

STRATEGIES COMBINING CELLS AND SCAFFOLDS FOR BONE TISSUE ENGINEERING

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There is increasing interest in new biomaterials and new culture methods for bone tissue engineering, in order to produce, *in vitro*, living constructs able to integrate in the surrounding tissue. Static culture environments suffer from limited diffusion and often result in inhomogeneous cell and extracellular matrix distribution. In order to overcome the drawbacks associated with static culture systems, several bioreactors have been designed[1].

Using an electromagnetic bioreactor (magnetic field intensity, 2 mT; frequency, 75 Hz), we investigated the effects of electromagnetic stimulation on SAOS-2 human osteoblasts seeded onto a 3D titanium alloy scaffold. In comparison with control conditions, the electromagnetic stimulation caused increased surface coating with decorin, type I and III collagen, osteopontin, osteocalcin, osteonectin, alkaline phosphatase and fibronectin. The immunolocalization of the above proteins showed their co-localization in the cell-rich areas. RT-PCR analysis revealed the electromagnetically up-regulated transcription specific for the foregoing matrix proteins and for the growth factor TGF-b. Furthermore, the sample exposed to an electromagnetic bioreactor showed an higher cell proliferation as confirmed by the measurement of DNA content and SEM observations. In order to overcome the total immunocompatibility with the patient, the use of mesenchymal stem cells (MSCs) could be promising. For this purpose, human bone marrow-derived MSCs (BMMSCs) were isolated from adult patient and their osteogenic potential was evaluated onto the same 3D titanium alloy scaffold in terms of cell adhesion, proliferation and differentiation in static conditions. We will be setting the same experiment with BMMSCs evaluating the effects of an electromagnetic stimulation.

The use of an electromagnetic bioreactor aims at obtaining the surface modification of the 3D scaffolds in terms of cell colonization and coating with calcified matrix; in this way the superficially modified biomaterial could be used, in clinical applications, as an implant for bone repair.

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8.1

ANTIOXIDANT ACTIVITY OF *OLEA EUROPAEA* DERIVATIVES

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At the present time it is easy to observe an increasing interest for the utilization of natural products as opposed to synthetic ones. The drug, food and cosmetic industry mostly use synthetic phenolic compounds with the aim of scavenging free radicals. In the cosmetic industry, in particular, these compounds are used to delay the skin ageing process.

Grape, olive and orange are fruits rich in antioxidants which are poorly exploited by the industry[1-4].

Persuing our interest in the field of natural antioxidant discovery, we have evaluated "in vitro" in different experimental models (Erythrocytes, Phosphatidylcholine liposomes, DPPH) the radical scavenging activity of raw extracts coming from manufacturing process of olive oil[5].

The products exhibit a marked antioxidant activity with an IC_{50} ranging from 1 to 5 mg/ml which is largely over to that of vitamin E.

In addition the compounds show a sinergistic interaction with this important antioxidant contained in olive oil..

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8.2

NEW APPROACHES TO THE EXPANDED NEWBORN SCREENING PROGRAMS BY TANDEM MASS SPECTROMETRY: REDUCTION OF FALSE-POSITIVES FOR C5, C5OH, C6DC.

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The expansion of newborn screening programs has increased the number of newborns diagnosed with inborn errors of metabolism in the presymptomatic phase but it has also increased false-positive results. False positive results are costly for Public Health Resources and causes unnecessary parental stress. We report an update on the latest developments in the expanded newborn screening programs. Acyl-carnitines C5, C5OH, C6DC are one of the analytes most frequently responsible for false-positive results. We developed a rapid liquid chromatography-tandem mass spectrometry (LC-MS/MS) method that identifies free acids and glicine derivatives: 3-idroxisovaleryl-acids (3-OH-IVA), 2-idroxisovaleryl-acids (2-OH-IVA), 3- MethylcrotonylGlycine (3MCCGly), Isovalerylglycine (IVAGly), PropionylGlycine (C3Gly), 3-idroxMethyl-Glutaric acid (3-OH-3-MetGlut), 3-Methyl-Glutaconic acid (3-MetGlutac), Lactic acid (LA), Metylmalonic acid (MMA), Succinyl acid (Suc) and TiglylGlycine (TiglilGly) in blood spots thus reducing false-positive rates due to C5, C5OH, C6DC during expanded newborn screening programs. We studied newborn screening spots from 92 healthy controls; 28 from false positives for abnormal C5, C5OH, C6DC and 23 from inborn truly affected. Analytical method consists of chromatographic separation on a C6-Phenyl column of an extracted 3.2 mm dried blood spot and injection into triple quadrupole mass spectrometer equipped with a Turbo Ion Spray Ionization Source. Specific Multiple Reaction Monitoring were carried out and labelled isotopic reagent were used as an internal standard. No derivatization is required and total analysis time is 5 minutes per sample. Intra- and interassay imprecision data were 3.5%-8% and 3.2%-6% for MMA. Limit of detection and limit of quantitation were 0.01 and 0.05 micromol/L, respectively, for C3Gly and IVAGLy. The recoveries were 92.9%-106.1%. No deterioration was noted on the columns after 500 chromatographic runs. The application of this method as second tier test allows to reduce false-positive results, the retesting and the consequent recalls inborn. In addition, the test allows us to diagnose with greater certainty diseases like Isovaleric acidemia (IVA), 3-Methylcrotonyl-CoA carboxylase deficiency or MethylcrotonylGlicinurie (MCC), Biotinidase (BIO) because on the other hand, false-negative cases have been reported by several newborn screening laboratories. We found that in experimental conditions developed specifically is that the following picture of metabolic alteration:

- ∞ IVA increase 3-OH-IVA, IVAGly
- ∞ MCG increase 3-OH-IVA, 3MCCGly
- ∞ BIO increase 3-OH-IVA, IVAGly, C3Gly

CONCLUSIONS: This method has the potential to markedly reduce false-positive results and the associated costs and anxiety. It may also be suitable for diagnosing and routinely monitoring blood spots IVA, BIO, MCC.

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8.3

EFFECTS OF HISTONE DEACETYLASE INHIBITORS ON ENERGY METABOLISM

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Recent experimental evidences reported in the literature have revealed that the control of metabolic pathways occurs at the transcriptional level also through epigenetic mechanisms, and particularly by chromatin remodeling. Previous results obtained in our laboratory showed that the treatment with histone deacetylase inhibitors (HDACi) induces the increase in CYP7A1 gene expression, a key actor in cholesterol homeostasis, in mice and the reduction in plasma cholesterol level. In addition we observed a net decreased concentration of serum triglycerides and of body weight, despite increased food intake.

Collectively, these changes could be due to increased fatty acid oxidation, altered fatty acid synthesis or increased energy expenditure. The aim of this work was to analyze the mechanisms underlying HDACi effects in the liver and in the muscle fibers, which are deeply involved in the regulation of the energy metabolism.

We first analyzed in the liver of treated mice the expression levels of relevant genes and we observed a general increase in mRNA levels of genes involved in lipid catabolism and its regulation, like LCAD, Fatty acid traslocase (CD36) and Apolipoprotein CII (ApoC-II), while we found decreased mRNA levels of ACC1, the rate limiting enzyme in lipogenesis. Interestingly, we observed a strong up-regulation of PGC-1α, a key player in mammalian energy metabolism involved in mitochondrial biogenesis. These data can also explain the net decrease in the triglyceride content observed in the liver of mice treated with HDACi.

Also in C2C12 myotubes, a murine model of muscle fibers we observed a strong increased expression of PGC-1α after HDACi treatment, which could contribute to improved oxidative ability. We observed in fact the upregulation of important genes involved in oxidative phosphorylation (Cyt C) and in lipolysis (MCAD, VLCAD) and CD36 in treated myotubes. Western Blot analysis also revealed that HDACi transiently increased AMPK phosphorylation, a signal that disappeared after several hours of treatment. We also reported an increased mRNA level of the insulin dependent glucose transporter GLUT4, whose expression and traslocation can be regulated by PGC-1α and AMPK.

These results suggest that HDACi treatment mimics an energetic stress condition to which cells respond with an increased demand of energy substrates, increased oxidation rate and enhancing mitochondrial biogenesis.

Administration of HDACi to mice fed high-fat diet, a model of insulin resistance, improves glucose tolerance test, thus suggesting that HDACi may be effective in reducing hyperglycemia.

In conclusion, HDACi reveal that the acetylation state of histones and other DNA associated proteins has a key role in the trascriptional regulation of genes involved in energy metabolism and therefore could be valuable tools to study molecular mechanisms implicated in metabolic diseases.

8.4

PHARMACOLOGICAL POTENTIAL OF INHIBITORS OF 6-PHOSPHOGLUCONATE DEHYDROGENASE FROM *T. brucei*: DIFFERENTIATION OF SUBSTRATE ANALOGUES FROM REACTION-INTERMEDIATE ANALOGUES BY ISOTHERMAL TITRATION CALORIMETRY

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6-Phosphogluconate dehydrogenase (6PGDH) is a validated target to develop new drugs against African trypanosomiasis. Kinetic assays have shown that 4-phospho-D-erythronate (4PE) and 5-phospho-D-ribonate (5PR) are substrate competitive inhibitors with K_i under the K_m for 6PG and a good selectivity for the parasite enzyme compared to the mammalian 6PGDH. The binding of the inhibitors has been further characterized by isothermal titration calorimetry (ITC) and, while the K_d of 5PR was found equal to the K_i (1.35 μM), the K_d for 4PE was found much higher than the K_i (2.86 μM versus 0.13 μM). The difference between the two inhibitors is larger but reversed in the ternary complexes with either NADP and NADPH. The number of NADP binding sites in the ternary complex with 4PE and 5PR is one per dimer, as it is in the ternary complex of the enzyme with 6PG and the nonoxidizing coenzyme analogue 3-amino-pyridine adenine dinucleotide phosphate. Thus the enzyme functions in a asymmetric way as 6PGDH from other species. However, while in the presence of 5PR NADP shows the same K_d observed in the binary complex, in the presence of 4PE the K_d decreases by two orders of magnitude, from 7.54 μM to 43 nM. The same difference is observed in the binding of NADPH, whose K_d slightly increases in the presence of 5PR and decreases from 1.05 μM to 20 nM in the presence of 4PE. The binding of 4PE to the enzyme-NADP complex shows two sequential binding sites, the first site with K_d 177 nM, very close to the K_i value of the inhibitor determined kinetically, and the second site with K_d in the μM range as in the binary complex. The observed half-site reactivity toward NADP means that the coenzyme at high concentration acts as a competitive inhibitor toward the 4PE. The 177 nM K_d is so an apparent K_d, while the true K_d is 17.7 nM. We conclude that 5PR simply mimics the substrate and supports the conformational changes accompanying the formation of the ternary complex, while 4PE is an analogue of the tightly bound, high-energy intermediate of the reaction. The large decrease of the K_d observed also in the presence of NADPH suggests that the 4PE could be an inhibitor more effective under physiological conditions, where NADPH concentrations are high, rather than in the in vitro assay performed in the absence of NADPH. Thus 4PE appears a lead compound in development of compounds with better pharmacological properties. The main obstacle in the use of phosphorylated sugar analogues as therapeutic agents is the impossibility to cross the cell membrane. However a recent report shows that chemical modification of the phosphate group can overcome this obstacle.

8.5

USE OF HUMAN RECOMBINANT PROLIDASE TO INVESTIGATE THE ENZYME ACTIVE SITE.

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Introduction. The latest stage of protein catabolism, particularly of those molecules rich in imino acids such as collagens, requires the activity of prolidase, the only Mn^{2+} dependent metalloenzyme that cleaves the iminodipeptides containing a C-terminal proline or hydroxyproline. Mutations in the prolidase gene (PEPD) cause the recessive connective tissue disorder Prolidase Deficiency. Beside its intracellular functions, prolidase has an antitoxic effect against some organophosphorus molecules, can be used in dietary industry as bitterness reducing agent and recently has been used as target enzyme for specific melanoma prodrug activation.

Results. We recently generated recombinant human prolidase characterized by substrate specificity, optimum pH and temperature of activity and ion dependence similar to the endogenous enzyme[1]. In solution recombinant prolidase, with or without Mn^{2+} activation, is present mainly (80-99%) as homodimer of 123 kDa as determined by gel filtration chromatography. ICP mass spectrometry revealed the predominant presence of Mn and Zn. While the presence of Mn^{2+} ions was expected, the presence of Zn^{2+} was surprising and the two metals were detected at a well defined concentration ratio in several preparations: [Prolidase dimer]: [Mn]=1:1 and [Prolidase dimer]:[Zn]=1:4. The five-fold metal coordination is in disagreement with the published data available on prolidase enzyme in other organisms containing only four metal binding sites, but confirms a X-Ray diffraction experiment performed on human prolidase which is reported to bind five Na^{+} ions. X-Ray Absorption Spectroscopy (XAS) study of the metal binding site structure of the prolidase was also performed. A preliminary analysis of XAS data confirmed that the dimeric protein in solution is capable of binding Zn^{2+} ions and Mn^{2+} ion. Our data showed two more likely situations: first one with Zn in A1, A2 and C3 metal sites while Mn was in site B1; the second with Zn in A1, B1 and A2 sites while Mn was in C2 site.

Conclusion. The complete analysis of XAS data could clarify whether the two sites belonging to the same monomer are both omo-loaded (Zn^{2+}-Zn^{2+}) or whether instead one is omo- and the second one etero- (Zn^{2+}-Mn^{2+}) and where is the binding site for an eventual fifth ion.

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8.6

THE COMBINED MUTATION OF INTERFACIAL AND ACTIVE-SITE RESIDUES OF *TREPONEMA DENTICOLA* CYSTALYSIN LEADS TO A FOLDED PLP-BOUND MONOMER

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Cystalysin is a dimeric pyridoxal 5'-phosphate dependent lyase produced by the oral pathogen Treponema denticola[1]. Recent studies have evidenced that apo- and holo-cystalysin exhibit dramatic structural differences and that PLP strongly influences the dimerization properties of the enzyme[2,3]. Site-directed mutagenesis experiments have been undertaken with the aim of getting a folded monomeric form of the enzyme suitable for investigating the role of the coenzyme on the associative/dissociative process of cystalysin. Based on molecular modelling analyses, the interfacial residues L57 and L62, as well as the Y64* residue, hydrogen-bonded to the PLP-phosphate, have been mutated to alanine. The L57A, L62A, Y64*A, L57A/L62A, L57A/Y64*A, L57A/L62A/Y64*A variants have been constructed, expressed and purified. By size-exclusion chromatography, the effects of the mutations on the quaternary structure of apo- and holo-cystalysin have been elucidated. The results indicate that L57A mutation, more that L62A mutation, affects the quaternary structure of apocystalysin, while the Y64*A mutation only interferes with holocystalysin dimerization. Moreover, the L57A mutant, which is monomeric in the apo-form and dimerizes in the presence of PLP, has been chosen as a model to investigate the effect of coenzyme binding on the dimerization process. Data show that i) the L57A monomer is able to bind PLP, even if with an affinity lower than that of the dimer; ii) PLP induces the association of monomers in a slow-equilibrium process with a rate which parallels that of the regain of lyase activity. Finally, by a combination of the three mutations, a stable PLP-bound monomeric form of cystalysin has been generated. Kinetic analyses of this mutant indicate that: i) the lyase, racemase and D-alanine half-transaminase activities are suppressed in the monomer; ii) monomeric cystalysin retains an L-alanine half-transaminase activity whose catalytic efficiency value is increased by ~2-fold with respect to that of the wild-type enzyme.

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8.7

CYSTATHIONINE GAMMA-LYASE IN BOVINE LENS

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The relevance of proper glutathione (GSH) levels in maintaining lens transparency is well assessed. Cysteine (Cys) represents the limiting amino acid for the synthesis of GSH and its availability is controlled by the flux through the transulfuration pathway. This metabolic route allows the conversion of methionine into Cys and is composed of two sequential steps catalyzed by cystathionine beta-synthase and cystathionine gamma-lyase (CGL), respectively.

CGL, a PLP-dependent enzyme, catalyses the rate limiting step of the transulfuration pathway, i.e. the conversion of L-cystathionine into L-Cys. CGL has been shown to be essential in liver and kidney for an adequate supply of Cys for GSH synthesis, thus its deficiency plays a key role in GSH depletion in several physiological and pathological situations. Recently, it has been shown that the transulfuration pathway exists also in the lens and that can be up regulated in oxidative conditions. Moreover, it has been hypothized that CGL activity may be particularly relevant for Cys availability in the aging lens. Despite this potential crucial role of CGL in affecting the redox potential of the lens, very limited information is available on the CGL from lens. This study is devoted to the isolation of CGL from bovine lens, in order to perform a characterization of the enzyme, useful to disclose features of the enzyme able to modulate Cys availability for GSH synthesis.

Extremely low levels of CGL activity are detectable in bovine lens crude extracts, in which the Western blot analysis reveals the presence of a single protein cross-reactive with anti-CGL antibody, with a molecular weight, in denaturing and reducing conditions, of approximately 25 KDa. It is worth noting that, in the same conditions, CGL from sources different from bovine lens is detected as a 40 KDa band. After partial purification of the crude extract, also a 40 KDa band, cross-reactive with anti-CGL antibody becomes detectable. The enzyme activity seems to be associated only with the latter molecular species. The two CGL proteins can be completely separated by hydrophobic interaction chromatography, and their further characterization reveals that their molecular weight in native conditions is compatible with a homotetrameric structure.

A well detectable CGL activity can be measured in cultured bovine lens epithelial cells. The measured specific activity results two orders of magnitude higher than what observed in the whole lens. Moreover in epithelial cells only the 40KDa cross-reactive band is present.

The possibilities that in lens CGL undergoes limited proteolysis catalyzed by proteolytic activities not present (or present at low levels) in the epithelial cells, or that oxidative modifications occurring on CGL makes the enzyme susceptible to proteolysis, have been investigated.

8.8

STREPTOZOTOCIN-INDUCED DIABETES REDUCES ACTIVITY AND EXPRESSION OF MITOCHONDRIAL CITRATE CARRIER IN RAT LIVER.

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Diabetes mellitus is characterized by substantial alterations in hepatic intermediary metabolism. In diabetic state, reduced enzyme activities in the glycolytic and pentose phosphate pathways together with an increase in gluconeogenic, glycogenolytic and lipolytic pathways were observed[1]. In absolute insulin deficiency, the plasma concentration and turnover of fatty acids increase, whereas *de novo* fatty acid synthesis and proportion of fatty acyl-CoA that is esterified as opposed to oxidized, decrease[1]. Lipogenesis requires cooperation between mitochondrial and cytoplasmic enzymes and involves fluxes of metabolites across mitochondrial membranes[2]. Citrate carrier (CiC), an integral protein of the inner mitochondrial membrane, plays an important role in hepatic intermediary metabolism, translocating mitochondrial citrate into the cytosol, where it supplies acetyl-CoA for *de novo* fatty acid and cholesterol syntheses. The aim of the present work was to investigate the effect of streptozotocin-induced diabetes on mitochondrial CiC activity and expression in rat liver and to characterize the molecular step(s) of this hormonal modulation. The rate of citrate transport was reduced by ~35% in mitochondria from diabetic versus control rats. Kinetic studies in diabetic mitochondria showed a reduction in the V_{max} and almost unchanged CiC K_m value. Diabetes led to changes in mitochondrial membrane fatty acid composition. In particular, a noticeable decrease in arachidonic acid (C20:4, n-6) and a simultaneous increase in docosahexaenoic acid (C22:6, n-3) was detected in mitochondria from diabetic animals. In the latters, linoleic acid (C18:2, n-6) level significantly accumulated. In spite of these changes there was no significant variation in the sum of saturated and unsaturated fatty acids and in their unsaturation index. Mitochondrial phospholipid composition was not significantly affected, while an increase in the cholesterol amount and in the cholesterol/phospholipid ratio was observed. To deeply investigate into the mechanism responsible for the reduced CiC activity in diabetic state, molecular studies were performed. RNase protection assay and Western blotting analysis indicated that both hepatic CiC mRNA accumulation and protein level decreased similarly to the CiC activity. The reduced mRNA level and the lower content of the mitochondrial CiC protein, might account for the decline of CiC activity in diabetic animals. In the latters, CiC activity and expression changed in covariance with those of *de novo* fatty acid synthesis enzymes.

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8.9

SILDENAFIL INHIBITS THE ROS PRODUCTION BY XANTHINE OXIDASE

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Xanthine oxidase (XO) catalyzes the hydroxylation of a wide variety of substrates, including purines, pirimidines, pterins d aldehydes, to acids[1]. At relatively high oxygen pressure, it generates reactive oxygen species (ROS) as superoxides and hydroxyl radicals. The XO, detected in endothelial and epithelial cell outer surface, has been involved in ischemia/reperfusion injury[1,2]. Furthermore, XO-ROS production has been implicated in chronic hearth failure, inflammatory diseases, LDL oxidation, atherosclerosis, hypertension, cancer, aging[1].

Allopurinol, a hypoxanthine analogue developed as xanthine oxidase inhibitor 30 years ago, and oxypurinol, its oxidation product, have proved to be effective in the treatment of these conditions both in experimental animals and human clinical trials[1]. Recent studies have shown the significant benefits of sildenafil, an inhibitor of type 5 phosphodiesterase, in patients with pulmonary hypertension, and an endothelium enhancing effect in preconditioning prior to ischemia/reperfusion[3,4]. As allopurinol/oxypurinol and sildenafil exhibit a marked structural analogy, we assayed the effect of this drug on the purified enzyme and in human prostatic cell cultures. The 80-100% inhibition of the ROS production by the enzyme bound to the external membrane of prostatic cells suggests that this mechanism may be of primary importance for the protective effects of the drug on epithelial cells.

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9.1

PROTEOGLYCAN UNDERSULFATION OF THE GROWTH PLATE IN A SULFATE TRANSPORTER KNOCK-IN MOUSE

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Mutations in the *diastrophic dysplasia sulfate transporter (DTDST or SLC26A2)* cause a family of recessively inherited chondrodysplasias including, in order of decreasing severity, achondrogenesis 1B, atelosteogenesis 2, diastrophic dysplasia (DTD) and a recessive form of multiple epiphyseal dysplasia. The gene encodes a widely distributed sulfate/chloride antiporter of the cell membrane. Its function is crucial for the uptake of inorganic sulfate, which is needed for sulfation of the glycosaminoglycan (GAG) chains of proteoglycans (PGs). We have already demonstrated undersulfation of GAGs in articular cartilage from patients and from a dtd mouse model generated in our laboratory. However, cartilage sulfation at the growth plate level has never been measured due to the low amount of tissue available and the difficulties in isolating this small area.

In this work we measured PG sulfation of the growth plate on mutant dtd and wild-type mice and we checked whether undersulfation affects chondrocyte proliferation.

The growth plate was obtained from sections of the tibia of 2 and 3 weeks old mice by manual microdissection and disaccharides, released from PGs after enzymatic digestion, were labeled with a fluorescent dye and analysed by FACE (fluorophore assisted carbohydrate electrophoresis) or reverse phase HPLC. Results obtained with both techniques demonstrated a significant undersulfation of the dtd growth plates compared to those of wild-type animals.

Chondrocyte proliferation was measured in 3 weeks old mutants and wild-type mice injected intraperitoneally with bromodeoxyuridine (BrdU). Animals were sacrificed 2 hours after injection. BrdU incorporated during the labeling time into replicating cells was detected by immunohistochemistry on the tibia growth plate. Our results demonstrate a significant decrease of chondrocyte proliferation in the growth plate of mutant 3 weeks old mice compared to wild type animals, demonstrating that PG undersulfation affects chondrocyte proliferation/differentiation causing reduced bone growth.

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9.2

EXPRESSION AND SUBCELLULAR LOCALIZATION OF SIALIDASE NEU3 IN MDCK CELLS.

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Among the four different forms of mammalian sialidases, Neu3 is characterized by its high substrate specificity toward gangliosides and by its association to cellular membranes. Moreover, Neu3 is known to co-fractionate with caveolin-1, indicating its presence inside lipid rafts. Recently we demonstrated that Neu3 behaves as a peripheral membrane protein and, in COS7 and HeLa cells, is associated with the extracellular leaflet of the plasma membrane and co-localizes with markers of the endocytic route[1]. The latter observation suggests that the protein cycles between the plasma membrane and the endosomal compartment.

Under specific growth conditions, MDCK cells can differentiate into a polarized cellular monolayer where the plasma membrane can be distinguished into an apical and a basolateral domain. Interestingly, the protein and lipid composition of the two domains is different and unique. Thus, in MDCK cells protein delivery to the apical or basolateral membranes is a specifically controlled cellular mechanism.

We expressed Neu3 in MDCK cells by stable transfection of the murine form of the protein and characterized transfectants by immunofluorescence and confocal microscopy analysis. As expected, indirect immunofluorescence experiments demonstrate that in non-polarized MDCK cells Neu3 localizes at the plasma membrane and in endosomes, with a specific enrichment of the protein at the cell-cell contact areas. When MDCK cells were allowed to polarize, Neu3 was detected only at the basolateral domain and, more specifically, at the lateral side of the membrane where cell-cell interactions take place. Moreover, intracellular vesicular structures labeled for Neu3 were found, especially enriched at the basal portion of the polarized monolayer. Association of Neu3 to lipid rafts was also investigated. When MDCK cells were grown in a non-polarized system a small, though significant, amount of Neu3 was found in light Optiprep density gradient fractions, corresponding to the microdomains enriched fractions. Analysis of the distribution of the protein after polarization showed that Neu3 does not co-fractionates with caveolin-1 positive light fractions.

Our results suggest that Neu3 undergoes specific mechanisms of delivery to the basolateral membrane of polarized cells. Whether the plasma membrane and the endosomal pools of the protein are in a dynamic equilibrium remains to be investigated. Experiments are in progress in our laboratory in order to identify possible mechanisms that regulate Neu3 delivery to the cell surface and exposure to the cell-cell contact areas.

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9.3

PURIFIED HUMAN GLYCOSAMINOGLYCANS REDUCED NF-kB ACTIVATION, PRO-INFLAMMATORY CYTOKINE PRODUCTION AND APOPTOSIS INDUCED BY LPS IN MOUSE CHONDROCYTES.

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Chondroitinsulphates (CS) are the main glycosaminoglycan (GAGs) component in human organism, and of plasma GAGs. A significant increase, with respect to normal values, of plasma GAG concentration was observed in patients with different types of diseases, although the meaning of the change is still unclear[1]. CS may modulate inflammatory responses and ,especially chondroitin-4-sulphate (C4S) exerts antioxidant activity[2]. Interleukin 1beta (IL-1β), interleukin-6 (IL-6), tumor necrosis-alpha (TNF-α), and interferon-gamma (IFN-γ) are pro-inflammatory cytokines that play an important role in immune regulation and inflammatory processes by inducing the expression of many effector proteins, such as cytokines/chemokines, inducible nitric oxide synthetase (iNOS) and matrix metalloproteinases (MMPs)[3]. Excessive and/or dysregulated activity of these mediators is associated with tissue destruction and cell death. Nitric oxide (NO) is spontaneously produced by chondocytes in osteoarthritis, and contributes to progressive cartilage degradation[4].

The aim of this study was to evaluate the effects of GAGs, purified from normal human plasma, on reducing inflammation in a model of LPS-induced increase in pro-inflammatory cytokines in mouse articular chondrocyte cultures. Chondrocyte stimulation with LPS for 24 hours generated high TNF-α, IL-1β, IL-6, IFN-γ gene expression and their related protein levels, nuclear factor kB (NF-kB) activation, IkBα phosphorylation and apoptosis evaluated by the increase in caspase-3 expression and its related protein amount. LPS treatment generated also high concentrations of iNOS and the detrimental oxidant species NO evaluated in terms of nitrites and nitrates.

Purified human GAGs, at three different doses, reduced inflammatory cytokines and iNOS produced by chondrocytes at mRNA and protein levels, blocked NF-kB activation and cytoplasmatic IkBα phosphorylation, limited cell death by inhibiting apoptosis, and finally reduced the NO concentrations. By capillary electrophoresis analysis of purified human plasma GAGs unsaturated disaccharides, C4S was identified as the main component. The positive modulatory effect exerted by plasma GAGs on all considered parameters may be due to its efficiency to bind protein structures thereby exerting an inhibitory activity. These results further support the hypothesis that circulating GAGs may function as immunomodulators and their increased release and degradation could be a biological response that acts to modulate inflammation during disease.

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9.4

THE ANTIOXIDANT ACTIVITY OF CHONDROITIN-4-SULPHATE, IN CARBON TETRACHLORIDE-INDUCED ACUTE TOXIC HEPATITIS IN MICE, INVOLVES NF-kB AND CASPASE ACTIVATION

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Although the pathogenesis of acute cirrhosis is not fully understood, it is clear that ROS play a key role in pathological changes in the liver[1]. Carbon tetrachloride (CCl_4), a hepatotoxin, has been used extensively to induce liver injury in various animal models. The experimentally induced cirrhotic response by CCl_4 in rats and mice has been shown to be similar to human cirrhosis of the liver[2].

The up-regulation of the nuclear factor kB (NF-kB) has been implicated in the pathogenesis of several diseases, including inflammation and liver diseases. Animal models of acute hepatic damage support a role for NF-kB in the development and progression of cirrhosis. Although oxidants are commonly considered to exert their effects via direct toxic action on target cells, recent findings suggest their contributory role in gene induction. NF-kB may be activated by low levels of ROS and inhibited by antioxidants[3].

Glycosaminoglycans (GAGs), especially chondroitin-4-sulphate (C4S), show antioxidant activity [4].

Aim of this study is to investigate whether the administration of C4S, besides exerting antioxidant activity, is able to modulate NF-kB and apoptosis activation in CCl_4-induced liver injury in mice.

Acute toxic hepatitis was induced in mice by an intraperitoneal injection of 1.0 ml/kg of CCl_4 in mineral oil. Varying doses of C4S were administered intraperitoneally 1 h before, 6 and 12 h after CCl_4 injection. 24 h after CCl_4 injection, the mice were sacrificed and biochemical and histological analysis performed.

CCl_4 injection produced: marked elevation of alanine and aspartate aminotransferases; hepatic membrane lipid peroxidation, assayed by 8-isoprostane levels; depletion of the antioxidants GSH and SOD activity; NF-kB translocation and reduction of IkBα; increase in metalloproteinase(MMP)-2, MMP-9, caspase-3 and caspase-7 gene expression and their related protein; induction of liver polymorphonuclear infiltration, evaluated by elastase assay, and hepatic cell disruption.

C4S administration: inhibited lipid peroxidation; blocked NF-kB activation and IkBα protein loss; reduced mRNA MMPs and caspase expression and their related protein; restored endogenous antioxidants; limited hepatic polymorphonuclear accumulation and hepatic damage.

This study is a further confirmation that the antioxidant effect of C4S, and show that C4S is able to inhibit NF-kB and apoptosis activation, due to the CS induced decrease of ROS production.

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9.5

NEU4L SIALIDASE ACCELERATES THE PROLIFERATION RATE IN THE HUMAN NEUROBLASTOMA CELLS, SK-N-BE, THROUGH CELL CYCLE DEREGULATION

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Neuroblastoma is a highly malignant tumour derived from neural crest cells that accounts for approximately 50 % of childhood cancer death. Among several typical genetic changes of this disease, the amplification of the oncogene MYCN is significantly associated to a poor prognosis[1]. In this report, we demonstrated that the expression of the long form of the sialidase Neu4 (Neu4L) is connected to the distorted control of proliferation characteristic of neuroblastoma and, in particular synergizes with MYCN to accelerate the G1/S phase progression.

The human neuroblastoma SK-N-BE cell line, known to possess MYCN amplification, was transfected with Neu4L cDNA. The first important consequence was a marked acceleration of the proliferation rate assessed by an increase of [3H]thymidine incorporation (+ 45%) and by cell count for up to 4 days (+ 36%). After cell synchronization through serum deprivation, Neu4-L over-expressing SK-N-BE cells failed to block in the G1 phase, in contrast to mock cells and proceeded, massively, to the S phase. Important alterations concerning the expression of the main molecules which rule the transition into the S phase could explain the bypassing of the G1/S checkpoint: the activation of MAPK cascade, the decrease of the cyclin dependent kinase (CDK) inhibitor p21 and p27, the activation of CDK2, and the phosphorylation of Rb. These events triggered the transcription of key S-phase-promoting genes such as cyclin A1 (+ 83%) and cyclin E (+ 61%). Significantly, also the expression of MYCN resulted to be more expressed (+ 36%). The direct substrates of Neu4L in SK-N-BE cells seem to be soluble glycoproteins in the range of 60 kDa. Therefore, the expression of Neu4L in neuroblastoma cells could synergize and further enhance MYCN cell cycle alteration, through the desialylation of key glycoproteins which possibly concur to regulate the MEK-ERK axis. These findings suggest that Neu4L expression is directly correlated to the malignancy degree of neuroblastoma.

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9.6

A GLANCE AT THE PHOSPHOPROTEOME OF AZT-TREATED K562 CELLS.

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Post translational modifications of proteins are considered to be one of the major determinants regarding the complexity of higher organisms[1]. More than 200 different types of post translational modi-fications are known[2] of which only a limited number are reversible and important for the regulation of biological processes. Among these modifications, the most studied is protein phosphorylation.

The aim of the present investigation was to identify the main differentially expressed phosphoproteins in human erythroleukemia cells (K562) upon treatment with AZT, the first anti-retroviral drug approved and yet used in combined AIDS therapy[3]. For this purpose we used K562 cells untreated (control), or exposed to 20 μM AZT for 3 h. This drug concentration was chosen because is higher but not far from that found in the blood of AIDS patients under AZT therapy. In addition, AZT concen-tration and treatment time were selected also to enhance the changes of new proteins under drug exposure to obtain the major differences between control and treated samples without affecting cells growth and cell viability. Then, by using specific biotinylated mAb (anti-phospho-serine, anti-phosphothreonine, anti-phosphotyrosine) and by means of ELISA, FACS, and confocal micro-scopy a generalized screen was performed on the *O*-phosphorylation level. In particular, the main results we obtained are the following:

a) an increase of *O*-phosphorylation level in K562 AZT-treated cells when analyzed by ELISA was observed (about 20%). However, after cells permeabilization, as FACS and confocal microscopy analyses revealed, the increase was mainly due to the intracellular phospho-proteins; in fact, AZT-treated but non-permeabilized cells showed a decrease of surface *O*-phosphorylation (about 12%).

b) proteomic analysis based on two-dimensional gel electrophoresis and subsequent protein identification by MALDI-ToF-MS, evidenced 27 phosphoproteins diffe-rentially expressed in AZT-treated K562 cells (up- or downregulated). Many of the differentially expressed proteins found in this study may be associated with important cellular activities such as protein biosynthesis, folding and degradation, energetic metabolism, cyto-skeleton motility, nucleotide biosynthesis and salvage, detoxification and redox processes and signal trans-duction.

Thus, many protein components of relevant cellular metabolic processes may be involved in AZT treatment of HIV-affected patients. Our findings also indicate that the proteomic approach could represent a simple and useful tool for monitoring cellular AZT response(s) which could be employed as a potentially useful implement in the management of HIV patients under antiretroviral therapy.

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9.7

SODIUM CARBONATE TREATMENT CAUSES THE RELEASE OF ACIDIC SIALIDASE PRESENT ON HUMAN ERYTHROCYTE MEMBRANE

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Sialidases or neuraminidases (EC 3.2.1.18) are enzymes widely distributed in nature from viruses to vertebrates. They play a key role in the metabolism of sialoglycoconjugates. In particular, they are able to remove sialic acid residues from gangliosides, sialoglycoproteins and sialoligosaccharides. It is becoming more and more evident that the partial desialylation of senescent erythrocyte membrane sialoglycoconjugates constitutes a primary or preliminary signal for erythrophagocytosis. The removal of sialic acid from the sialoglycoconjugates is assumed to be promoted by the sialidases present on the membranes. The presence of two sialidases in human erythrocytes has been previously reported. One acts optimally at acidic pH (4.2-4.7) and the other at neutral pH. In order to identify the exact nature of these two sialidases and the enzyme-membrane leaflet interaction, we incubated the erythrocyte membranes with different solutions. We obtained interesting results when we treated the membranes with 0,1 M sodium carbonate buffer pH 11,5. In literature it is reported that sodium carbonate treatment is one of the methods used to discriminate between peripheral and integral cell membrane proteins (Fujiki et al., 1982). The sodium carbonate treatment led to the release of 50% acidic sialidase into the supernatant, whereas the neutral sialidase remained totally in the pellet. Our results suggest that only alkaline pH is responsible for the release of this sialidase from the membrane and that the enzyme is a peripheral protein. Then we analyzed the stability of the acidic sialidase released after the treatment: the enzymatic activity decreases by 40% after 24 h at 4°C. The enzyme looses 25% of its activity after the first freeze-thaw cycle, while seems to be stable after the other five freeze-thaw cycles. The released enzyme has been analyzed also using polyclonal anti-Neu1 antibodies. The western blot analyses reveal that Neu1 sialidase is present on the erythrocyte membranes and that the alkaline treatment causes the partial release of this sialidase from the membranes. This is the first evidence of the presence on erythrocyte membranes of Neu1 sialidase, an enzyme that is usually located in a multienzymatic complex in the lysosomes of mammalian cells. So we investigated if the multienzymatic complex was present on erythrocyte membranes using antibodies against the other components of the complex. Our results suggest that Neu1 sialidase is alone on the erythrocyte membrane and it does not need the other complex components to expound its enzymatic activity.

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9.8

SILENCING OF THE MEMBRANE SIALIDASE NEU3 TRIGGERS MEGAKARYOCYTIC DIFFERENTIATION IN CHRONIC MYELOID LEUKEMIC CELLS, K562, THROUGH THE INCREASE OF GANGLIOSIDE GM3 CONTENT

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Chronic myeloid leukaemia (CML) is a paradigmatic example of neoplasia in which a differentiation arrest occurs during the myeloid lineage and a highly proliferative malignant clone originates[1]. In the context of cancer disease and differentiation, an ever-increasing interest is being focused on sialidases and sialoglycoconjugates since alterations in these fields are directly interconnected to neoplastic transformation[2]. In particular, the plasma-membrane associated sialidase Neu3 over-expression, reported in several tumours, is linked to apoptosis resistance phenomena. In this report, we demonstrated that the silencing of Neu3 in the CML K562 cells decreases proliferation rate and apoptosis resistance in favour of a differentiation process.

K562 cells were transduced with a shRNA targeting the coding region of Neu3, inserted in a lentiviral vector. Neu3 silencing (-70 % as protein content and – 93 %, as catalytic activity) gave rise to significant events. First of all, cyclin D2 and Myc were much less expressed (- 40 % and – 30 %, respectively) while p21 increased (+ 60 %); as consequence, cell growth and [3H]thymidine incorporation (- 47 %) diminished. Apoptosis resistance toward chemotherapeutic molecules such as etoposide and staurosporine decreased, accordingly to a concurrent decrease of the anti-apoptotic protein Bcl2 (- 30 %) and to an increase of the pro-apoptotic proteins Bax and Bad (+ 17 % and + 32 %, respectively). Moreover, K562 cells became able to differentiate toward the megakaryocytic lineage as proved by the appearance of the megakaryocytic markers CD10, CD44, CD41, CD61. This important cascade of events was triggered by the activation of the signalling pathways PLC-b2, PKC, RAF, ERK1/2, RSK90, and JNK. The molecular connection between Neu3 silencing and the activation of PLC-b2 has to be searched in the significant increase of GM3 (+ 81 %), as demonstrated by the treatment of K562 cells with brefeldin A which simulated a GM3-rich conformation. Therefore, these results indicate that Neu3 plays a decisive role in CML and could be an interesting target for developing therapeutic strategies.

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9.9

NEU3 SIALIDASE STRICTLY MODULATES GM3 LEVELS IN SKELETAL MYOBLASTS C2C12 THUS FAVORING THEIR DIFFERENTIATION AND PROTECTING THEM FROM APOPTOSIS

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Membrane-bound sialidase NEU3, often referred to as the “ganglioside sialidase”, has a critical regulatory function on the sialoglycosphingolipid pattern of the cell membrane, with an anti-apoptotic function, especially in cancer cells. Although other sialidases have been shown to be involved in skeletal muscle differentiation, the role of NEU3 had yet to be disclosed. Nevertheless, NEU3 involvement seems quite plausible, as the enzyme has a critical regulatory function on the sialo-glycosphingolipid pattern of the cell plasma membrane. For instance, NEU3 of COS7 cells is able to modify the sialo-glycosphingolipid pattern of adjacent cells, supporting its involvement in cell-cell interactions[1]. On these bases, we decided to investigate the effects of NEU3 on muscle differentiation by constitutively silencing NEU3 with small interference RNA (shRNA), using murine C2C12 myoblasts as the cell model. Our results show that: (a) the induced down-regulation of the enzyme in murine myoblasts C2C12 completely inhibited their ability to enter the differentiation process; (b) upon induction of differentiation, or when grown to confluence, NEU3-silenced myoblasts underwent a massive apoptotic cell death; (c) NEU3 silencing caused EGFR inhibition and down-regulation due to the increased levels of endogenous ganglioside GM3; (d) supplementation of GM3 in the culture medium of wild-type C2C12 strongly reduced their differentiation capability; (e) NEU3-silenced myoblasts, when co-cultured with wild-type C2C12, re-acquired the capability to differentiate and fused to form MHC-expressing myotubes.

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9.10

CHARACTERIZATION OF HUMAN LYSOSOMAL a-D-MANNOSIDASE IN LEUKAEMIC AND NON LEUKAEMIC CELLS

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Lysosomal a-D-mannosidase (EC 3.2.1.24) is an exoglycosidase involved in the ordered degradation of N-linked oligosaccharides. Lack of a-D-mannosidase activity leads to the lysosomal storage disorder a-mannosidosis (MIM No 248500), characterized by neurological symptoms. Abnormal levels of a-D-mannosidase activity have been previously reported in some pathological conditions, such as cancer (Hakomori S., PNAS 99: 10231–3) and neurological disorders. We observed that a-D mannosidase enzymatic activity is high in blasts from Acute Myeloid Leukaemia (AML) M2 and M3 subtype patients, and in cell lines derived from a AML-M2 patient, such as HL60. Gene expression analysis demonstrated increased levels of MAN2B1 transcript in HL60, as compared to HEK cells used as non leukaemic control. To elucidate molecular events underlying human lysosomal a-D-mannosidase expression, we characterized a-D-mannosidase promoter. Regulatory regions crucial for promoter activity were determined by reporter gene assay in HEK cells and highest promoter activity was found in the region -101/-53 with respect to the first ATG. Mutational analysis confirmed that this region was of basic importance for MAN2B1 gene promoter activity. Electrophoretic mobility-shift assays demonstrated that Sp1 bound to this sequence in both HEK and HL60 cells but in HL60 other transcription factors binding upstream are responsible of the increased level of a-D-mannosidase transcript.

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9.11

RAS MUTANTS SPECIFICALLY REGULATES LYSOSOMAL GLYCOHYDROLASES ACTIVITY IN DIFFERENT CELL MODELS

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Ras is a monomeric GTPase which transduces signals from membrane receptors to downstream effectors, thus regulating transcription factors state of activation. Several Ras mutants have been characterized. In particular, RasV12 is a constitutively active Ras protein, while double mutants RasV12S35, RasV12G37 and RasV12C40 are loss of function mutants that maintain the ability to activate specific downstream effectors, i.e. MAPK, RalGEF and PI3K respectively. Previous investigations showed that active Ras up-regulates lysosomal glycohydrolases a-D-mannosidase, b-D-galattosidase and b-D-hexosaminidase in human primary fibroblasts. To investigate signal transduction pathways linking Ras activation to lysosomal enzymes activity, we prepared plasmid and retroviral vector constructs encoding RasV12, RasV12S35, RasV12G37 and RasV12C40 to express transgenes in a transient and stable manner. We tested these constructs in different human cell lines, fully differentiated (fibroblasts), immortalized (HEK, HUDE) and of tumor origin (MCF7). Transfected cells were analysed for their lysosomal glycoidrolases activity content with fluorogenic substrates (4MU-a-mann, 4MU-GlcNAc, 4MU-b-gal). Results demonstrated that lysosomal glycohydrolases were differently regulated. a-D-mannosidase activity was significantly increased only in primary fibroblasts over expressing RasV12 while b-D-galactosidase and b-D-hexosaminidase activity was specifically up regulated by double mutants in a cell model depending manner. Taken together, these evidences demonstrated that lysosomal enzyme activity is regulated by Ras activation through specific signaling pathways in different cell models. Interestingly, they show that Ras oncogene double mutants mainly affect the activity of b-D-galactosidase and b-D-hexosaminidase, both involved in glycosphingolipid catabolism.

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9.12

THE CYTOKINE REDUCTION BY GLYCOSAMINOGLYCANS IN LPS-STIMULATED MOUSE CHONDROCYTES MAY INVOLVE TOLL-LIKE RECEPTOR-4 MODULATION

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Toll-like receptors (TLRs) ligand activation elicits production of pro-inflammatory cytokines. The role of TLRs in the exacerbation of the inflammatory response has been postulated. TLR-4 has a key role in the inflammation induced by lipopolysaccharide (LPS). TLR-4 activation by LPS involves many adapters, as the myeloid differentiation primary response protein (MyD88) and the tumor necrosis factor receptor-associated factor 6 (TRAF6), involving the phosphorylation of several kinases that in turn led to liberation of nuclear factor kB (NF-kB)/Rel family members into the nucleus. Chain fragments of acid glycosaminoglycans (GAGs) hyaluronan (HA) and heparan sulphate (HS) are also ligands able to activate TLR-4 [1,2].

Both HA and HS, and other GAGs, as chondroitin-4-sulphate (C4S) and chondroitin-6-sulphate (C6S), are suggested to protect cells from oxidative stress[3]. It was also reported that HA and C4S were able to reduce cell damage by inhibiting the NF-kB and apoptosis[4,5].

The aim of this study was to investigate whether GAGs may exert anti-inflammatory activity due to an antagonist effect on TLR-4 receptor in a model of LPS-induced increase of pro-inflammatory cytokines in mouse articular chondrocyte cultures.

Chondrocyte stimulation with LPS for 24 hours increased TLR-4, MyD88 and TRAF6 mRNA expression and their related proteins, induced NF-kB activation and generated high levels of tumor necrosis factor alpha (TNF-α), interleukin beta (IL-1β), and the inducible nitric oxide synthase (iNOS), evaluated in terms of gene expression and protein production.

The treatment of chondrocytes with two different doses (25 and 50 µg/ml) of HA, C4S, C6S and HS produced various effects: HA reduced MyD88 and TRAF6 levels and NF-kB activation with the higher dose only, and exerted a very low anti-inflammatory effect; C4S, and to less extent C6S, significantly inhibited MyD88, TRAF6 production and NF-kB activation and the inflammation mediators TNF-α and IL-β; iNOS expression and activity were also significantly reduced; HS, like C4S, was able to significantly reduce MyD88, TRAF6 and NF-kB activation and inflammation. The use of a specific TLR-4 blocking antibody confirmed these results, suggesting that the inhibitory effect exerted by GAGs on NF-kB activation may follow the block of TLR-4. Since GAGs are able to bind a variety of biological molecules, especially proteins, the block of TLR-4, together with their antioxidant activity and an eventual direct inhibition of NF-kB, may represent a further step of GAG fine modulation of the inflammatory mechanism.

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9.13

STIMULATION/BLOCK OF CD44 RECEPTOR DURING INFLAMMATION BY HYALURONAN AT DIFFERENT MOLECULAR SIZE

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Hyaluronan (HA) plays a dynamic regulation during inflammation[1]. In its native state, HA exists as high molecular weight (MW) polymer, in excess of 10^6 Da. However, shorter HA chains are frequently found, with different biological effects on cells and tissues. Low MW HA showed high inflammatory activity, while high MW HA a marked anti-inflammatory effect[2,3]. HA fragments elicit the expression of pro-inflammatory cytokines, inducible nitric oxide synthase (iNOS) and metalloproteases (MMPs) through a mechanism involving the cell-surface receptor for HA, CD44. The CD44 stimulation activates protein kinase C (PKC) family that in turn activates the transcriptional nuclear factor kB (NF-kB), responsible of the expression of inflammation mediators such as tumor necrosis factor alpha (TNF-α), interleukin beta (IL-β) and iNOS[4]. CD44 interaction with its ligands strongly depends upon PKC which modulates the phosphorylation state of CD44 and the various anchoring proteins[5]. The aim of this study was to investigate the effects of HA at different MW (50, 1000 and 5000 kDa) on inflammation and especially on PKC expression in a model of phorbol 12-myristate 13-acetate (PMA) -induced PKC/inflammation in mouse articular chondrocyte cultures.

High CD44 and $PKC_{\delta\delta}$ and PKC_{η} mRNA and their related proteins levels were found in chondrocytes 24 hours after PMA treatment. NF-kB up-regulation, and increased levels of TNF-α, MMP-13 and iNOS gene expression and their related protein production were also induced by the PMA stimulation.

The treatment of chondrocytes with HA at various MW (two doses for each MW, 0.15 and 0.30 mg/ml) produced different effects: low MW HA up-regulated CD44 expression, increased $PKC_{\delta\delta}$ and PKC_{η} mRNA levels and protein production, and enhanced the inflammatory activity in untreated chondrocytes; while in PMA-treated cells it increased CD44, $PKC_{\delta\delta}$ PKC_{η}, NF-kB, TNF-α, MMP-13, and iNOS with enhancement of PMA effects; medium MW HA did not exert any inflammatory activity in untreated chondrocytes and was unable to reduce PMA effects; high MW HA had no effect in untreated chondrocytes while it reduced $PKC_{\delta\delta}$ PKC_{η}, NF-kB activation and all the inflammatory mediators stimulated by PMA treatment. The use of a specific CD44 blocking antibody supported the results.

These data confirm the multifactorial role played by HA in living organisms and especially its regulatory effect exerted at different states of aggregation. The HA stimulation/block of CD44 during inflammation needs to be taken into account to develop new strategies against inflammation.

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10.1

PROTEIN CHANGES INDUCED BY GROUP B STREPTO-COCCUS INFECTION OF PERITONEAL MACROPHAGES

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Group B Streptococcus (GBS), a pathogen involved in serious neonatal infections, has evolved different strategies to evade immune defences of host organisms. For example, interaction of GBS with macrophages causes defects in membrane permeability, massive increase in intracellular calcium and apoptosis, paralleled by profound changes in protein expression.

In order to investigate proteome alterations induced in this host-pathogen model, mouse peritoneal macrophages (3×10^6 cells) were infected either with active 3×10^8 GBS cells or with heat-inactivated GBS. Non infected macrophages, incubated in the same conditions, were used as control cultures (C).

After two hours, macrophages were recovered and repeatedly washed with PBS. Macrophage proteins, solubilised in a small volume of aqueous solution containing chaotropes and amphionic detergent, were separated by two-dimensional electrophoresis (2DE).

Preliminary tests were performed to demonstrate that contamination of protein preparations by streptococcal proteins was negligible using the solubilisation procedure outlined.

Sypro-Ruby stained gel images obtained from three separate experiments were analysed by using a dedicated software (PD-Quest, BioRad). After normalisation, matching polypeptide spots whose intensity was significantly different ($p<0.05$ Kruskal-Wallis test) in the three experimental groups were submitted to trypsinisation and identified by LC-MS/MS.

The expression of 74 spots, corresponding to 61 unique proteins, was significantly affected by the interaction with GBS.

The identified proteins were clustered according to Gene Ontology (GO) categories by using web tools for functional annotation (Babelomics, DAVID Bioinformatics Resources) with the aim of identifying common pathways involved in cell-pathogen interaction.

About 30% of the polypeptides whose expression was changed in macrophages following GBS infection, are subunits of mitochondrial proteins.

Significant alterations of the expression of proteins involved in regulation of metabolic processes were induced by co-culturing macrophages with GBS. The abundance of selected enzymes and coenzymes involved in glucose and carboxylic acid metabolism was significantly lowered in GBS-infected macrophages.

In addition, DNA-binding proteins and proteins involved in nucleocytoplasmic traffic as well as proteins implicated in intracellular signalling cascades, cell communication, intracellular transport, cytoskeletal organisation, antioxidant defense, calcium-binding and apoptosis were differentially expressed in GBS-infected macrophages and controls.

10.2

A FAST MUTAGENESIS APPROACH TO BYPASS INTERNAL AMBER STOP CODONS IN scFvs AND PURIFY RECOMBINANT ANTIBODIES

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Single chain fragment variable (scFv) antibodies are a great tool for understanding the immunological properties of molecules. They have been developed in the form of phage displayed proteins, allowing to rapidly select a battery of mono-specific and structure sensitive probes against the antigen of interests starting from a library of randomized Ig molecules containing hundreds of billions of individuals. The most recent and frequently used scFv libraries are the Tomlinson I+J one, available at www.geneservice.co.uk and based on a human framework. The selection is made possible in amber-suppressor *E.coli* strains (e.g TG1) that allows to bypass an amber stop codon between the coding sequences of the scFv and a specific protein of the phage envelope, generating a phage displayed fusion protein. After the biopanning, a simple infection by the selected phages of *E.coli* amber-sensitive strains allows to express and purify an scFv molecule in the form of a soluble protein of about 27 KDa.

From the time of their development a number of cases have been reported[1,2] in which additional amber stop codons have been found inside the scFv molecules (a natural and never obscured consequence of the maturation of the randomized libraries inside amber suppressor stains), leading to the impossibility of switching to soluble scFv purification in amber sensitive stains.

Here we report a simple and fast solution that allowed us to bypass internal amber stop codons and to express up to three milligrams/liter of soluble and fully functional scFv antibody.

Our procedure is based on the mutagenesis of the amber stop codon dividing scFv and pIII-phage protein from amber (TAG) to normal (TAA) stop codon. This single point mutagenesis is made by a couple of primers that can be considered as universal for the Tomlinson library, being the mutagenised region conserved in all the library sequence. We then transformed the mutagenised vector into XL1-Blue amber-suppressor strain (supplied by the mutagenesis kit from Stratagene) and induced, after proper set up of the conditions, the scFv over-expression with IPTG. Protein purification was with Ni-NTA affinity columns after sonication based bacterial lysis.

When tested by SDS-PAGE, western blot, dot blot, and ELISA, the purified scFv proved to be integer, stable and well performing as those resulting from classic expression protocols.

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10.3

EFFECT OF pH ON MMP-9 RELEASE BY LPS-STIMULATED NEUTROPHILS

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Acidosis and alkalosis are complications that may be causes of poor prognosis in critically ill patients. In particular, the mortality rate of patients that go under alkalosis conditions is greater than that in acidosis.

Certain neutrophil responses can be affected by changes in the external pH: extracellular acidic conditions enhance neutrophil proinflammatory responses triggered by conventional agonists[1].

Neutrophils have different kind of granules: primary granules are Myeloperoxidase (MPO) positive and MMP-9 negative; secondary granules contain lactoferrin, a little amount of MMP-9 and other molecules; tertiary granules contain the larger amount of MMP-9. The aim of this study is to verify if MMP-9 released by stimulated neutrophils may be higher in extracellular alkalosis respect acidosis conditions. This may have implications in severity of alkalosis and acidosis.

Neutrophils were isolated from buffycoats of healthy subjects by dextran sedimentation and Ficoll-Hypaque gradient centrifugation. Contaminating RBC were removed by hypotonic lysis. Cell pellets were resuspended in NaCl 0.9% and than counted. $2x10^6$ cells were placed in bicarbonate-buffered RPMI 1640 medium at various pH (7.0 to 7.8) with 1% heat-inactivated FBS and LPS 10 µg/ml and placed at 37 °C for 30 minutes; conditioned mediums were collected and stored at –80 °C until MMP-9 assay. Total MMP-9 was measured using commercially Activity Assay System.

Extracellular acidosis and alkalosis enhance neutrophil proinflammatory response. We have studied the release of MPO and MMP-9 “in vitro” in the pH range from 7.0 to 7.8. After LPS-stimulation, MPO released doesn’t show significative difference in the pH range studied. Instead, the amount of MMP-9 released increases both under acidic and basic conditions ($P<0.001$) compared to physiological pH (7.4). Furthermore, the amount of MMP-9 released at basic pH is greater than that released at acidic pH ($P<0.001$).We have also analysed the pH dependence of MMP-9 proteolytic activity. We have observed that in the pH range from 7.0 to 7.8 MMP-9 shows an higher activity when the pH is over 7.4 (physiological pH).

Neutrophil response to a proinfammatory stimulus is differentially affected by the environmental pH. While the release of primary granules seems to be insensitive to the analysed pH, the release of tertiary granules is sensitive to small pH changes and in particular when the pH shifts to slightly basic conditions.

The increased release of MMP-9 at higher pH together with its proteolytic activity dependence from the pH suggest that MMP-9 could be one of the adverse factors in the prognosis of alkalosis and acidosis conditions.

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11.1

DEFICIENCY OF POLYCYSTIN-1 LEADS TO UPREGULATION OF ADENOSIN A_3 RECEPTOR EXPRESSION IN HUMAN KIDNEY CELL LINES: POSSIBLE MODULATION OF CELL PROLIFERATION BY SPECIFIC RECEPTOR AGONIST.

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Autosomal Dominant Polycystic Kidney Disease (ADPKD) is mainly caused by mutations in the PKD1 gene encoding for polycystin-1 (PC1). PC1 is a large G−protein coupled receptor of the plasma membrane, playing important roles in cell-cell, cell-matrix interactions and signalling pathways. Between these latter, Ca^{2+} and cAMP signalling are particularly involved in the abnormal cell proliferation typical of PKD kidney cysts. In particular, ADPKD cystic cells respond to cAMP with increased cell proliferation through activation of the b-Raf/ERK pathway[1], and reduction of cAMP has been reported to normalize cell growth in cystic cells[1]. On the other hand, we have found that abnormally increased Ca^{2+} levels and oscillations result in increased cell proliferation, via PKCalpha and NFAT signalling, in PC1-depleted HEK293 cells and in PKD1-mutated cystic cell lines[2]. Thus, the discovery of molecules capable to inhibit the adenylyl cyclase (AC) or to normalize Ca^{2+} signals could function as new therapeutic agents for the ADPKD treatment.

Here we demonstrate that the PKD1 knockdown by siRNA in HEK293 cells is also associated to modulation of A_3 adenosine receptor expression, which are G-protein coupled receptors that negatively modulate AC activity. In particular, in PC1-depleted HEK293 cells as well as in PKD1-mutated cystic cells, we have found a significant increase in the expression of A_3 adenosine receptors. This A_3R increase caused a bigger inhibition of AC activity, after treatment with the selective agonist Cl-IB-MECA, in PC1-depleted than in control cells. Consistently, the Cl-IB-MECA stimulation, induced a higher reduction of cell proliferation in PC1-depleted cells compared to normal cells. The reduction of cell growth by Cl-IB-MECA treatment is associated to increase of $p21^{WAF1}$ expression and reduction in ERK1/2 kinase activity. Interestingly, the effects on ERK1/2 are reverted by the A_3 receptor antagonist, supporting the role of these receptors in the growth inhibition. Cl-IB-MECA could be, therefore, considered an interesting molecule in ADPKD treatment.

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11.2

ANTIOXIDANTS PREVENTS MITOCHONDRIAL DAMAGE AND CELL DEATH IN KERATINOCYTES FROM PERILESIONAL SKIN IN VITILIGO

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Vitiligo is a chronic acquired hypomelanotic disorder affecting 0,5%-2% of the world population. The two major pathogenetic hypotheses are focused on immune-mediated or toxic-mediated cell damage primarily directed on melanocytes. Recent experimental data underline the complex interactions that exist between melanocytes and other cells found in the skin. Among these cells, keratinocytes are able to influence both the survival and the functional activity of melanocytes.

Oxidative stress has been suggested to be the initial pathogenetic event in melanocyte degeneration[1] with H_2O_2 accumulation in the epidermis of patients with active disease. Defective recycling of tetrahydrobiopterin has been reported in vitiligo epidermis, associated to the intracellular production of H_2O_2. In addition, an alteration in the antioxidant pattern, with a significant reduction of catalase activity, has been demonstrated in both lesional and non lesional epidermis of patients[2], as well as in melanocytes. However, the antioxidant imbalance has been confirmed also in peripheral blood mononuclear cells of active vitiligo patients; it was correlated to an increased intracellular production of reactive oxygen species and appeared to be due to a mitochondrial impairment[3]. These findings support the concept of a possible systemic oxidative stress in vitiligo.

In order to gain insights on the involvement of oxidative stress in the pathogenesis of vitiligo, we have grown cultures of keratinocytes from lesional, perilesional and healthy skin, evaluating the presence of oxidative damage and apoptotic markers in the cells.

Results indicate that keratinocytes from perilesional skin show the features of damaged cells. Pretreatment with antioxidants inhibited caspase activation, increased total antioxidant capacity, repressed intracellular ROS generation, lipid peroxidation and ameliorated mitochondrial activity.

Our data, besides considering the achromic patch as the terminal event of a chain of biological processes which take place in the perilesional skin, give to keratinocytes a role of primary importance in vitiligo development. We suggest that the presence of an imbalance in the oxidant-antioxidant system might play a role in the pathogenesis of vitiligo and support the concept that free radical-mediated damage may be the initial pathogenic event in melanocyte degeneration in generalized vitiligo.

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11.3

CYTOSTATIC EFFECT INDUCED BY 2-CHLOROADENOSINE SENSITISES PC3 CELLS TO DOCETAXEL.

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2-Chloroadenosine (2-CADO) is an adenosine analog capable of inducing apoptosis in several cell lines by acting either via adenosine receptors or via uptake that is followed by metabolic transformations leading to nucleotide analogs. DNA-directed nucleotide analogs are antimetabolites effective in the treatment of a variety of malignancies. Docetaxel-based chemotherapy is the only treatment that demonstrated an overall survival benefit in men with hormone refractory prostate cancer. The study showed that 2-CADO inhibits the growth of PC3 cells, through a mechanism involving cellular uptake. The nucleoside analog induces apoptosis and accumulation of cells in the S-phase of the cell cycle. 2-CADO pre-treatment followed by docetaxel at subclinical dosage reduces the viability of either PC3 or LNCaP while it does not enhance docetaxel-induced cytotoxicity in adherent non-neoplastic HECV suggesting that the treatment is effective for prostate cancer cells independently on their androgen sensitiveness. Down-regulation of PAR-1 gene expression results in a slightly lower metastatic potential, whereas up-regulation of IL-23 induces the activation of the immune system. On the whole the results suggest a beneficial cytotoxic effect of 2-CADO and Docetaxel in PC3 cells and the information gathered from this experimental study might be exploited to design clinical polytherapeutic protocols for the management of prostate cancer.

11.4

PROTEASOMES ARE NOT A TARGET OF DOXORUBICIN IN FELINE-INJECTION SITE SARCOMA

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Doxorubicin is a potent anti-cancer agent of the anthracycline family widely used in the chemotherapeutic treatment of different tumors. The exact mechanism by which Doxorubicin induces death of neoplastic cells remains to be fully elucidated. Although anthracycline can exert cytotoxic action by directly liberating free oxygen radicals, Doxorubicin was also shown to bind and inhibit proteasomes. Notably, proteasomal inhibition is known to induce apoptosis of rapidly proliferating cells and, consistent with this notion, proteasome inhibitors are emerging as powerful tools against many tumors, especially plasma cell malignancies. Since recent work from our lab demonstrated an enhanced expression and activities of immunoproteasomes in feline injection-site sarcoma (FISS)[1], a spontaneously occurring tumor of cats that is an informative model for the study of tumour biology in other species, including humans, we undertook this study to assess if clinical Doxorubicin treatment induces modulation of proteasomes level and functions *in vivo*. To this end, we measured proteasomal subunits expression levels and catalytic activities in tissue extracts from primary fibrosarcoma lesions and related healthy subcutis of nine cats affected by FISS who received two Doxorubicin treatments 31 and 10 days before surgery. By this approach we demonstrated that the enhanced immunoproteasomal expression and enzymatic activity characteristic of FISS is not at all affected by standard Doxorubicin administration. This unexpected finding might account for the reported low clinical effectiveness of such a treatment in FISS and provides the rationale for developing new therapeutic protocols aimed at achieving a better proteasomal inhibition in this and others poorly Doxorubicin-sensitive tumors.

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11.5

NEW LOW CALCEMIC VITAMIN D ANALOGS: 2-METHYLENE-22E-19,26-DINOR-1A,25-DIHYDROXY VITAMIND3 COMPOUNDS, SYNTHESIS AND BIOLOGICAL PROPERTIES.

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The natural hormone, 1α,25-dihydroxyvitamin D_3 and its analog in the ergosterol series, i.e. 1α,25-dihydroxyvitamin D_2 are known to be highly potent regulators of calcium homeostasis in animals and humans, and their activity in cellular differentiation has also been established[1]. Many structural analogs of these metabolites have been prepared and tested, including 1α-hydroxyvitamin D_3, 1α-hydroxyvitamin D_2, various side chain homologated vitamins and fluorinated analogs. Some of these compounds exhibit an interesting separation of activities in cell differentiation and calcium regulation. This difference in activity may be useful in the treatment of a variety of diseases such as renal osteodystrophy, vitamin D-resistant rickets, osteoporosis, psoriasis, and certain malignancies.

We describe here the synthesis and the biological profile of a small series of new 2-methylene-19-nor-1a,25$(OH)_2D_3$ compounds: RR-22 (20*R*,25*R*), SOR-1(20*S*,25*R*), REN (20*R*,25*S*) and SS-22 (20*S*,25*S*), structurally characterized by the presence of a methylene substituent at the carbon 2 (C-2), a hydroxyl substituent attached to the 25-position (C-25) in the side chain, the methyl group normally located at the 26 position (C-26) in the side chain replaced with a hydrogen atom and a double bond located between carbon atoms 22 and 23 (C-22 and C-23) in the side chain.

In order to synthesize these new 2-methylene-19,26-dinor-22E-1a,25$(OH)_2D_3$ compounds, we have taken advantage of the Lythgoe-type Wittig-Horner coupling approach, which we have previously used in the preparation of other 2-substituted 19-norvitamins[2].

When tested *in vitro* these new 2-methylene-19,26-dinor-22E-1a,25$(OH)_2D_3$ compounds bind to the vitamin D nuclear receptor (VDR), and their binding is comparable to 1a,25$(OH)_2D_3$, they are equally active as 1a,25$(OH)_2D_3$ in inducing differentiation of HL-60 cells as well as in stimulating 24-hydroxylase transcription. Importantly, they all have limited calcemic activity when measured *in vivo*, either by intestinal calcium transport or bone calcium mobilization, at equimolar quantities or even when given at consistently higher dosages as compared to 1a,25$(OH)_2D_3$.

Accordingly, they may find use in the treatment of a large variety of diseases, such as autoimmune diseases, secondary hyperparathyroidism and cancer.

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11.6

SURFACE-ENHANCED RAMAN SPECTROSCOPY (SERS) INVESTIGATION ON ANTI-INFLAMMATORY DRUGS INTERACTING WITH METAL IONS

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Introduction: It is well-known that anti-inflammatory drugs are usually carboxylic acids in which the carboxylate group is available for metal–ligand interactions; the formation of such complexes has been hypothesized to play a role to develop and enhance the pharmacological properties of the drug[1]. Surface Enhanced Raman Spectroscopy (SERS) technique, with an enhancement up to 10^6 of the intensity of Raman spectra, gives the opportunity to study the mechanism of the action of a drug at concentrations mimicking the physiological condition. In the present paper we present a SERS study on four non-steroidal anti-inflammatory drugs (NSAIDs), namely the Na salts of Ibuprofen, Tolmetin, Piroxicam and Diclofenac, both alone and in presence of Co^{++} and Zn^{++} ions, at concentrations lower than 500 ppm.

Materials and METHODS: Raman spectra were obtained using a Jasco NRS-2000C instrument. All spectra were recorded using the 514.5 nm [Ar^+] line and the laser power on the samples was 15 mW. The systems investigated by SERS technique consisted in solutions containing both NSAID and the metal ion in a Ag colloidal suspension, resulting from the controlled hydroxylammine reduction of silver nitrate.

RESULTS: All SERS spectra exhibited intense bands in the region 1350 - 1600 cm^{-1}, due to vibrational modes of the aromatic ring and to the asymmetrical and symmetrical stretching of COO^- group. It should be noted that the overall feature of the spectra changes noticeably comparing the Raman spectra of NSAIDs concentrated solutions with the SERS spectra of NSAIDs adsorbed on the Ag colloidal suspension. The differences are mainly due to the presence of highly polarizable groups that are strongly adsorbed on the metal surface. As a consequence, only the bands related to the groups directly interacting with the Ag surface are enhanced. On the contrary, the general feature of the SERS spectra does not change noticeably after the addition of an equimolar concentration of Zn^{++} and Co^{++} ions. In fact, the $\Delta\nu$ ($\nu_{as}CCO^-$ - ν_sCOO^-) deduced from SERS spectra appear to be, 161 cm^{-1} for Na-ibuprofen; 158 cm^{-1} for Zn-Ibuprofen and 155 cm^{-1} for Co-Ibuprofen. Similar results were observed in other NSAIDs, were the $\Delta\nu$ difference was always within 10 cm^{-1}. Moreover, the SERS low frequency region (ν < 600 cm^{-1}) does not exhibit noticeable differences before and after the metal ions addition.

The experimental data seemed to support the hypothesis that both with Na^+ ion as well as with divalent Co^{++} and Zn^{++} ions, at the low concentration considered, the formation of the same monomeric monodentate structure prevailed, reshuffling thus the role of the metal complex formation to enhance the therapeutic activity.

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11.7

PROTEOMICS OF PARATHYROID GLANDS: A PRELIMINARY STUDY

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In the last years a growing interest has arisen in the application of proteomic approach to discover additional biomarkers in many types of cancer but at this time not even one proteomic study has been performed regard to the search of biomarkers in parathyroid diseases. Particularly, parathyroid carcinoma is a rare cause of parathyroid hormone dependent hypercalcaemia (PTHP) with incidence value in PTHP patients less than 1 % of cases. However, the diagnosis of a parathyroid malignancy is notoriously difficult to make on both gross and histological examination. It is often impossible to distinguish between benign and malignant disease without clear evidence that the tumor is invasive. Local or distant metastases firmly establish the diagnosis of parathyroid malignancy. However at this stage cure is impossible. Until now, no genetic or proteic markers that reliably distinguish carcinoma from adenoma have been identified. Recently, different reports have demonstrated in parathyroid carcinoma the presence of mutations of HRPT2 tumor suppressor gene in patients affected by parathyroid carcinoma and a loss of immunoreactivity to parafibromin, the protein encoded by HRPT2.

In the present work, for the first time, proteomic analysis has been performed to obtain the parathyroid tissue protein map of adenoma. All patients included in the study have been submitted to a surgical procedure to remove hyperplastic gland. Fifteen patients were enrolled in the study and were classified in three groups depending of their calcaemia levels. The proteins profile of parathyroid tissue was obtained for each sample by two-dimensional electrophoresis (2DE) in a non linear range of pH 3-10. About 1050 proteins spots have been detected by analysis Image Master 2D platinum software, while comparison of each group of pathological samples with respect to controls (normal parathyroid) showed qualitative and quantitative differences of protein expression.

Our preliminary results demonstrate the applicability of a proteomic approach in the study of parathyroid diseases and suggest it as a start point to investigate, the presence of potential markers implicated in parathyroid cancer progression.

11.8

HUMAN COLORECTAL CANCER: GENE EXPRESSION OF PURINE METABOLISM.

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Colorectal cancer is the third commonest cause of cancer-related death in the western world. Early diagnosis and surgical treatment ensure a five-year survival of 70-90%. It is essential to understand the genetic factors and molecular mechanisms that, together with life-style related risk factors, cause the development of adenomatous polyps and their malignant transformation. Molecular diagnostics in cancer patients can be useful for prognostic assay and for monitoring response to therapy. In this study, we used qRT-PCR to evaluate expression of four enzymes of purine metabolism in human intestinal biopsy specimens. The enzymes were adenosine kinase (AdK: EC 2.7.1.20), adenylate kinase (MK: EC 2.7.4.3), adenosine deaminase (ADA: E.C. 3.5.4.4) and 5'nucleotidase (5'N: E.C. 3.1.3.5). The above quoted enzymes are involved in the biosynthesis, interconversion and degradation of purine compounds and play important functional roles in the life of the cells. The former contributes to the steady-state maintenance of adenosine, the well known cytoprotective functions of which include stimulation of angiogenesis and inhibition of inflammatory reactions. 5'N is considered to be a key enzyme in the generation of adenosine, a potential vasodilator; ADA seems to be associated with the differentiation of epithelial cells; MK equilibrates the adenylate energy charge and AdK, together with ADA, controls adenosine concentration. The four enzymes may play major roles in tumor progression and their expression may be a sufficiently accurate prognostic indicator for individual colorectal cancer patients.

We analysed 30 patients (15 males and 15 females, age 48-82 years) classified according to cancer location and TNM staging. Fragments of neoplastic and normal-appearing mucosa close to (less than 3 cm) and distant from (at least 10 cm) the tumour were removed during surgical resection and immediately placed in RNA-later solution. Quantitative mRNA expression levels were normalized against the reference gene β-actine, which showed minimal variation between paired normal colon and cancer tissue and the analysis for relative gene expression was carried out using the 2^{-DDCt} method.

The obtained results clearly show that AdK[1], ADA and 5'N gene expression were significantly higher in cancer tissues than in normal-appearing mucosa, while MK did not vary; a similar trend was previously observed assaying the enzymatic activity of the enzymes in the same pathology[2]. AdK significantly vary according to tumor position and with the progression of the disease. These interesting findings represent a novel approach to monitoring tumor progression and contribute to enlarge our knowledge on purine metabolism in cancer.

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11.9

APPLICATION OF PROTEOMICS IN THE STUDY OF ANTIAGGREGANT THERAPY EFFECT IN PATIENTS UNDERWENT PERCUTANEOUS CORONARY INTERVENTION (PCI)

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The efficacy of antiplatelet therapy to prevent cardiovascular and athero-thrombotic events in patients underwent percutaneous coronary intervention (PCI) has been demonstrated by different clinical trials. Nevertheless at this time 10-20% of patients treated with clopidogrel and/or aspirin seems to not take advantage from this therapy and occur in thrombotic events in the first year of follow-up. This phenomenon, that has been called "resistance", usually is connected with a persistent hyper-activation at platelet level.

With the aim to investigate the potential causes of this "resistance" and the physiopathological mechanisms responsible of platelet function, in this study, for the first time, we used a proteomic approach to compare expression changes in protein patterns of platelets from patients with stable coronary ischemic disease before PCI (T0), 12 h after 600 mg of clopidogrel (T1) and 24 h after PCI (T2).

Twenty patients with stable angina taking aspirin (100 mg/die) for at last a month were enrolled in the study. Blood samples from patients were collected and processed to obtain platelet isolation and purification. The proteins profile of platelets was obtained for each sample by two-dimensional electrophoresis (2DE) and the difference of proteins expression of platelets obtained from patients at different times was evaluated using Image-Master 2D Platinum. Approximately 1000 protein spots on each gel using an ammoniacal-silver staining were detected. Quantitative and qualitative differences were found from comparison of synthetic gels obtained from each group. Our results suggest that the platelet proteomic profile can be used not only to evaluate the platelet activation state and the potential drug target proteins but also to investigate the mechanisms responsible of the antiplatelet drugs "resistance".

11.10

DEHYDROEPIANDROSTERONE INHIBITS PLATELET AGGREGATION WITH A cGMP-DEPENDENT MECHANISM

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Dehydroepiandrosterone (DHEA) and its sulfate ester (DHEA-S) are the major circulating steroid hormones in humans and serve as precursors to both androgens and estrogens. Epidemiological studies suggest that high concentrations of DHEA(S) play a protective role against atherosclerosis and coronary artery disease. Accordingly, in vitro DHEA(S) stimulates NO production by endothelial cells, and platelets from DHEA treated patients contain a higher amount of cGMP, marker of platelet inhibition. Pioneering studies showed that DHEA(S) inhibited arachidonate-dependent platelet aggregation. The aim of this study was to investigate *in vitro* the effect of DHEA(S) on human platelets and to characterize the signal transduction pathways involved. Gel-filtreted platelets were incubated with different concentrations of DHEA-S or pre-incubated with DHEA-S and then stimulated with different doses of agonists. We found that DHEA-S inhibited the aggregation induced by thrombin in a dose-dependent manner. This effect was due to DHEA and not to DHEA-S, as demonstrated by the treatment of platelets with the sulphatase inhibitor Estrone Sulphamate that prevented the DHEA-dependent inhibition of aggregation of thrombin-stimulated platelets. The signal transduction pathways triggered in human platelets by DHEA were investigated in immunoblotting experiments. We found that DHEA caused inhibition of thrombin-induced activation of ERK1/2 and Akt, however this effect was observed only when DHEA was added to platelets few seconds before thrombin and not for longer treatment of platelets with the hormone. DHEA caused in platelets a rapid and dose-dependent VASP phosphorylation on Ser239, but not on Ser157. This finding suggests that DHEA activates guanyly cyclase and cGMP-dependent PKG in platelets, presumably with a NO-dependent mechanism. In fact in DHEA-S-treated platelets a significant increase of cGMP was detected, whilst intracellular cAMP levels remained unchanged with respect to the control. However, the NOS inhibitors L-NMMA and L-NAME did not prevent DHEA-dependent inhibition of platelet aggregation, therefore DHEA caused activation of guanyly cyclase with a different mechanism. This evidence requires more investigation, however we have found that in DHEA-S-treated platelets occurred the activation of Src that recently has been proposed as a mechanism alternative to NO to activate guanyly cyclase in platelets (1). In conclusion, our results indicate that in human platelets DHEA plays an anti-aggregating role acting through a cGMP-dependent signal transduction pathway.

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11.11

NICOTINAMIDE N-METHYLTRANSFERASE: A new PROGNOSTIC MARKER FOR ORAL SQUAMOUS cell CARCINOMA.

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Oral squamous cell carcinoma (OSCC) is the most frequent malignant tumour of the oral cavity with about 30,000 new cases and 8,000 related deaths per year in the United States. Despite refinement of surgical techniques and adjuvant therapies, the mortality rate of OSCC has shown little improvement over the last three decades. The optimal management of cervical lymph node metastases is very important to improve survival, and the identification of reliable and clinically applicable markers which allow their preoperative detection is crucial.

In the present study, we wished to focus on the expression of genes critical in the drug metabolism process, namely on Nicotinamide N-Methyltransferase (NNMT), enzyme belonging to Phase II Metabolizing Enzymes and involved in the biotransformation and detoxification of many xenobiotics. To explore the involvement of NNMT in OSCC, we analysed the enzyme expression in paired tumour (T) and non-tumour (NT) tissues obtained at surgery by semiquantitative RT-PCR, Real-Time PCR, western blot and immunohistochemical analyses. Compared with normal mucosa, OSCC exhibited significantly increased expression of NNMT in 11 of 22 (50 %) examined patients. Interestingly, NNMT was upregulated in most of the favourable OSCCs (N 0), while no marked NNMT expression alterations between tumour and normal mucosa were detected in most of the unfavourable OSCCs (N+). Both, pT and pathological staging showed an inverse correlation with NNMT mRNA levels, and a significant negative association of the amount of NNMT expressed by tumour tissue compared to the adjacent normal mucosa was found with metastasis [1]. To observe the effect of NNMT silencing on cell proliferation and cell cycle distribution, four shRNA plasmid vectors against NNMT were constructed and transfected into human oral cancer cell line PE/CA-PJ15. NNMT down-regulation was detected by Real-Time PCR and western blot analysis. The cell proliferation inhibition was determined by MTT and soft agar colony formation assays; cell cycle distribution and apoptosis were examined by flow cytometry. ShRNA vectors targeted against NNMT efficiently suppressed gene expression, showing inhibition rates around 70 %, observed at both the mRNA and protein levels. The shRNA-mediated gene silencing of NNMT resulted in a significant rise in apoptosis rate.

The present data support the hypothesis that the enzyme plays a role in tumour expansion, and NNMT expression level measurements would provide a rapid and useful method of identifying patients at high risk of lymph node metastases. Therefore, NNMT may have potential as a new prognostic marker, and its inhibition could represent a possible molecular approach to the treatment of OSCC.

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11.12

STREPTOZOTOCIN-INDUCED DIABETES AFFECTS TWO REGULATIVE STEPS OF THE CITRATE CARRIER GENE EXPRESSION IN RAT LIVER

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The present study was focused on defining how streptozotocin (STZ)-induced diabetes down-regulates mitochondrial citrate carrier (CiC) activity and expression in rat liver. CiC, an integral protein of the inner mitochondrial membrane, plays an important role in intermediary metabolism catalyzing the transport of acetyl-CoA, primer for both fatty acid and cholesterol syntheses, from mitochondria to the cytosol. Male Wistar rats (150-200 g) were used throughout this study. Diabetes was induced by a single intraperitoneal (i.p.) injection to rats of buffered solution of STZ at a dosage of 70 mg/kg b.w. We showed that in the diabetic rats CiC activity was significantly reduced in covariance with those of lipogenic enzymes. CiC kinetic characterization indicated that diabetes decreased V_{max} of this transport without affecting K_m.

CiC activity, assayed as in[1], significantly decreased in diabetic rats (approx. 35%) as compared to control animals. The molecular mechanism for the diabetes-induced CiC modulation was then investigated. We found that the decreased CiC activity observed in diabetic animals can be most likely ascribed to a lower content of both CiC immunoreactive protein and mRNA, measured as in[1] by RNase protection assays (RPA) and Western blotting analysis, respectively. CiC expression changed in parallel with those of *de novo* fatty acid synthesis enzymes. Adenine Nucleotide Translocase, isoform 2 (ANT2) and porin showed different behaviour.

The estimated half-life of CiC mRNA was the same in the hepatocytes from diabetic and control rats, whereas the transcriptional rate of CiC mRNA, tested by nuclear run-on assay, decreased by about 30% in the nuclei from diabetic vs control rats. In addition, RPA showed that in the nuclei of diabetic rats the splicing of CiC RNA is affected.

Finally, the ratio polyadenylated/unpolyadenylated CiC RNA as well as the length of the CiC RNA poly(A) tail were similar in diabetic and control rats.

Overall these results suggest that: i) reduced mRNA level and the lower content of the mitochondrial CiC protein, might account for the decline of CiC activity in diabetic animals; ii) CiC expression is affected by diabetes at transcriptional and post-transcriptional level; iii) diabetes exerts a gene specific effect.

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11.13

A NOVEL ND5 GENE NUCLEOTIDE VARIANTS ASSOCIATED WHIT OPTICAL SUBATROPHY AND RENAL INVOLVEMENT

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The mitochondrial DNA (mtDNA) encoded subunit of NADH dehydrogenase ND5 is a the largest mitochondrial complex I gene and has turned out to be a hotspot for mutations in several mitochondrial pathologies including Leber hereditary optic neuropathy (LHON)[1].

Herein, we present the genetic characterization of a patient with an unusual clinical association of optic subatrophy and nephropathy. Total DNA was extracted from peripheral blood lymphocytes, fibroblasts and skin biopsy. The presence of the LHON primary mutations (11778/ND4, 3460/ND1, and 14484/ND6) was evaluated by RFLP-PCR. Automated sequencing by ABI Prism 310 Analyzer was performed on overlapping PCR products encompassing the entire mtDNA. Haplogroup was defined by sequencing analysis. PCR-RFLP assays were developed to quantify the novel ND5 mutations versus wild-type mtDNA. Skin biopsy was obtained from patient to cultivate fibroblast. After excluding the presence of primary LHON mutations in the proband, sequencing of the entire mtDNA revealed two nucleotide changes, A13528G and C13565T, both present also in the mother and sister. The same association of ND5 mutations has recently been reported in a MELAS patient[3].

We present a novel genotype/phenotype correlation in a patient who harbors two ND5 mutations on a haplogroup U4b mtDNA, suggesting that the overall mtDNA background may modulate the penetrance and affect the significance of specific mutations. We are investigating the enzymatic activities and the membrane potential in fibroblasts and transmitochondrial cybrids to evaluate the role of the ND5 variants in affecting the mitochondrial metabolism.

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11.14

LHON "*PLUS*" PHENOTYPE IN A FAMILY WITH A 3460/ND1 MITOCHONDRIAL DNA MUTATION

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Introduction. Leber Hereditary Optic Neuropathy (LHON) is a maternally inherited disorder characterized by loss of central vision and optic atrophy, affecting most frequently young males. Classical LHON phenotype is associated to three primary mitochondrial DNA (mtDNA) mutations mostly homoplasmic in ND1, ND4 and ND6 genes, encoding for complex I subunits of respiratory chain[1]. The syndromic form of this mitochondrial disorder, referred to as LHON "plus" phenotype, can occur with variable involvement of peripheral and central nervous systems and of cardiac and skeletal muscle. It has been hypothesized that the 3460/ND1 primary mutation, when associated with specific cytochrome b (cytb) variants, may contribute to the clinical expression of LHON "plus" phenotype[2]. In the present work we report on a 3460 LHON plus family, characterized by mental retardation and epilepsy as extra-ocular features.

Material and Methods. Total DNA samples were extracted from peripheral blood lymphocytes. LHON mutations was screened by RFLP-PCR analysis. Haplogroup assignment was carried out by sequencing of mitochondrial marker regions by ABI PRISM 310 sequencing analysis system.

Results and Conclusion. Family members harbour the 3460 mutation partly in homoplasmy and partly in heteroplasmy according to the severity of symptoms. Polymorphisms analysis assigned our family to the haplougroup U4. Cytb gene sequence revealed the presence of the T15693C variant. Sequencing of the entire mtDNA will be performed on skeletal muscle biopsy from the proband to evaluate other possible variants in mitochondrial genes acting synergistically with the 3460/ND1 mutation.

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12.1

PROBING STRUCTURAL AND DYNAMIC FEATURES IN THE MITOCHONDRIAL OXOGLUTARATE CARRIER BY SITE DIRECTED SPIN-LABELING.

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The mitochondrial oxoglutarate carrier (OGC) catalyzes the transport of 2-oxoglutarate across the mitochondrial inner membrane in exchange for malate, or other dicarboxylates, and belongs to a large family of related transport proteins called the mitochondrial carrier family (MCF). The primary structures of the family members are made up of three tandemly-repeated homologous domains of about 100 amino acids in length, each containing a characteristic sequence motif and two hydrophobic stretches[1]. No 3D structure is available for OGC yet, but several spectroscopic and functional studies have unveiled some of its features. An important contribution to the description of the structural characteristics of MCF has been provided by EM studies on two dimensional crystals of yeast ADP/ATP carrier $(AAC)^2$ and by elucidation of the crystal structure of bovine AAC[3]. The OGC seems to be folded into 6 transmembrane segments too in the inner mitochondrial membrane. Site directed spin-labeling (SDSL) has been used to probe the structural and dynamic features of residues comprising the sixth transmembrane segment of the mitochondrial oxoglutarate carrier. Starting from a functional carrier, where cysteines have been replaced by serines, 18 consecutive residues (from G281 to I298) have been mutated to cysteine and subsequently labeled with a thiol-selective nitroxide probe. The labeled proteins, reconstituted into liposomes, have been assayed for their transport activity and analyzed with continuous-wave electron paramagnetic resonance. Linewidth analysis, that is correlated to local probe mobility, indicates a well defined periodicity of the whole segment from G281 to I298, indicating that it has an a-helical structure. Saturation behaviour, in presence of paramagnetic perturbants of different hydrophobicities, allow the definition of the polarity of the individual residues and to assign their orientation with respect to the lipid bilayer or to the water accessible translocation channel. Comparison of the EPR data, homology model and activity data indicate that the segment is made by an a-helix, accommodated in an amphipathic environment, partially distorted in the middle at the level of L289, probably because of the presence of a proline residue (P291). The C-terminal region of the segment is less restrained and more flexible than the N-terminus.

This work puts together another piece of the puzzle in the quest for elucidating the structure-function relationship of the mitochondrial oxoglutarate carrier.

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12.2

EFFECT OF IBUPROFEN ON THE THERMAL BEHAVIOUR OF DMPC LIPOSOMES AS A FUNCTION OF pH

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Introduction: The effect of amphiphilic non-steroidal anti-inflammatory drugs (NSAIDs), like Ibuprofen (Ibu) a white insoluble powder with pK_a = 4.31, on liposomal bilayer as biomembrane model, plays a crucial role in understanding their mechanism at the molecular level[1]. To explain their activity and correlate it with the chemico-physical properties of the drug, the thermal behaviour of the hydrated multilamellar vesicles (liposomes) of dimyristoylphosphatidylcholine (DMPC) in the presence of increasing amounts of both Ibu as well of its Na-salt (Na-Ibu) have been studied at pH = 3.0 and at pH = 7.0 by means of Differential Scanning Calorimetry (DSC) technique.

Materials and METHODS: Liposomes were prepared by mixing the appropriate amount of Ibu or Na-Ibu with DMPC in a NaCl 0.9 % w/w solution buffered at the request pH value up to a final lipid concentration of about 20 % w/w. DSC scans were performed on a Mettler-Toledo DSC 821^e calorimeter at a heating rate of 2.0°C/min.

RESULTS: The results on Ibu/DMPC liposomes at pH = 3.0 show significant T_m decrease and a $\Delta T_{1/2}$ increase when Ibu si added, suggesting that the hydrophobic core is strongly affected by the presence of the drug unionized molecules. Moreover, in the samples where Ibu content is 20 % w/w, the transition disappeared. The ΔH behaviour is more complex; indeed initially, up a 2.0 % w/w of added substance, it increases and successively, up to 20 % w/w Ibu content, its values decreased lineally. On the contrary, the measurements on the Ibu/DMPC systems at pH = 7.0 showed noticeably lower values both in T_m decrease as well as in $\Delta T_{1/2}$ increase, suggesting that the hydrophobic core is not so strongly affected by the presence of Ibu as it was at pH = 3.0. Moreover the transition is present at any Ibu concentration up to 30 % w/w and also ΔH increased up to 20 % w/w system, decreasing only in the most concentrated Ibu containing sample (30 % w/w). Similar results were obtained starting from the very soluble Na-Ibu salt.

The data suggest that the interaction Ibu-liposomes is strongly pH-dependent, suggesting that at pH lower than pK_a the interaction involves mainly the deeper part of the bilayer. On the contrary, when pH is greater than pK_a, the setting up of strong electrostatic interactions between the negative ion on Ibu molecule and the polar moiety of the lipid localize the interaction on the external part of the bilayer. Moreover, the obtaining of the same results even starting from Na-Ibu water solution, confirm the capability of the of liposomes to strip the drug molecules out from water and concentrate within the bilayer.

The perturbing effect of Ibu on the membrane structure would alter, indirectly, the function of the membrane proteins, whose function is highly dependent on the membrane structure.

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12.3

OPA1 LINKS MITOCHONDRIAL GENOME MAINTENANCE TO NETWORK DYNAMICS

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OPA1 is an intra mitochondrial dynamin showed to be a major regulator of pleiotropic mechanisms associated to mitochondrial inner and outer membrane dynamics[1].

Eight OPA1 isoforms resulting from alternative splicing combinations of exon 4, 4b and 5b have been described. The different spliced exons encode domains included in the N-terminal region and contribute to determine OPA1 functions[2].

Similarly to its yeast orthologue protein mitochondrial genome maintenance-1 (Mgm1), OPA1 is relevant for mitochondrial network dynamics and for cristae structuring[3, 4]. But to date, there is no clue suggesting that OPA1, as Mgm1, might be involved in human mitochondrial DNA maintenance[5].

Using a siRNA approach, cell biology and quantitative PCR, and analysing HeLa cells, we show that a specific OPA1 isoform is involved in mtDNA maintenance. Silencing of this variant is responsible for mtDNA depletion and its abundance correlate to the amount of mtDNA. Furthermore this variant is responsible for mtDNA distribution into the mitochondrial network. Interestingly we evidenced that the N-terminal region of OPA1 is important for this function.

So, in addition to the recent discovery linking mutation of OPA1 GTPase domain to mtDNA deletion[6,7], our data suggest that mutation in OPA1 can be responsible for mtDNA depletion syndromes, and that OPA1 plays a fundamental role in mitochondrial genome maintenance.

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12.4

BCL-2 ANTIOXIDANT FUNCTION IN CELLS WITH DEFECTIVE RESPIRATORY COMPLEX I

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Bcl-2 protects cells from apoptosis and necrosis, although the molecular mechanisms by which it mediates cell survival are not well defined. Bcl-2 can regulate the mitochondrial metabolism and its overexpression has been reported to significantly improve oxidative phosphorylation in cells bearing pathogenic mitochondrial DNA mutations[1].

Furthermore, Bcl-2 was shown to display an antioxidant action, inhibiting mitochondrial dysfunction and cell death elicited by GSH-depleting reagents[2]. Finally, Bcl-2 has also been reported to stabilize microtubule network[3].

Here we have investigated the effect of Bcl-2 overexpression in XTC.UC1 cells bearing a disruptive mutation in the ND1 subunit of complex I leading to a severe energetic impairment[4]. When forced to use exclusively oxidative phosphorylation for energy production by inhibiting glycolysis, these cells triggered a caspase-independent cell death pathway, which was associated to a significant imbalance in glutathione homeostasis and a cleavage of the actin cytoskeleton.

Overexpression of the antiapoptotic Bcl-2 protein significantly increased the level of endogenous reduced glutathione, thus preventing its oxidation after the metabolic stress. Furthermore, Bcl-2 completely inhibited actin cleavage and increased cell adhesion, but was unable to improve cellular viability. Similar effects were obtained when XTC.UC1 cells were incubated with exogenous GSH. We hence propose that Bcl-2 can safeguard actin cytoskeleton stability through an antioxidant function.

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12.5

LIPID RAFT ISOLATION AND POTENTIAL CORRELATION WITH GLUCOSE TRANSPORT AND NOX ACTIVITY IN ACUTE LEUKAEMIA CELLS

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Lipid rafts (LRs) are detergent-resistant microdomains containing glycosphingolipids, cholesterol and glycosylphosphatidylinositol-linked proteins; they seems to be actively involved in many cellular processes including signal transduction, apoptosis, cell adhesion and migration[1].

Membrane lipid rafts may represent the important functional platforms by which redox signals are produced and transmitted in response to various agonists or stimuli. In addition, a new concept is emerging that could be used to define the interactions or amplification of both redox signalling and lipid raft-associated signalling. This concept is characterized by redox-mediated feed forward amplification in lipid platforms. It is proposed that lipid rafts are formed in response to various stimuli; NAD(P)H oxidase subunits are aggregated or recruited in these platforms, increasing Nox activity. Superoxide and hydrogen peroxide generation could induce various regulatory activities, for instance the induction of further lipid-raft platforms formation.

The aim of our study is to probe the involvement of lipid rafts in the modulation of the glucose transporter Glut1 and NAD(P)H oxidase activities of human acute leukaemia cells, where a direct correlation between Nox-derived ROS and glucose uptake is already established, as we previously published[2,3].

At first, we performed a sucrose density gradient centrifugation of the cell lysates, and collected the subcellular fractions. Flotillin-2, which is strongly associated with LRs, was considered as the LRs marker protein[1].

To investigate the potential functional role of LRs in the regulation of NAD(P)H oxidase and Glut1 activities, methyl-β-Cyclodextrin, a well-established cholesterol depleting reagent, was used to disrupt lipid rafts.

Our findings suggest that the important role played by Nox-derived ROS in the regulation of glucose uptake and proliferation of leukaemia cells could likely occur through the control of lipid-raft-associated signalling and therefore they may hint at novel targets for cancer treatment.

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12.6

THE EFFECTS OF SERUM AND CAMP PATHWAY MODULATION ON MITOCHONDRIAL RESPIRATORY CHAIN: TRANSCRIPTIONAL AND POST-TRANSLATIONAL PROCESSES.

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Introduction: The cellular metabolism has to adapt to several changes for energetic demand. In this work, the effects of serum modulation upon signal transduction systems (NF-kB and c-Jun pathways), respiratory chain protein expression, enzymes activity and ROS generation in human fibroblasts, were studied.

Material and Methods: Cells were serum starved 3 days to induce metabolic quiescence and to synchronize cell cycle as shown by growth curve analysis. Reintroduction of serum in the culture medium was followed by functional changes which appeared to be associated with transcriptional and post-translational events. The possible involvement of cAMP signalling, was analysed using PKA inhibitor H89.

Results: Results show that serum starvation caused a significant inactivation of NF-kB pathway. Confocal microscopy analysis revealed that NF-kB migrated from cytosol to nucleus after 1 hour of serum induction; c-Jun, instead, showed an opposite behaviour.

Time-course measurement of enzymatic activities (6, 12, 24, 48 hours) of citrate synthase and respiratory chain complexes I and IV in serum-induced cells showed an increase in complex I activity after 24 hours. Western-blot analysis in both whole cell lysate and in mitoplast fraction resolved by Blue native/ SDS PAGE showed that the increased activity was accompanied by an enhanced expression of complex I subunits (39 kDa and GRIM 19) after 24 hours.

In serum-induced cells treated with H89 we found no induction of complex I subunits, showing a role of NF-kB pathway in complex I subunits expression which is under control of growth factors and cAMP cascade. In fact is reported that NF-kB phosphorylation by PKA was essential for NF-kB transactivation and DNA binding.

Conclusions: The early activation of NF-kB, which was obtained with serum induction treatment, promotes cell survival, cell growth and also interfere with ROS induction and JNK signalling. NF-kB pathway may regulate complex I activities and biogenesis, possibles by enhancing Tfam transcription cAMP signal can play a role in regulation of complex I activity both at transcriptional level as well as post-translational.

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12.7

ANALYSIS OF *D-AKAP1* GENE EXPRESSION IN HUMAN CELLS

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INTRODUCTION. D-AKAP1 (also known as AKAP1, S-AKAP84, AKAP121 and AKAP149) focuses distinct signalling events by binding and targeting cAMP-dependent protein kinase (PKA), protein tyrosine phosphatase (PTPD1), and mRNA[1,2]; variation in the N-terminus can alter the localization of D-AKAP1 to either ER or mitochondria thereby changing the distribution of PKA in the cell[3,4].

It is note that AKAP149, AKAP121, and AKAP84 arise from a single gene by alternative mRNA splicing through a fine mechanism of gene expression[1,5]. In the present work, we show a preliminary study on the expression of this gene and the localization of protein in different subfractions of HeLa and HepG2 cells.

METHODS. HeLa and HepG2 cells were grown in supplemented DMEM. Total RNA was extracted and reverse transcribed. RT-PCR was performed with specific primer. To silencing *D-Akap1* gene, cells were transfected with four plasmid pLKO.1-puro clones that allow expression of four different shRNAs. Proteins AKAP121/149 and AKAP84 were analyzed by Western blot with specific antibodies.

RESULTS AND DISCUSSION. *D-Akap1* gene produces only the transcript encoding for the protein AKAP121/149. RT-PCR showed, infact, that the transcript corresponding to AKAP121/AKAP149 was expressed at significantly level in the cells analyzed. On the contrary, low level of the transcript corresponding to AKAP84, was detected. In addition, western blotting analysis, revealed only the AKAP121/149 protein band in both HeLa and HepG2 cell lines, which is associated to the mithocondrial membranes. Silencing showed that this protein is essential for the viability of both cell lines.

Further investigations will be direct to elucidate the role of AKAP proteins on the mitochondrial function.

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12.8

GENETIC AND PATHOGENETIC MECHANISMS OF TWO TYPES OF HEREDITARY DYSFUNCTION OF COMPLEX I

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INTRODUCTION. Genetic deficiency of complex I is a most frequent case of inborn mitochondrial disease. This study reports the cases of three patients with pathological deficiency of complex I. The first patient has a pathological intronic mutation IVS1nt-1,G >A in the *ndufs4* gene, which causes the transcription of a shorter mRNA, without the exon 2, non coding for a functioning protein. The other two patients, *K* and *C*, have no pathological mutations in all 46 CI subunits nor in CI assembly factors known at the moment.

In these patients we have analyzed the assembly state, the protein profile and the activity of complex I.

METHODS: All measurements and analysis were carried out on patient fibroblasts. Respiratory complexes activities were assayed as in[2]; protein content of respiratory chain was evaluated by BN/SDS PAGE of mitoplast fraction as described in[2]; mitochondrial proteolysis was analyzed by LSCM following the degradation of calpain-specific substrate SLLVY-AMC[1].

RESULTS: 2D electrophoresis showed in patient *ndufs4* IVS1nt-1,G >A the lost of a normally assembled CI and a small amount of CI subcomplex (39kDa, 20kDa and Grim-19), while patients K and C have a significant decrease of the content of normally assembled and immunodetectable complex I (39kDa, 20kDa, 17kDa) which was more severe in one of two brothers *(patient C*). In all patients, a marked decrease of complex I activity was found, in particular patient *ndufs4* IVS1nt-1,G >A CI activity was almost lacking.

While for *ndufs4* IVS1nt-1,G >A patient the lost of the enzymatic activity was attributable to the missing of the wild type NDUF4 protein, causing CI misassembly, for patient K and C the explanation was more difficult. Treatment of patients K and C cell culture with protease inhibitors prevented the decrease of the assembled complex I and restored the enzymatic activity. Using the peptide probe SLVVY-AMC, whose fluorescence emission of proteolytic product is used to measure the activity of calpains and other chemotrypsin-like enzymes, we found a significant production of the fluorescent proteolytic product of the probe essentially localized at the mitochondrial level. This data indicated that the decrease of the functional and structural level of complex I in the two patients can be due to an enhanced proteolytic cleavage of the complex, even if further investigations are necessary to identify the gene responsible for the decreased stability of complex I. These data indicate that genetic and pathogenetic mechanisms of hereditary dysfunction of complex I can be divided in two types. The first type includes mutations in assembly factors and in structural subunits, both mitochondrial and nuclear, the second type, mutations or altered functioning of proteases, causing compex I instability.

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12.9

N-ARACHIDONYL-GLYCINE INTERACTION WITH THE RESPIRATORY CHAIN OF RAT LIVER MITOCHONDRIA

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Introduction. The N-arachidonyl-amino acids (NAAAs) are a recently characterized family of arachidonic acid derivatives that include N-arachidonyl-glycine (NA-Gly), N-arachidonyl-alanine (NA-L-Ala) and N-arachidonyl-γ-aminobutyric acid (NA-GABA). These compounds are structurally related to anandamide (arachidonyl-ethanolamine) which is considered an endogenous ligand of cannabinoid receptor. NA-Gly is present at relatively high levels in the spinal cord, small intestine, kidneys and at lower, but remarkable, levels in testes, lungs and liver. This wide distribution suggests multiple functions, in addition to the reported anti-inflammatory and pain suppression action (Huang et al, J. Biol. Chem. 276, 2001, 42639-44). Here we report on the interaction of NA-Gly with isolated rat liver mitochondria.

Materials and methods. The respiratory activity of liver mitochondria was measured polarographically with a Clark-type electrode. The membrane potential in intact mitochondria was measured following the safranin-O fluorescence quenching at 525 nm (excitation), 575 nm (emission). ROS production was detected spectrofluorimetrically using the oxidation-sensitive dye DCF-DA.

Results. Micromolar concentrations of NA-Gly cause: i) a substantial increase of state 4 respiration with both glutamate + malate and succinate as substrate; ii) a decrease of ADP or CCCP stimulated respiration with a consequent drop of the respiratory control ratio. The membrane potential generated by either succinate oxidation or ATP hydrolysis was only weakly dissipated by NA-Gly concentrations increasing state 4 respiration. Experiments are also presented showing that NA-Gly caused a respiration dependent large ROS production and cytochrome c release.

12.10

β-ADRENERGIC SIGNALLING PATHWAY IN THE MODULATION OF COMPLEX I AND ROS BALANCE

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Introduction The cAMP cascade regulates a variety of cellular processes such as the utilization of glycogen and lipid stores, neuronal activity, transcription of specific genes, cell proliferation, differentiation and death. It has been shown that the increasing of cellular cAMP in various serum-limited (SL) cell cultures stimulates the complex I activity and reverts ROS accumulation[1]. Oxidative stress can regulate protein thiols redox state and it is reported that the complex I activity is also modulated by glutathionylation. In this work we investigated the modulation of complex I activity by modulation of β-adrenergic signalling pathway and we examined the involvement of glutathionylation in cAMP-dependent complex I activity and ROS removing effect.

Materials and methods The NADH–UQ oxidoreductase and cytochrome c oxidase activities were determined spectrophotometrically. ROS production was measured by DCF-DA. Glutathionylation was analyzed using GSH-antibody.

Results and Discussion The activation of the β-adrenergic receptor by isoproterenol (ISO) reverted the accumulation of H_2O_2 and depression of complex I activity in serum-limited fibroblasts (NHDF-neo). Treatment of cardiomyocytes H9c2 with isoproterenol promotes complex I activity and basal ROS reduction; pre-exposure of H9c2 to β1-blocker metoprolol abolished this effect. Howewer, direct treatment with β-blockers metoprolol and propranolol promoted enhanced complex I activity and ROS reduction, probably due to their antioxidant activity[2]. The analysis by 2D-electrophoresis of the complex I subunits in mitoplast isolated from NHDF-neo cells in exponential growth phase, serum limited and serum limited treated with bcAMP with anti-GSH antibody revealed that the glutathionylation not influence cAMP activation of complex I. In conclusion our data show that the activation of complex I and prevention of oxygen free radicals production is not due to changes in the glutathionylation of the complex. On the other hand these effects of cAMP can be associated with phosphorylation of structural subunits of complex I.

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12.11

TRANS- RETINOIC ACID EFFECT ON RESPIRATORY CHAIN COMPLEX I: PROTEOLYTIC STABILIZATION OF GRIM-19 SUBUNIT IN HUMAN KERATINOCYTES

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INTRODUCTION: All-*trans*-retinoic acid (RA), a metabolite of vitamin A, binds to specific nuclear receptors, induces expression of various genes, and inhibits growth of certain types of cancer[1-2]. The product of the gene GRIM 19 is a component of complex I that is essential for the assembly of complex I and the integrity of the whole mitochondrial electron transfer chain[3-4]. In this work the effect of all-trans-retinoic acid (ATRA) on cellular growth and complex I activity, of human keratinocytes are presented.

MATERIAL AND METHODS: Cells were treated up to 72 hours with 20μM ATRA and/or protease inhibitors. The dynamics of complex assembly was examined using 2D Blue Native/SDS PAGE of mitoplasts from cultured cells.

RESULTS AND DISCUSSION: Keratinocytes treatment with RA resulted in a strong depression of cells growth. A decrease of the catalytic activity of NADH-UQ oxidoreductase in RA treated cells was also found. Cytocrome c oxidase and citrate synthase activities were not affected by RA treatment.

2D Blue-Native/SDS PAGE of mitoplast fraction revealed, on the other hand, significant increase in the level of GRIM-19 as well as of the overall complex I. Cell treatment with RA had no effect on the level of complex III, IV and V.

Treatment with protease inhibitors resulted in an electrophoretic pattern similar to that of retinoic acid treatment. The present result indicate that the enhanced level of the GRIM-19 and depression of complex I activity caused by ATRA result from defective proteolytic degradation of damage unproductive subunits of the complex. The consequence of complex I increased stability and decreased turnover could lead to the accumulation of an “aged” enzyme exhibiting lower functional activity. Balance between life and death might depend on the ability of the cells to sustain activation of transcription factor of the NF-kB family[5]. We observed activation of NF-kB by retinoid acid which could lead to an extension of the cellular life possibly related to JNK deactivation.

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12.12

RESHAPING OF LIPID MICRODOMAINS IN BOAR SPERM DURING CAPACITATION

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Studies of the last 15 years showed the presence in plasmamembrane of lipid domains, restricted areas having lipid and protein composition different from the environment. Two types of lipid microdomains coexist in non-neural cells: caveolae, flask-shaped invaginations of plasma membrane rich in caveolin (cav-1) and lipid rafts, highly dynamic heterogeneous structures rich in cholesterol and GPI-anchored proteins. In different mammalian species, in sperm plasma membrane coexist both type of microdomains and a recent work (Cross, 2004) shows their role in "capacitation", process indispensable for sperm maturation. This process is also regulated by receptors like CBR1 (cannabinoid receptor 1) and VR1 (vanilloid receptor 1), that respond to endocannabinoids.

The aim of this work was to investigate lipid microdomains organization in sperm during capacitation in order to assess if their molecular composition, in particular CBR1 and VR1 receptors, changes.

Semen samples were collected from 3 boars of proven fertility. After remove of seminal plasma, the sperm sample was immediately processed for biochemical analysis (NC sperm) or was diluted in TCM 199 to a final concentration of $2x10^8$ spermatozoa/ml to reach *in-vitro* capacitation (C sperm). The incubation was carried out at 38.5°C in 5% CO_2 humidified atmosphere for up to 4 h. Successively the detergent-resistant membrane fraction (DRM), that includes *caveolae* and *lipid rafts*, was prepared (Palestini et al., 2000). We determined the protein content in DRM by SDS-PAGE followed by WB and lipid composition (Cholesterol and GM1) by TLC.

The total protein content in DRM increase after capacitation but the protein markers of microdomains, cav-1, CD55 and flot-2 are not enriched in DRM, and these proteins are present also in the other fractions of the gradient. We found an acrosomal marker in different fractions of the gradient, including DRM, indicating that this distribution is the cause of the presence of cav-1, flot-2 and CD55 in all the gradient fractions. CB1 and VR1 are present in DRM and their content increase after capacitation, suggesting a shift of CBR1 and VR1 from fluid membrane areas to domains. Conversely, the total amount of cholesterol and GM1, after capacitation, decreases significantly in DRM. The modification of DRM in sperm plasma membrane after capacitation and in particular the increase in endocannabinoid receptors, indicate an important role of these microdomains in signalling events that induce capacitation. Moreover, it is possible that these series of events are extended also to the acrosomal membrane, like it is testified from the variation in DRM of the acrosine amount. In conclusion, it is possible to suppose that the DRM molecule reshaping has a crucial function in the fusion process between plasma membrane and acrosome, crucial phase and real functional end-point of the capacitation.

12.13

FUNCTIONAL ANALYSIS OF UCP3 NATURAL MUTANTS IDENTIFIED IN SEVERELY OBESE CHILDREN LIVING IN SOUTHERN ITALY

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Uncoupling proteins (UCPs) are carrier proteins that uncouple the electron transport chain from ATP synthesis localized in the inner mitochondrial membrane. Five UCP homologues (UCP1–UCP5) are present in mammals[1]. Apart from UCP1, which is involved in thermogenesis, the physiological role of UCP isoforms is unclear. UCP3 has recently been implicated in fatty acid transport and metabolism. In fact, it promotes β-oxidation and reduces their storage. Therefore, UCP3 has been proposed as a candidate gene for susceptibility to obesity[2]. Thus far, no data have been reported about UCP3 mutations associated to obesity in humans. We recently identified four novel mutations in the heterozygous state in 200 severely obese (BMI-SDS>2.5) children with early-onset obesity (<4 y) living in Southern Italy: three missense mutations V56M, A111V, V192I, and one non-sense mutation, Q252X, that generates a truncated protein. Here we report preliminary results of an *in vitro* functional analysis of the V56M, V192I and Q252X mutant proteins. Constructs expressing human UCP3 cDNA *wt* and mutants were transiently transfected in HEK 293 cells, which lack endogenous UCP3. *Wt* and mutant UCP3 proteins were correctly localized in mitochondria as assayed by western blotting experiments. The activity of *wt* and mutant UCP3 was indirectly measured by incubating cells with a 100 μM palmitic acid mix containing 16.7 μCi/ml of ^{3}H-labeled palmitic acid for 2 h with and without mitochondrial fatty acid oxidation inhibitors. ^{3}H-labeled water, produced by palmitate oxidation, was collected and counted in a scintillation counter (3). Mutants V192I and Q252X retained only 40% of *wt* protein activity and V56M only 20%. Interestingly, children carrying mutations V192I and Q252X had a higher respiratory quotient at rest (0.9 and 1.05 respectively) than obese children without UCP3 mutations (0.85). Therefore, it appears that carbohydrates are used more than fatty acids in the metabolic mixture. Furthermore, children carrying mutations V56M and Q252X have mild steatosis, which suggests an increase in fat storage. Further experiments are underway to better clarify the involvement of UCP3 in fat storage.

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12.14

METABOLIC CONTROL ANALYSIS REVEALS A SUPERASSEMBLED RESPIRATORY CHAIN IN POTATO TUBER MITOCHONDRIA.

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Besides the "liquid state" model for the organization of the respiratory chain, depicting the redox enzymes as complexes independently embedded in the lipid membrane bilayer, another condition can be envisaged based on direct channelling between the redox enzymes ("solid state") since stable multicomplex units have been observed in several mitochondrial species[1].

Considering the lack of functional data, here we report our efforts to understand the physiological implications of the existence of supercomplexes. Particularly, we address the problem in mitochondria (POM) from freshly harvested potato tubers (*Solanum tuberosum)* by focusing on the kinetic properties of the respiratory complexes involved in the NADH- and succinate-dependent respiration.

For the first time, we show that all the enzyme steps in the rotenone-sensitive respiratory machinery in POM exert the same metabolic control and that their flux coefficients are close to unity (1.0, 1.1, 1.2 for Complex I, Complex III and Complex IV, respectively) therefore we can demonstrate that, contrarily to animal mitochondria[2], the respiratory complexes behave as a functional supramolecular unit where, according to the principles of the Metabolic Control Analysis[3], also Complex IV molecules are included. Furthermore, we discuss the presence of alternative dehydrogenases as functionally independent components not comprised in the supercomplex assembly.

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12.15

CHANGES IN MEMBRANE PHOSPHOLIPIDS COMPOSITION AFFECT ABETA PEPTIDE TOXICITY ON NEURONAL CELL .

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Biochemically, Alzheimer's disease is characterized by the formation of plaques of Abeta that participates to the progressive deterioration of memory, language and other cognitive functions which lead to dementia. A number of observations indicate that the primary target of amyloid peptide (Abeta) is neuronal cell membrane and specific lipids, e.g. ganglioside GM1[1]. Alteration of the ratio cholesterol/phospholipids are involved in membrane-peptide interactions, including peptide insertion, cytotoxicity, and pore formation[2]. *In vitro* experiments using atomic force microscopy and transmission electron microscopy indicate that the interaction with the membrane lipids induces a structural change of the peptide, from mature fibrils into soluble oligomers[3], influencing membrane fluidity and neurodegeneration. Recently, it has been reported that Abeta (1-42) oligomers inhibit neuronal viability 10-fold more than fibrils and 40-fold more than unaggregated peptide[4]. In this context, the comprehension of Abeta-lipid interaction mechanisms would be useful to develop strategies against Abeta toxicity to prevent neuronal damage.

Experiments of immunochromatography on lipids extracted from SH-SY5Y human neuroblastoma cells showed that phosphatidylethanolamine (PE) is able to bind oligomeric Abeta 1-42 more efficiently than other membrane phospholipids, suggesting that this phospholipid could be involved in Abeta/membrane interaction and toxicity. Therefore, inhibition of the specific PE biosynthetic pathway may affect the poisonous effects of the peptide on neuronal cells. Exploiting this hypothesis, choline and phosphocholine were tested as inhibitors of PE biosyntesis. Experiments were carried out on SH-SY5Y human neuroblastoma cells differentiated for 5 days with retinoic acid. Cells were treated for 6, 16 or 24 hours with 1 mM or 2.5 mM choline or 0.5 mM or 1 mM phosphocholine. A statistically significant ($p < 0.05$) reduction of PE level (42%) was obtained with 2.5 mM choline treatment after 24 hours, without affecting cell viability (data obtained by MTT test) and slightly affecting PC level. On the other side, treatment with phosphocholine did not show alteration in PC and PE levels. Noteworthy, cells treated with 5 µM Abeta 1-42 showed a decrease of cell viability of 48%, against a value of 31% when pre-treated with 2.5 mM choline. Such treatment or membrane lipid modulation by assumption of food rich in choline (liver, cauliflower, brown bread, yolk, peanuts) or soy lecithin supplements, could reduce the toxicity of the peptide.

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12.16

STRUCTURAL STUDIES ON THE HUMAN ABCC6 TRANSPORTER

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The human ABCC6, formerly called MRP6, is a member of the adenosine triphosphate-binding cassette (ABC) gene superfamily[1]. The exact function and natural substrate(s) of ABCC6 are currently unknown. Mutations in ABCC6 gene cause Pxeudoxanthoma elasticum (PXE), an autosomal recessive disorder of the connective tissue characterized by progressive calcification of elastic structures in the skin, the eyes and the cardiovascular system[2].

The proteins of this superfamily are involved in active transport of a wide variety of substrates, including aminoacids, lipids, peptides, saccharides and drugs, into and out of the cell and in the intracellular compartment. The structure of these proteins includes two transmembrane domains not much conserved, TMD1 and TMD2 that bind substrates, and two hydrofilic domains, NBD1 and NBD2, able to bind and hydrolyze ATP. The MRP6 protein, as some other transporters belonging to the subfamily ABCC, contains an additional sequence (TMD0) in the N-terminal region of about two hundred residues. The function of TMD0 remains poorly defined. In this report, the structure of the N-terminal region and the NBD1 domain was investigated. To study the structure and the function of TMD0, the polypeptide sequence corresponding to the aminoacids 1-102 of N-terminal region have been synthesized by recombinant DNA technology. CD spectroscopy showed a strong tendency of the N terminal region to assume an α-helical conformation in hydrophobic solutions and in micelles, suggesting a transmembrane localization for this sequence. The ATP binding to the NBD1 domain was also studied.

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12.17

NEW ASPECTS OF INHIBITORY EFFECT OF NITRIC OXIDE ON THE RESPIRATORY ACTIVITY OF LIVER MITOCHONDRIA

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In the last ten years a great attention has been paid to the interaction of nitric oxide (NO) with the respiratory chain activity, essentially after reports showing the existence of a specific mitochondrial nitric oxide synthase (mtNOS) distinct from the well known three isoforms: endothelial (eNOS), neuronal (nNOS) and inducible (iNOS). Moreover, different research groups have questioned the existence of an mtNOS. In our lab we have characterized the activity of the cytosolic NADH/cyto-c system, an electron transport pathway additional to that of the respiratory chain. In isolated liver mitochondria the addition of a catalytic amount of cytochrome c (cyto-c) activates this system which promotes the oxidation of NADH-dependent substrates added outside and generates an electrochemical proton gradient useful for ATP synthesis.

This report describes the effect of NO on the activity of the cytosolic NADH/cyto-c system. Our original research program was aimed to compare the effect of NO generated inside the mitochondria by mtNOS to that of NO added to the incubation medium. Since we were unable to promote any mtNOS activity, the data obtained with NO generated by the dissociation of GSNO or added as water solutions saturated with gaseous NO molecules, are presented. The inhibition of NADH/cyto-c system by NO is reversible, is fully recovered as NO is completely metabolised and can be ascribed essentially to NO interaction with the cytochrome oxidase (Cox), as suggested by the data obtained with intact and solubilized mitochondria. A direct interaction of NO with the free molecules of cyto-c with the formation of a quite stable adduct, has been also found. This interaction, at least in part, may contribute to the overall inhibition of Cox observed in the presence of NO. At constant oxygen concentration, increasing NO up to 12 micro molar the time life of inhibition state increases but the entity of inhibition remains the same. This is consistent with an inhibitory mechanism of “all or nothing” type. In the presence of NO, Cox preferentially works as NO oxidase rather than as cyto-c oxidase, behaving as an efficient “NO scavenger”. On the metabolic point of view, this opens a new scenario on the role and function of NO/Cox interaction. Very relevant is also the finding that the membrane potential generated by the oxidation of exogenous NADH is not affected by a relatively low concentration of NO, while the oxidation rate is strongly inhibited. Thus we suggest that this behaviour could be correlated to the protective effect observed in the presence of NO in the ischemia reperfusion injury.

12.18

STRUCTURAL STUDIES ON THE HUMAN MRP6

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The MRP6 is codified from the ABCC6 gene, a member of the adenosine triphosphate-binding cassette (ABC) gene superfamily . The proteins of this family are involved in active transport of intracellular compounds to the extracellular environment. Mutations in ABCC6 cause Pxeudoxanthoma elasticum (PXE), an autosomal recessive disorder of the connective tissue characterized by progressive calcification of elastic structures in the skin, the eyes and the cardiovascular system.

The structure of these proteins includes two transmembrane domains, TMS1 and TMS2, not much conserved, that bind the substrates, and two hydrofilic domains, NBD1 and NBD2, able to bind ATP. The MRP6 protein, as some other transporters belonging to this family, contains an additional sequence in the N-terminal region of about three hundred residues, perhaps responsible of a regulatory function. To study the structure of this protein, some short sequences, corresponding to the N-terminal and the NBD1 regions, have been synthesized by recombinant DNA technology and/or chemical synthesis. CD spectroscopy showed a strong tendency of the N terminal region to assume an α-helical conformation in hydrophobic solutions and in micelles, suggesting a transmembrane localization for this sequence.

12.19

EVIDENCE FOR AEROBIC METABOLISM IN RETINAL ROD OUTER SEGMENT DISKS.

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Visual transduction in retinal rod Outer Segments (OS) is an energy demanding process for which ATP supply is not yet clarified. Toward the goal of increasing our understanding of the molecular basis of OS energetic metabolism, we have presented a comprehensive proteomic study of purified rod disks. Proteins involved in vision as well as mitochondria-specific proteins not known to be part of the disk, were identified (respiratory chain complexes I to IV and oxidative phosphorylation complex V (F_1F_o-ATP synthase). Results suggested that F_1F_o-ATP synthase is catalytically active on disk surface. We also reported that the OS selectively stains with mitochondrial vital dyes, by an *ex vivo* technique on living retinas.

Now we report a consistent ATP synthesis by purified bovine disks which accounts for about 200 μM ATP/sec, sufficient for the phototransduction energy need. ATP synthesis was inhibited by mitochondrial ATP synthase inhibitors (oligomycin, nigericin, DCCD, Antimycin A). Inhibition by oligomycin and nigericin, suggests that disk ATP synthase employs a transmembrane electrochemical proton potential difference to synthesize ATP. The presence of a proton gradient across disks is also demonstrated by fluorescence quenching experiments of Rhodamine 123. Moreover, Rhodamine 123 selectively stained OS in a whole living retina, *ex vivo*. The four respiratory chain complexes display an activity comparable to that of mitochondria and are sensitive to the common inhibitors, such as Antimycin A, Rotenone or KCN. Moreover, intact disks consume oxygen when are energized with NADH or succinate, at a rate similar to that of mitochondria. All the above results suggest that ATP is generated through oxidative phosphorylation in these organelles, that are devoid of mitochondria, likely by a recruitment of mitochondrial proteins, but not mitochondria.

13.1

EFFECTS OF PEROXYNITRITE ON LCAT ACTIVITY IN CEREBROSPINAL FLUID

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AIMS: The enzyme Lecithin-Cholesterol Acyl-Transferase (LCAT) produces cholesteryl esters (CE) in plasma but its activity in cerebrospinal fluid (CSF), although detected, has not yet been characterized. LCAT, secreted by hepatocytes and neuroglial cells, might play an important role in brain cholesterol transport. Oxidative stress severely affects brain function, and might impair LCAT activity. Peroxynitrite is a major oxidant in CSF, and the footprint of its attack to proteins is represented by 3-Nitrotyrosine (NT) formation. The aims of this work were to asses whether peroxynitrite can affect LCAT activity in CSF.

METHODS:. CSF samples from healthy donors were processed by SDS-PAGE and Western Blotting (WB), and the membrane was stained with rabbit anti-NT IgG. Digital images were analyzed by the Gel-Pro Analyser software (Media Cybernetics, Silver Spring, MA), and the band intensities were recorded as peaks on a densitogram. The LCAT activity ex vivo, represented by the ratio of CE with unesterified cholesterol (C) in HDL[1], was determined in the HDL-like fraction of CSF by measuring the C levels before and after saponification. LCAT assays in vitro (using liposomes containing ApoE3 for stimulation, and tritiated C as substrate) were carried out with/without 100 μM 3-Morpholinosydnonimine hydrochloride (SIN, peroxynitrite donor of peroxynitrite) as published[2].

RESULTS: Two CSF samples with the same amount of protein (namely A and B) were analyzed for their NT level, and CE/C ratio. Densitometry of WB patterns indicated 1.5-fold higher NT level in A. CE/C was higher in A than in B (1.5± 0.01 and 0.82± 0.03 respectively). LCAT activity in vitro was 4.82 or 1.87 units in the absence or presence of SIN, respectively. This result indicates that peroxynitrite damages the enzyme activity in vitro

DISCUSSION:

We here report preliminary data showing that, in CSF, higher C esterification might be associated to lower NT formation. This result is supported by the finding of decreased LCAT activity by peroxynitrite in vitro. It can thus be hypothesised that peroxynitrite, produced under conditions of oxidative stress such as those associated to neurodegeneration, might impair lipoprotein-mediated C removal from brain. LCAT-dependent C transport might actually play a crucial role for neuron function, when C accumulates in the plasma membrane by reduced hydroxylation and causes ß-amyloid release in CSF.

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13.2

EFFECT OF NEUROSTEROIDS ON NESTIN, NEUROFILAMENT, -TUBULIN AND MAP-KINASE ESPRESSION ON RAT BONE MARROW MESENCHYMAL STEM CELL CULTURES

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The aim of present investigation was to study the effects of dexamethasone (DEX) or estradiol (E_2) on proliferation and differentiation of rat bone marrow mesenchymal stem cells (MSCs) in culture, evaluating by western blotting and immunocitochemical analysis some specific neural proliferative and differentiative markers. MSCs are multipotent cells in the bone marrow stromal line, known for their potential to differentiate into several types of cells.

MSCs were harvested from bone marrow of femurs of 4 to 8 month-old rats. Cytofluorimetric analysis revealed that MSCs were negative for CD45, CD34 and positive for CD90, CD105.

After 24h starvation period, MSC cultures were treated for 48h with DEX 10^{-9} M or E_2 5 x 10^{-9} M. Qualitative and quantitative analysis were performed by immunocytochemical and western blot analysis respectively for nestin, neurofilament, β-tubulin and MAP-kinase.

Our results show an enhancement of the above mentioned neural markers and MAP-Kinase in MSCs cultures treated with DEX. E_2-treatment increased MAP-Kinase and β-tubulin expression, but it decreased nestin and neurofilament expression.

Collectively, our results indicate that DEX induces an up regulation of some neural protein expression indicating an its possible role played on differentiation of these MSCs to neural line. In addition, it shows an involvement on signal transduction patways. On the contrary, E_2 treatment induces up and down modulation of nestin, neurofilament, β-tubulin and MAP-kinase expression. Moreover, are in progress experiments on DEX-growth factors crosstalk on GFAP, vimentin, nestin, PARP and MAP-Kinase expression in astroglial cell cultures at 15 DIV, in order to compare the different effects evaluated in both in vitro models.

13.3

CHARACTERIZATION OF BIOTIN-ANANDAMIDE, A NOVEL TOOL FOR THE VISUALIZATION OF ANANDAMIDE ACCUMULATION.

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Anandamide (arachidonoylethanolamide, AEA) acts as endogenous agonist of both cannabinoid and vanilloid receptors. During the last two decades, its metabolic pathways and biological activity have been extensively investigated and relatively well-characterized. In contrast, the effective nature and mechanism of AEA transport remain at present a controversial and still unsolved issue. We have reported the characterization of a biotinylated analogue of AEA (b-AEA), that has the same lipophilicity of the parent compound. In addition, by means of biochemical assays and fluorescence microscopy, we have shown that b-AEA is accumulated inside the cells in a way superimposable on that of AEA. Conversely, b-AEA doesn't interact nor interfere with the other components of the endocannabinoid system, i.e. type-1 and type-2 cannabinoid receptors, vanilloid receptor, AEA synthetase (NAPE-PLD) or AEA hydrolase (FAAH). Taken together, our data suggest that b-AEA could be a very useful probe for visualizing the accumulation and intracellular distribution of this endocannabinoid.

13.4

EFFECT OF CARNOSINE AND TREHALOSE ON PARP-1 AND 2 EXPRESSION UNDER PROINFLAMMATORY STRESS CONDITIONS

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INTRODUCTION: A large body of evidences have indicated a relevant protective properties of carnosine and trehalose against many antioxidative stress conditions. The neuropeptide carnosine (β-alanyl-L-histidine), is involved in many processes of cellular defence such as inhibition of protein cross-linking and glycation, metal chelation, free radicals and NO detoxification[1].

The disaccharide trehalose is produced in large amounts under stress conditions and can counteract protein mysfolding and aggregation in neurodegenerative disorders.

In the present study we examined L and D- carnosine and trehalose effect against NO induced cell death in primary rat astroglial cell cultures treated with lipopolysaccharide (LPS) and interferon gamma (INFγ). To better evaluate the molecular mechanism underlying the role of carnosine and trehalose we measured cell viability, nitrite production and the LDH release. PARP expression was also determined to verify its involvement in the mechanisms of protection and/or cell death during proinflammatory commitment.

MATERIALS AND METHODS: Interferon-γ (100U/ml) and lipopolysaccharide (LPS 1 μg/ml) were used to induce iNOS dependent stress conditions in primary rat astroglial cell cultures with or without carnosine and trehalose (20mM).

RESULTS aAND DISCUSSION: An increase of nitrite production and LDH release and a decrease of cell viability (MTT) after 24 or 48 hrs of LPS and INF-γ treatment was observed. Carnosine as well trehalose addition separately or tougether was able to decrease nitrite and LDH release and to increase cell viability in a dose-response manner. Apart the NO binding activity of carnosine, such effect is also attributable to the observed PARP 1 and 2 down-regulation by carnosine and trehalose treatment under stress conditions.

In the same experimental conditions D-carnosine a "synthetic peptide" shows a more remarkable effect, than L-carnosine. This result can be attributed to the effect of carnosinase activity wich is able to hydrolize the L-carnosine brt not D-carnosine.The data obtained suggest that, beside the antioxidant role of carnosine and trehalose, these molecules can also modify the PARPs expression pathway. Such hypothesis is under further investigation to better understand such critical step of many neurodegenerative disorders[2].

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2. V. Spina-Purrello et al, 2008 Neurochemical Research Submitted for publication

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13.5

AGGREGATION AND ASSEMBLY OF Aβ(25-35) IN WATER

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Several lines of evidence suggest a relationship between the aggregation state of amyloid β peptides and their ability to promote cell degeneration *in vitro*, an aspect which might also govern their toxicity *in vivo*[1]. The very toxic Aβ(25-35) is the shortest peptide sequence that retains a biological activity comparable with that of full-length Aβ(1-42)[2]. This fragment differs from all the other commonly studied β-amyloid peptides because of its extremely rapid aggregation properties so that it cannot be studied with conventional electrophoretic techniques[3]. Mounting evidence shows that synthetic Aβ, in an aggregated state is toxic to neurons in cultures. The assembly phenomenon is dependent on *p*H, concentration, and incubation time in solution. Several Aβ fragments have been reported to form such assemblies, but only those that include a substantial portion of a transmembrane sequence assemble into aggregates that are stable at *p*H 7.4 and resistant to disruption by SDS. We provided the first information on the very rapid aggregation of Aβ(25-35) in water at *p*H 7.4. Adopting UV-Vis spectroscopy, Congo Red spectrophotometry and thioflavin-T fluorimetry, we were able to quantify in water the role of initial solvent in determining the aggregation rate, extent and, thanks to thioflavin-T data, modality of Aβ(25-35) self-assembly to form stable insoluble aggregates. Moreover, the percentage of Aβ(25-35) aggregation at 37°C in water was evaluated by UV-Vis spectroscopy and we assessed that it reached 37% of aggregation in 30 minutes of incubation, far higher than the aggregation percentages of the very amyloidogenic Aβ(1-42) peptide. Aβ(25-35) aggregation tendency was then confirmed by the evaluation of its percentage of sedimentation. Congruently with aggregation quantification, Aβ(25-35) showed the highest sedimentation percentage (56%) respecting to the reference Aβ(1-42) peptide (24%). The basic understanding of the modality of Aβ(25-35) self-assembly will help to comprehend the pathway and key steps of Aβ association.

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13.6

TSPO EXPRESSION ON MURINE MODEL OF OBESITY

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The *ob/ob* murine line, mutant for leptin gene, represents a good experimental model to study several pathologies as diabetes and obesity. The ob/ob mouse is obese, hyperphagic, hyperinsulinemic and become significantly hyperglycaemic during stress, presumably as a direct consequence of leptin deficiency at hypothalamic level. In fact, leptin stimulates melanotropic hormone (α MSH) release, inhibits food intake, increases parasympathetic nervous system tone and energy expenditure; on the other hand, it inhibits neuropeptide Y (NPY) release, that increases parasympathetic tone, appetite and reduces energy consumption.

Ob/ob mice lack these functions at hypothalamic level. In particular, these mice present an adrenal hypertrophy and increased corticosteroid secretion during diurnal rhythms. One consequence of increased glucocorticoids is a diminished muscle glucose uptake. An increased serum cholesterol, associated to a moderate hypertryglicemia is also observed in ob/ob mice.

TSPO (translocation protein or PBR - peripheral benzodiazepine receptor) is richly expressed in tissues producing steroids, implying that one of its main functions is represented by regulation of the synthesis process of these hormones.

Cytoplasmic cholesterol binds with high affinity to TSPO and this interaction is involved on cholesterol transport inside mitochondria: its translocation from outer to inner mitochondrial membrane is the rate determining step in the whole synthesis process.

Therefore, our aim was to evaluate TSPO expression in mutant ob/ob mice in comparison with wild-type ones to individuate a possible translocator alteration, associated with the hypercolesterolemic status of the murine leptin mutant.

TSPO expression was evaluated either in central nervous system or in periphery, by means of [^{3}H]PK11195 binding assay, carried out on brain and kidney membranes.

A significant statistical increase of [^{3}H]PK11195 Bmax values (maximal receptor density) was evidenced in the brain of mutant ob/ob mice without significant alterations of Kd values (dissociation constant); by contrast, there were no significant variations of either [^{3}H]PK11195 Bmax or Kd in kidneys of both control or leptin mutant mice.

These results showing an increased TSPO expression in mutant mouse brain need further investigations by autoradiographic tecniques, which will evidence the translocator localization and distribution in the different cerebral areas.

13.7

PIN1: A NEW OUTLOOK IN THE STUDY OF ALZHEIMER'S DISEASE

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Alzheimer's disease (AD) is characterized by extracellular plaques deposits of amyloid-beta (Aβ) oligomers and intracellular hyper-phosphorylated microtubule-binding protein Tau neurofibrillary tangles (NFTs). Recent data suggest that Pin1, a peptidyl-prolyl cis/trans isomerase, might be involved in AD pathogenesis, because of its role in Tau and APP (beta-amyloid precursor protein) *cis/trans* isomerisation on Ser(P)/Thr(P)-Pro sequences. Pin1 may take part in promoting APP cleavage by α-secretase rather than the amiloydogenic pathway[1]. Moreover, it has been demonstrated that Pin1 is implicated in oxidative stress-induced Tau dephosphorylation[2] on Thr231 by protein phosphatase 2A (PP2A). The Thr231 residue plays a critical regulatory role because its phosphorylation greatly diminishes the ability of Tau to bind and stabilize microtubules in the cell[3]. Since oxidative stress is an early event in AD pathogenesis, we investigated the Pin1 involvement in modulation of tau phosphorylation in rat cultured hippocampal cells after exposition to β-amyloid oligomers.

Hippocampal neurons cultured for 7-8 days are treated with Aβ (1-42) oligomers[4] 2,5 μM for different times (up to 24 hours). Western blotting analysis revealed that Tau phosphorylation level on Thr231 seems to decrease after 3-8 hours of 2,5 μM Aβ (1-42) oligomers treatment, increasing again in 24 hours of exposition, although we observe caspases activation and 40% of cell apoptotic death within 24 hours. The early Tau dephosphorylation event observed may probably due to protein isomerisation by Pin1, since that Aβ-induced Tau phosphorylation is recovered by pre-treatment with a specific Pin1 inhibitor (juglone 10 μM). Furthmore, in cells pre-treated with okadaic acid (20nM), that mainly inhibits PP2A, Tau^{Thr231} results hyper-phosphorylated, suggesting a correlation between Pin1 and PP2A activity. Additionally, we established that, after Aβ treatment, some of other Tau phosphorylation sites under Gsk3β/PP2A regulation (Ser199, Ser396) undergo to dephosphorylation indirectly induced by Pin1 activity.

Taken together, these results suggest an intriguing hypothesis of Pin1 direct involvement in early mechanisms activated during Aβ-induced cytotoxic insult and modulating Tau phosphorylation state.

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13.8

DISTRIBUTION OF THE NUCLEAR PROTEIN TDP-43 IN THE CYTOPLASM AS FULL LENGTH MOLECULE AND UBIQUITIN-TAGGED-DERIVATIVE IS A FEATURE OF BRAIN CORTEX AND SPINAL CORD IN SPORADIC AMYOTROPHIC LATERAL SCLEROSIS.

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Sporadic Amyotrophic Lateral Sclerosis (sALS) is a progressive disorder ending into voluntary muscle paralysis caused by selective and progressive death of motor neurons in brain and spinal cord, the reason of which is still poorly known. Cytoplasmic ubiquitin-positive round or skein-like inclusions are a hallmark of sALS. TDP-43, a 43 kDa nuclear protein involved in gene expression and RNA splicing regulation, was uncovered to be a component of these inclusions in which it is described to exist in the native form, as a 43 kDa protein, and in a variety of high molecular mass derivatives predictive of its abnormal distribution as a protein tagged with ubiquitin polymers[1].

We were interested in going in deep into the nature and subcellular distribution of TDP-43 in sALS. Brain frontal cortex (FC) and spinal cord (SP) autoptic specimens from three heavily affected patients and three age-matched neurologically healthy subjects were evaluated by western-immunoblotting carried out on a cleared cytoplasmic fraction and a pellet enriched in the nuclear fraction and the insoluble inclusions (hereafter called nuclear pellet) using TDP-43 and ubiquitin selective antibodies. An immunohistochemical analysis on formalin-fixed, paraffin-embedded tissue sections was performed in parallel on 30 other sALS cases.

Biochemically, in FC and SP of controls TDP-43 was distributed exclusively as a nuclear protein and only as a 43 kDa protein; surprisingly in FC and SP of sALS it was expressed in the cytoplasmic fraction, that was free from inclusions, in which it was easily appreciable not only as 43 kDa full length protein but even as high molecular mass derivatives tagged with various ubiquitin monomers; in the nuclear pellet, in addition to the normal 43 kDa form, TDP-43, here too, was clearly expressed as a highly ubiquitinated abnormal component. Immunohistochemically, in the sALS cytoplasm TDP-43 was distributed diffusely as well as inside insoluble aggregates.

These dysfunctions highlight a perturbation of nuclear trafficking and/or solubility of TDP-43 ending into loss of the nuclear functions and gain of the cytoplasmic ones, both exerting a negative impact on neuron metabolic activity and lastly vitality.

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13.9

PROTEOMIC ANALISYS OF GLYCOPROTEIC PATTERN IN MILD COGNITIVE IMPAIRMENT AND ALZHEIMER DISEASE HIPPOCAMPUS

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Alzheimer's disease (AD), an age-related neurodegenerative disorder, is characterized clinically by a progressive loss of memory and cognitive functions. Neuropathologically, AD is defined by the accumulation of extracellular amyloid protein deposited senile plaques and intracellular neurofibrillary tangles made of abnormal and hyperphosphorylated tau protein, regionalized neuronal death and loss of synaptic connections within selective brain regions. Mild Cognitive Impairment (MCI) is generally referred to the transitional zone between normal cognitive aging and early dementia or clinically probable AD. Protein glycosylation is one of the most common post-translational modifications of proteins in eukaryotes. It is critical to growth control, cell migration, cell adhesiveness, tissue differentiation and inflammatory reaction cascades. Protein glycosylation modifies the processing of several key proteins involved in the molecular pathogenesis of AD. We employed a proteomic approach coupled to lectin-affinity chromatography, using two different lectin Con A and WGA, to elucidate possible differential expression in the total pattern of glycoproteins in AD and MCI hippocampus compared to age-matched control. This technique allows to focus the interest only to glycosilated proteins identifying which might be involved in the pathogenesis and the progression of the AD. We show many differences in glycoproteins expression, which demonstrate the alteration of several cellular systems, molecular pathways or processes during AD development. Alterations on γ-enolase and glutamate dehydrogenase suggest an impairment of energy metabolism pathway both in MCI and AD, as previously reported[1]. Downregulation of both GRP 78 (glucose related protein 78) and GRP 94, HSP90 and α-glucosidase indicates an impairment of the protein folding machinery in MCI and AD. Moreover we show that also neuron arrangement undergoes to several changes as indexed by the alteration of structural and neuronal growth proteins (TPM 2-3, DRP-2, 14-3-3) in MCI and AD and of synaptic vesicles trafficking in MCI (XAP-4). In conclusion, our data enrich previous knowledge on the molecular mechanisms involved in the onset, progression and pathogenesis of AD and provide a contribute to explain the biological events that may lead MCI patients to progress to AD.

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13.10

PROTEIN OXIDATION AND CELLULAR STRESS RESPONSE IN AGING BRAIN: A REDOX PROTEOMICS APPROACH

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Increasing evidence supports the notion that reduction of cellular expression and activity of antioxidant proteins and the resulting increase of oxidative stress are fundamental causes in the aging processes and neurodegenerative diseases. Several conditions including protein, lipid or glucose oxidation disrupt redox homeostasis and lead to accumulation of unfolded or misfolded proteins in aging brain. To cope with the accumulation of misfolded proteins and to activate the repair processes, the central nervous system has evolved the conserved mechanism of unfolded protein responce. One of the main intracellular redox systems involved is the *vitagene* system[1]. Vitagenes encode for cytoprotective heat shock proteins (Hsp) Hsp70 and heme oxygenase-1, as well as thioredoxin reductase (TRXred).

In the present study, we investigated, in rats 12 (adult) and 28 (senescent) months old, the role of Hsp expression on aging-induced changes in the antioxidant redox status. In the brain, expression of Hsp72 and HO-1 increased with age. The maximum induction was observed in the hippocampus and substantia nigra (SN) followed by cerebellum, cortex, septum and striatum. We also evaluated levels of expression of Hsp90, thioredoxin and TRXred as function of aging process and we observed in senescent rats compared to adults a significant decrease of Hsp90 in the cortex and cerebellum, an increase in the SN, while no significant changes were found in septum, striatum and hippocampus between senescent and adult animals. TRXred activity and expression was elevated in senescent rats compared to adults in all brain regions examined while the protein expression of thioredoxin decreased in all brain regions but the SN and cerebellum. We used a redox proteomics approach to identify proteins which are oxidatively modified as a specific target in the hippocampus of senenscent rats compared with adults. Many of these are energy-related proteins such as pyruvate kinase, ATP synthase, aldosase, creatine kinase and alpha-enolase. The oxidative modification of these enzymes likely leads to their inactivation. These results are in line with current literature data showing that free radical damage and decreased energy production are characteristic hallmarks of the aging process. We posit that increase of Hsp expression might promote functional recovery of oxidatively damaged proteins thus protecting neural cells from progressive age-related damage.

Conceivably, heat shock signal pathway by increasing cellular stress resistance may represent a crucial vitagene controlled mechanism operating against free radical-induced damage occurring in aging brain and in neurodegenerative disorders.

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13.11

RESTRICTED EXPRESSION OF RELAXIN 3 GENE IN THE BRAIN OF DEVELOPING ZEBRAFISH EMBRYO

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The relaxin 3 (Rln3) is a neurotransmitter abundantly expressed in a restricted cell group of brainstem region, known as the nucleus incertus (NI)[1]; this neuron cluster is positioned in a behavior control network that integrates information related to memory, attentional state and stress response[2]. To date, the role of *rln*3 has been only investigated in mammalian species. In our study we analyzed *rln*3 expression in the brain of a non-mammalian vertebrate, zebrafish, which has emerged as vertebrate model for genetic, molecular and behavioral studies. We demonstrated, by whole mount in situ hybridization experiments, that, at late pharyngula period, two discrete symmetric *rln*3-expressing cell groups are present in developing zebrafish brain in the midbrain tegmentum region. This restricted signal was still evident later in developing embryos. These cell clusters are distributed latero-dorsally to the central midbrain tegmentum in the putative periaqueductal gray matter (PAG). The expression of zebrafish *rln*3 gene in the putative PAG neurons is very appealing since these neurons have been correlated to the production of vocal communication, which is an important feature for social behaviour[3]. Interestingly, starting from larval stage, a new more posterior signal was detected in a smaller cell group in tegmentum/medulla region. We hypothesize that this cell cluster might correspond to mammalian NI, reporting the first molecular evidence of existence of nucleus incertus in fish. We support our hypothesis comparing the anatomical position of *rln*3-expressing cells and dorsal raphe neurons finding the same anatomical relationship reported in rat brain. NI acts in a complex control network together with other neural components such as locus coeruleus and raphe nuclei that are emerging sites of the brainstem neural circuitry involved in stress response. Our analyses, based on double in situ hybridization experiments using rln3 antisense riboprobe and marker genes for locus coeruleus and raphe nuclei, indicate that these brainstem circuitries, could take place shortly after hatching, when the embryo starts to interact with the environment and is easily accessible to behavioural studies. In conclusion, our findings provide the basis to analyze the involvement of Rln3 peptide and of PAG and NI in different neural mechanisms in early life stage of a vertebrate model organism.

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13.12

GLIOMA AND MITOCHONDRIAL PERMEABILITY TRANSITION: NEW CHEMOTHERAPY TARGET.

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Among the brain tumors, the glioblastoma multiforme (GBM) is the most incurable form of cancer. It is defined by uncontrolled proliferation, infiltration, propensity for necrosis, robust angiogenesis and resistance to apoptosis. Despite surgery, radiotherapy and chemotherapy, the prognosis of GMB patients is very poor. New findings in this field will be fundamental to develop targeted therapies against this untreatable cancer.

The mitochondrial permeability transition (MPT), due to the opening of a multiprotein pore (MPTP) shows a central role in mediating cell death. Moreover, some of the MPTP components are selectively up-regulated in cancer, such as the TSPO and ANT proteins. Mitochondria-directed cytotoxic agents, combined with traditional chemotherapy, could be emerging tools to fight GBM.

We planned to extend the knowledge about the therapy combination of the MPT-inducing drugs and the traditional chemotherapy, with the aim to improve the GMB therapeutic protocols.

The ability to induce cell death of MPT-inducing agents, selected among those targeting the TSPO and ANT proteins, has been tested in murine and human glioma cells by MTS assay. Moreover, the induction of MPT has been tested, assaying the mitochondrial potential, using the specific fluorescent probe JC1 and the apoptosis has been investigated by cytochrome C release and DNA fragmentation assays and electron microscopy.

Our results indicated the ability of the tested TSPO and ANT drugs to trigger cell death in murine and human glioblastoma cell lines as a consequence of MPT induction, and such drugs were also potently effective in glioma cells resistant to the usual chemotherapy drugs.
Studies are undergoing to test MPT-inducing agents, or combination of MPT-inducing agents and conventional chemotherapy drugs, on GMB cells.

13.13

IMPAIRMENT OF METHYLATION CYCLE IN TREATED PATIENTS WITH PARKINSON'S DISEASE

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L-3,4-dihydroxyphenylalanine (L-DOPA) alone or in combination with a peripheral dopa decarboxylase inhibitor (DDI) is the most effective therapeutic agent to improve motor function in most of patients with Parkinson's disease (PPD). However, chronic L-DOPA therapy is associated with of side-effects arising particularly during long-term therapy. Only a small percentage of an exogenous dose of L-DOPA is converted into dopamine (DA) in the brain. The majority is either decarboxylated in peripheral tissues by aromatic amino acid decarboxylase (AAD) to DA, which does not cross the blood-brain barrier, or is *O*-methylated by catechol-*O*-methyltransferase (COMT) in both peripheral and brain tissue to yield 3-*O*-methyldopa (3-OMD). To effectively raise brain DA levels, a large amount of L-DOPA must be administered, often in an oral dose. It has been reported that such large doses of L-DOPA can significantly affect sulphur amino acid metabolite levels. During long-term clinical practice, the decarboxylation of L-DOPA is inhibited, the role of COMT is accentuated and circulating L-DOPA is largely converted into 3-OMD. Therefore, *O*-methylation of L-DOPA to 3-OMD is linked with conversion of SAM to S-adenosylhomocysteine (SAH). SAH is split into adenosine and Hcy.

We evaluated the impact of long-term application of L-DOPA/DDI formulations on plasma methionine (MET), SAM, SAH and tHcy levels in PPD.

Patients were from the Institute of Neuropsychiatry, Palermo University. All patients entering the study were examined by neurologists to confirm or exclude the diagnosis of Parkinson's disease. There were 10 PPD treated with L-DOPA/DDI formulation and 10 healthy controls. Peripheral blood samples were taken in the morning after the subjects had fasted and were off medication for at least 12 hrs. Thus we avoided impact of acute L-DOPA/DDI intake. Plasma tHcy and sulphur metabolite levels were determined by high-performance liquid chromatography (HPLC) as reported.

The levels of MET and SAM (approximately 1.21 and 1.32 fold, respectively) in the treated PPD were significantly lower than in the controls while the levels of tHcy (mean 16.6 µmol/L; SD 4.4) were higher compared with controls (mean 9.8 µmol/L; SD 3.4). No significant differences in SAH levels appeared.

Based on these findings, we hypothesized that another consequence of high-dose e/or long-term L-DOPA administration might be hyperhomocysteinaemia and may also represent a risk factor for both ischaemic heart and cerebrovascular disease in treated PPD. Besides, the resulting hyperhomocysteinaemia might be increased if L-DOPA therapy is superimposed on a condition known to impair Hcy metabolism, such an enzyme defect or B/acid folic vitamin deficiency.

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13.14

ANT1 EXPRESSION AND RAGE-NF-KB PATHWAY ACTIVATION IN SPORADIC INCLUSION BODY MYOSITIS

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Sporadic IBM (s-IBM) is the most common muscle disease beginning after age 50, which leads to severe disability. There is increasing evidence that free-radical toxicity may participate in the IBM pathogenesis. Indicators of oxidative stress, as well as enzymes participating in the cellular defense against oxidative stress, are accumulated in IBM muscle fibers. Mitochondrial abnormalities shown to be more prevalent in s-IBM than in other inflammatory myopathies. Reducted expression of adenine nucleotide translocator (ANT1), mitochondrial protein involved in the response to oxidative stress, has been associated with reactive oxigen species (ROS) production in muscular cell. The products of irreversible non-enzymatic glycation and oxidation have been termed advanced glycation end products (AGEs). Through their capacity to form cross-links and to liberate reactive oxygen intermediates, AGEs and their receptors (RAGE) can induce cellular damage. Recently it has been demonstrated that RAGE-NF-*κ*B pathway is involved in proinflammatory pathomechanism and in muscle fiber regeneration in inflammatory myopathies.

Our study want to analyze oxidative stress markers, ANT1 and RAGE-NF-kB pathway in s-IBM.

On muscle samples of five patients with s-IBM, five patients with polymyositis (PM) and five normal controls were done the following studies: immunocytochemistry for activated NF-κB; electrophoretic mobility shift assay (EMSA) of NF-κB DNA binding activity; western blot studies of RAGE and ANT1; hydrogen peroxide (HP), peroxidase and glutathione peroxidase (GPx) assays. Nuclear immunoreactivity for NF-*κ*B, NF-*κ*B-DNA binding activity, increased RAGE and GPx expression were found in muscle specimens from s-IBM and PM patients, whereas ANT1 reduction was present in s-IBM in comparision with PM and normal controls.

ANT1 reduction, high GPx activity and RAGE-NF-kB pathway activation indicate a redox system alteration in s-IBM.

13.15

SILENCING OF HEXA AND HEXB GENES REVEALES A ROLE OF b-HEXOSAMINIDASE IN NEUROINFLAMMATION

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Inflammation is an important component in the pathogenesis of many diseases of different origin. In most cases, the role of inflammation is a natural response to injury, and an important mechanism for healing and tissue repair. However, the immuno-response can either be inadequate or overwhelming, leading to direct injury or severe host disease. This is the case of several lysosomal storage disorders, such as GM2 gangliosidosis, where the inflammation is one of the most important features of neurodegeneration within the central nervous system (CNS). Using GM2 gangliosidosis as a neurodegenerative diseases model[1] we are exploring how the inflammation is related to the metabolic storage disorder.In particular, we investigated whether the genetic defects in the HEX (HEXA and HEXB) genes, which lead to the absence of Hexosaminidase (Hex) isoenzymes in GM2 gangliosidosis, are involved in an altered immuno-response.

To this end, we generated an *in vitro* model of human $CD1a^+$ Dendritic Cells (DCs) knockdown for HEX genes. Our data demonstrated that the HEXA and HEXB gene expression have a 90 and 85 percent of inhibition respectively after siRNAs transfection of WT-$CD1a^+$DCs. These results have a direct correspondence for the level of inhibition of the Hex activity in these cells. More importantly, using a specific fluorescent BRDU-MLR for the T-cell proliferation assay, we found that the HEX-knockdown $CD1a^+$-DCs express a tolerogenic-like phenotype and were unable to activate the T lymphocytes. As, these results are in agreement with highly reduction of the Hex activity that we observed in tolerogenic DCs generated in vitro from hematopoietic stem cells, we suggest a role for the enzyme within the cell-mediated immuno-response which is therefore likely linked to inflammatory processes during GM2 gangliosidosis.

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13.16

OXYGEN CONSUMPTION AND ATP SYNTHESIS IN ISOLATED MYELIN: A NEW TROPHIC ROLE OF MYELIN SHEATH.

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Brain functioning requires a lot of energy to restore and maintain its electro-physiological activity. It represents only 2-3% of the body weight, but its energy consumption is more than 20% of that of the whole organism. This is quite surprising because the mitochondrial density in brain is very low. Several Authors suggest that part of the energy may be supplied by the glia. Our attention has focused on myelin, the multilayered membrane protein/lipid structure surrounding axons, because there is growing evidence that it also has a neuro-trophic role. In fact, in demyelinising diseases like Multiple Sclerosis, the loss of myelin causes an axonal necrosis, cause of the neurological disability.

Following a challenging hypothesis that the aerobic production of ATP for axons takes place in myelin sheath, we conducted experiments on isolated myelin vesicles (IMV) obtained according to the method of Norton ad Poduslo. By semiquantitative WB analysis, biochemical assays and TEM and confocal microscopy techniques, we have observed that IMV: i) are able to consume oxygen when are energized with NADH and Succinate; ii) display a proton gradient on their surface; iii) contain Fo-F1 ATP Synthase and all the four respiration chain complexes, which are catalytically active and are sensitive to the common inhibitors, such as Olygomicine, Rotenone or KCN. The extent of mitochondrial contamination was assessed by semiquantitative WB with an Ab against ANT, TIM and TOM (proteins typical of the mitochondrial membranes) and by biochemical assay of Adenylate Kinase Isoform 3 (AK3,a typical matrix protein). ANT, TIM and TOM as well as AK3 activity were absent in IMV.

These data suggest that the whole redox chain is present in myelin sheath and that it is catalytically active in aerobic ATP production, functional to the high energy demands of CNS. Moreover this basic study will be pivotal for shedding some light on the etiophatogenesis of many demyelinating diseases.

13.17

pLG72 BINDING TO D-AMINO ACID OXIDASE MODULATES D-SERINE CONCENTRATION IN HUMAN BRAIN.

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In the past few years, the identification of relatively high concentration of glial derived D-serine shed new light on the role played by the flavoenzyme D-amino acid oxidase (DAAO). In several regions of the brain, the gliotransmitter D-serine acts as an endogenous allosteric modulator of the NMDA receptors. Since in brain DAAO is mainly expressed in astrocytes surrounding the synapses, this flavoenzyme has been proposed to regulate the synaptic concentration of D-serine, and thus indirectly to modulate glutamatergic neurotransmission. According to this proposal, it was not surprising that the genes coding for human DAAO (hDAAO) and its putative interactor pLG72 have been linked to the predisposition to schizophrenia[1], which onset has been related to an altered functionality of NMDA receptors.

We investigated the regulation of hDAAO exerted by pLG72 by using different approaches. In human brain cortex, immunohistochemical analyses revealed that both proteins are expressed in astrocytes, where they most likely interact, considering their partial overlapping subcellular distribution and their co-immunoprecipitation. The specific *in vitro* interaction of the two proteins (as was demonstrated by different techniques) yields a ~200 kDa complex composed by 2 hDAAO homodimers and 2 pLG72 molecules. The binding of pLG72 did not affect the kinetic properties and FAD binding ability of hDAAO, but resulted in the modification of its tertiary structure and in a time-dependent loss of activity. We then demonstrated that overexpression of hDAAO in human glioblastoma cells decreases the cellular level of D-serine, an effect which is nulled when pLG72 is co-expressed. These evidences strongly indicate that pLG72 acts as a negative effector of hDAAO.

Thus, we propose a molecular mechanism by which hDAAO and pLG72 are involved in schizophrenia susceptibility: an increase of hDAAO activity (e.g. due to the anomalous hypoexpression of pLG72 under pathological conditions) yields to a decrease of D-serine released at the synapse and, as a result, to the hypofunction of NMDA receptors[2]. The elucidation of the role played by hDAAO and pLG72 in such a complex psychiatric disorder and the availability of the hDAAO-pLG72 complex will provide an ideal system to test small molecules with potential therapeutic application.

This work was supported by a grant from Fondazione Cariplo to L.P.

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13.18

ALTERATION OF TRANSMETHYLATION REACTIONS IN AMYOTROPHIC LATERAL SCLEROSIS

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Amyotrophic Lateral Sclerosis (ALS) is an adult-onset neurodegenerative disorder characterized by death of motor neurons in the spinal cord. Approximately 90% of ALS cases are sporadic (SALS), while only a minority of cases shows a familiar inheritance (FALS). FALS is mainly caused by missense mutations in the gene encoding superoxide dismutase 1 (SOD1), an enzyme catalyzing the conversion of harmful superoxide radicals to hydrogen peroxide and oxygen.

It has been reported that oxidative damage plays a fundamental role not only in FALS, but also in SALS pathogenesis.

In proteins, L-asparaginyl residues flanked by small, non-bulky residues are subject to deamidation, a spontaneous post-translational modification generating L-isoaspartyl abnormal derivatives (IsoAsp), in turn responsible for structural and functional protein impairment. Under physiological conditions, isoAsp residues are recognized by protein L-isoaspartate (D-aspartate) O-methyltransferase (PIMT; EC 2.1.1.77), an enzyme involved in the repair of the damaged proteins. This protein is ubiquitous in all human tissues, but is more active in red blood cells (RBC), where the deamidation rate increases with aging and in response to oxidative stress. Owing to its kinetic features, PIMT's activity is highly sensitive to any significant alteration of transmethylation potential, a useful index of the propensity to transfer methyl groups from AdoMet. This value is expressed as the ratio AdoMet/AdoHcy, being AdoMet the methyl donor and AdoHcy a powerful methyltransferase inhibitor.

The present project aims to investigate the possible effect of oxidative injury, one of the main pathogenetic factors involved in SALS, on the efficiency of transmethylation reactions.

We found that methyl esterification of RBC membrane proteins is significantly increased in SALS patients, compared to healthy controls. This result indicates that an accumulation of abnormal IsoAsp residues occurs *in vivo* in these patients, presumably as a consequence of the oxidative microenvironment.

In addition, the intracellular concentration of AdoMet in SALS patients is 50% reduced compared to healthy controls. As a consequence, [AdoMet]/[AdoHcy] ratio is significantly decreased in the patients. This strong reduction might contribute to the accumulation of IsoAsp residues through the impairment of PIMT-mediated repair process. Moreover, an alteration in DNA methylation, an epigenetic modification involved in gene expression regulation, can be hypothesized, owing to the high sensitivity DNA methyltrasferases to variations of transmethylation potential. The possible reactivation of genes whose expression is allele-specific and methylation-dependent (genes X-inactivated or imprinted) is currently under investigation.

13.19

EVALUATION OF NEUROACTIVE STEROID LEVELS, BIOCHEMICAL AND FUNCTIONAL PARAMETERS IN AN EXPERIMENTAL MODEL OF NERVE CRUSH INJURY

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Peripheral neuropathy due to trauma of nerves is one of the common functional deficits in road and domestic accidents. Different therapeutic approaches are now available to promote the regeneration of damaged nerve, but a satisfactory management to ensure a full restoration of peripheral nerve after trauma is not yet available.

Using an experimental protocol in which crush injury was applied 1 cm above the bifurcation of the rat sciatic nerve for 20 seconds, we have evaluated whether neuroactive steroid levels as well as biochemical (i.e., myelin proteins and Na^+,K^+ ATPase pump) and functional (nociceptive response to thermal stimulation and walking track test) parameters were affected. By means of liquid chromatography coupled to tandem mass spectrometry, we here demonstrate that the levels of neuroactive steroids such as pregnenolone (PREG), progesterone (P) and testosterone (T) and their reduced metabolites (i.e., dihydroprogesterone, DHP, and tetrahydroprogesterone and dihydrotestosterone, DHT, respectively) present in injured sciatic nerve were significantly decreased with respect to those in control. The drop of DHP and DHT levels in crushed sciatic nerve does not seem to be the consequence of the reduced levels of the general precursor PREG, since the levels of P and T (i.e., their direct precursors) were not significantly affected. A possible hypothesis could be an impairment of the enzyme devoted to their synthesis (i.e., the enzyme 5α-reductase). In agreement with this hypothesis, we here observe that the mRNA levels of this enzyme were significantly decreased in crushed sciatic nerve. Crush nerve lesion is also able to induce other biochemical alterations, such as a decrease of myelin protein gene expression and an increase of Na^+,K^+ ATPase activity and expression of its catalytic subunits. Moreover, nociceptive and motor functions are deeply altered in crush animals.

Altogether these findings represent an important background to analyze whether treatment with neuroactive steroids might counteract functional and biochemical parameters affected in this experimental model.

13.20

TRANSLOCATOR PROTEIN (TSPO) AND IDENTIFICATION OF NEW MOLECULAR DIAGNOSIS TOOLS.

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The 18 kDa mitochondrial Translocator Protein (TSPO) is constitutively expressed in central and peripheral tissues. It is associated with a plethora of biological functions, such as cholesterol transport, steroidogenesis, cellular proliferation, apoptosis. Many studies reported its up-regulation in several forms of cancer, with a positive correlation between TSPO level and the grade of malignancy. For such reason, TSPO has become an interesting target in molecular imaging (i.e. PET, fluorescence microscopy and cytofluorimetry).

We aimed to develop new fluorescent or radio-labeled ligands targeting TSPO to create new imaging tools that can make easier to study TSPO expression and function in tumor cells.

Previously, we described a series of N,N-dialkyl-(2-phenylindol-3-yl)glyoxylamides as potent and selective TSPO ligands[1]. Pursuing our interest in this field, we obtained new indolylglyoxylamides as molecular probes labelled with a fluorescent moiety or with a Positron Emitter Carbon-11.

The fluorescent probes were obtained by linking the norbornadiene (NBD) moiety to the N-alkyl chain, and were monitored at the mitochondrial level in glioma cells to verify the specific TSPO labelling. Instead, to obtain the TSPO radioligands, the indolylglyoxylamides bearing a methyl group on the indole N-1 were synthesized and tested for their biological activity by radiobinding assays, showing high affinity for the TSPO. Such results make these compounds good candidates to be labelled with Carbon-11.

In conclusion, both fluorescent and radiolabelled indolylglyoxylamides are new molecules targeting this protein as promising imaging tools for cancer.

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13.21

LIPID RAFT CHOLESTEROL PROTECTS AGAINST AMYLOID CYTOTOXICITY.

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Increasing evidence supports the idea that the initial events of Aβ oligomerization and cytotoxicity in Alzheimer's disease involve the interaction of amyloid Aβ-derived diffusible ligands (ADDLs) with the cell membrane, indicating lipid rafts, ordered membrane microdomains enriched in cholesterol, sphingolipids and gangliosides, as likely primary interaction sites of ADDLs. To shed further light to the relation between Aβ oligomer interaction with the cell membrane and cytotoxicity, we investigated the dependence on membrane cholesterol content of ADDLs binding to lipid rafts in human neuroblastoma cells and in primary fibroblasts from familial Alzheimer's patients. In particular, confocal laser microscopy analysis showed that Aβ1-42 oligomers markedly interact with plasma membrane rafts and that Aβ1-42 oligomer- monosialoganglioside GM1 interaction was prevented by a moderate membrane cholesterol enrichment. Moreover, anisotropy fluorescence measurements of flotillin-1-positive rafts purified by sucrose density gradient suggested that the cholesterol content is inversely correlated with membrane perturbation by Aβ1-42 ADDLs. Finally, membrane microdomain morphology and size were imaged by Atomic Force Microscopy (AFM), before and after the exposure to monomeric and aggregated forms of Aβ. Contact mode AFM images of lipid rafts in liquid showed that ADDLs induce changes in raft morphology with the appearence of large pores. The pore size and depth were significantly reduced in cholesterol-enriched rafts, suggesting that cholesterol protects against amyloid-induced membrane damage at the lipid raft level by modifying raft physicochemical features.

13.22

EXTRACELLULAR UPTAKE OF AMYLOID BETA AGGREGATES BY A CLATHRIN-DEPENDENT, RAFT-MEDIATED MECHANISM.

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A leading theory on the molecular basis of amyloid toxicity considers that prefibrillar unstable assemblies interact with cell membranes destroying their ordered structure. Although considerable evidence indicates that extracellular Aβ contributes to the intracellular pool of Aβ, the mechanisms involved in Aβ uptake by neurons are poorly understood. Recent evidence suggests that amyloid Aβ-derived diffusible ligands (ADDLs) interact with the amyloid precursor protein (APP) present at the cell surface, which acts as a ligand of its own precursor to enhance APP complex formation, resulting in a cell death-related signal. To shed further light to the relation between Aβ oligomer interaction with the cell membrane and cytotoxicity, we investigated the dependence on APP distribution in the plasma membranes of Aβ aggregate binding to lipid rafts, ordered membrane microdomains enriched in cholesterol, in human neuroblastoma cells. Confocal laser microscopy analysis showed that ADDLs markedly interact with plasma membrane rafts. Moreover, anisotropy fluorescence measurements of flotillin-1-positive rafts purified by sucrose density gradient suggested that the APP content is significantly correlated with membrane perturbation by ADDLs. Finally, confocal microscopy studies suggested a caveolae-independent, clathrin-dependent, raft-mediated mechanism of extracellular ADDLs internalization inside neuronal cells, thus implying lipid rafts as contributors not only to Aβ biogenesis and accumulation but also to extracellular Aβ uptake.

13.23

POTENTIAL CYTOPROTECTIVE EFFECTS OF A NEW POLY(ADP-RIBOSE)POLYMERASE INHIBITOR

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Poly(ADP-ribose)polymerases (PARPs) are members of a family of enzymes that utilize nicotinamide adenine dinucleotide as substrate to form large ADP-ribose polymers (PAR) in the nucleus. PAR has a very short half-life due to its rapid degradation by poly(ADP-ribose) glycohydrolase. PARP-1 is involved in DNA repair and in maintenance of genome stability. Futhermore, it is becoming increasingly clear that the regulation by poly(ADP-ribosylation) contributes to a wide range of cellular functions. PARP-1 mediates acute neuronal cell death induced by a variety of insults including cerebral ischemia, parkinsonian neurotoxins and CNS trauma. The regulation of PAR metabolism by specific inhibitors has generated important insight into poly(ADP-ribosylation) functions and highlighted the therapeutic potential of PARP inhibitors in human diseases.

The PARP inhibitor capacity of a new 4-chinazolidonic derivative MC2050, has been characterized by evaluating enzyme activity both "in vitro" and in SH-SY5Y neuroblastoma cell line. Its effect has also been assessed in a cellular model which mimicks Alzheimer's degeneration, i.e. SH-SY5Y cells treated with Abeta 25-35 fragment.

The data obtained in vitro by PARP activity colorimetric kit (Trevigen) indicate that the new compound is highly active in the micromolar range (IC50 = 50 microM) compared to other well known inhibitors, chosen as reference drugs. In addition MC2050 is a more potent inhibitor than 3-ABA and PJ34 when assayed in SH-SY5Y cells. Cell viability assays revealed also that this compound is not cytotoxic at the tested concentrations.

When the SH-SY5Y cells were pretreated with 10 microM Abeta 25-35, the level of endogenous PARP activity increased up to 40% within 24 hours. In the presence of MC2050 enhancement of PARP activity seems to be blunted.

These results indicate that MC2050 is able to inhibit PARP probably by binding to the active site of the enzyme. Regulation of PARP activity may be a useful therapeutic intervention to reduce neurodegenerative damage.

14.1

PYRIMIDINE NUCLEOSIDE TRIPHOSPHATES ARE MAINTAINED AT HIGH LEVELS DURING MASSIVE ATP BREAKDOWN INDUCED BY ANOXIC-LIKE CONDITIONS IN CENTRAL NERVOUS SYSTEM CELLS.

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ATP is the primary high-energy phosphate compound produced by catabolism, in the processes of glycolysis and oxidative phosphorylation. In normoxic cells, its average concentration is 3 to 5 mM[1], depending on cell type and cell metabolic condition. A set of at least 100 kinases, including nucleoside-, nucleoside monophospho-, and nucleoside diphospho-kinases, then carry phosphoryl groups to other purine and pyrimidine free nucleosides and nucleotides, to form the other three main nucleoside triphosphates: GTP, UTP, and CTP. For these three ribonucleotides, the average cellular concentration is about one order of magnitude lower than that of ATP. It is a widely accepted tenet in neurobiology that, under normoxic conditions, intracellular nucleoside triphosphate levels are rigorously protected. However, in such events as ischemia or anoxia, massive ATP breakdown occurs. In this communication we present evidence that both in "post-mitochondrial" brain cell extracts, and in oligomycin-treated human astrocytoma cell line (ADF), the breakdown of ATP[2,3] is *not* accompanied by a concomitant breakdown of UTP and CTP.

The time courses of UTP and CTP breakdown, as catalyzed by rat brain extracts, were followed in the absence and in the presence of ATP, at physiological concentrations of the three nucleotides. In the absence of ATP, 0.6 mM UTP was rapidly broken down to UMP, uridine, and uracil, and CTP was broken down to CMP, cytidine, uridine and uracil (in the order). The addition of 3.6 mM ATP, almost completely prevented UTP and CTP breakdown. Strikingly, only when the concentration of ATP fell down around the Km values of nucleoside mono- and di-phosphokinases, UTP and CTP started to be broken down. The results obtained with brain extracts were sustained by experiments performed with cultured cells. In fact, the incubation of ADF cells with oligomycin, which inhibits oxidative phosphorylation, causes ATP breakdown at a more rapid rate as compared to CTP breakdown.

Our results suggest that nervous system cells have the capacity to maintain pyrimidine nucleotides at a high degree of phosphorylation, at least during the first anoxic stages, to avoid that certain nucleotides are lost, and to achieve better reperfusion recovery.

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14.2

TEMPERATURE DEPENDENCE AND KINETIC STUDIES ON THE HUMAN CYTIDINE DEAMINASE MUTANT ENZYME Y33G.

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Human cytidine deaminase (CDA) consists of four identical subunits (4 x 14,900 Da) with one active site per subunit. The four active sites, which lie at the subunit interface, operate independently and each subunit is equipped with a zinc atom that is involved in the covalent addition of water to cytidine, followed by elimination of ammonia[1]. Molecular modelling studies on human CDA[2] and the crystal structure of the tetrameric *B. subtilis* CDA[3] indicated that a complicate set of intersubunit interactions contributes to the building of the active site in each monomer. In this work the role exerted of the residue Y33, in the subunit interface and in the active site of human CDA was investigated. Y33 residue is part of a conserved region (32PYSHF36) in most tetrameric CDAs and it was supposed to be close to the active site in the CDA tertiary structure[3]. To verify this hypothesis on the purified Y33G mutant CDA, a kinetic study was performed and the effect of temperature on the protein activity and stability was also investigated.

The kinetic parameters calculated in presence of cytidine, deoxycytidine, azacytidine and cytosine arabinoside (Ara-C) revealed that deoxycytidine was the best substrate for Y33G whereas the Km value for cytidine and azacytidine was higher with respect to the wild-type CDA. The Vmax was significantly lower, indicating that the substitution of a tyrosine with a glycine had a dramatic effect on catalysis efficiency. Ara-C, an analog used in chemotherapy as antileukemic agent, was not recognized as substrate by the Y33G. The ability of a number of nucleosides and nucleotides to inhibit the mutant enzyme Y33G was tested, obtaining a lower Ki value with respect to the wild type CDA.

Differently to wild-type CDA[1], Y33G is a thermolable enzyme with optimal temperature around 40°C and a rapid decrease of enzymatic activity at 50°C due to protein denaturation. The temperature dependence of Vmax and Km values of Y33G mutant was analysed by using the empirical Arrhenius equation and the van't Hoff equation. The energy activation (Ea) value calculated for the mutant enzyme was 13,812 kcal/mol whereas for the wild type CDA the Ea was 9,038 kcal/mol. From these data it may be supposed that Y33G mutant CDA introduces strong interactions with the ligands (in particular deoxycytidine and inhibitors) as confirmed by its higher negative enthalpic value with respect to the wild-type enzyme. On the other hand Y33G mutant enzyme has difficulty to convert the substrate into the product because of the increasing Ea value that leads to a decrease of the Vmax value with respect to the wild-type CDA.

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14.3

EARLY PRESENCE OF A MYCOVIRUS IN CHERRY RUSTY SPOT DISEASE BEFORE EVIDENCE OF LEAF ALTERATION.

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The presence of mycovirus dsRNAs has been detected in cherry leaves showing symptoms of Chlorotic Rusty Spot disease (CCRS)[1]. In previous communications we provided evidence that the fungus *T. wiesneri* might be a possible host of that mycovirus because it is invariably present not only in affected but also in healthy leaves and also in immature buds[2]. We show here detailed location of *T. wiesneri* in cherry bud structures and provide evidence that mycotic *Chrysovirus* is an early presence in leaf areas that later will show the chlorotic rusty disease.

Leaves and buds of cherry trees were collected from plants closely inspected to detect the initial symptoms of CCRS. Samples were analysed for fungi presence by PCR amplification and sequencing of both 18S rDNA and ITS1 and ITS2 spacer segments. Mycovirus presence was revealed by RT-PCR amplification of 4 different segments corresponding to the 4 genomic dsRNAs of *Chrysovirus*. Analyses were performed on presumptive rusty spots and on adjacent areas of each leaf to establish possible boundaries of virus presence.

PCR results have confirmed the previous identification of *Taphrina wiesneri* as the most frequent fungus on cherry tree tissues[2]. Analyses of *Chrysovirus* dsRNAs have demonstrated its presence, at high titer, on leaves that showed faint spots of only early chlorotic modification, presumed to evolve in rusty spots. Similar analyses, on the closely adjacent areas of the leaf, and on its vascular conducts, gave negative results for virus genome. Also fungi collected and cultivated from the exterior of the affected areas of the leaf were negative for virus presence.

In conclusion, present data demonstrate that the *Chrysovirus* dsRNAs is a very early symptom of CCRS. The virus is detected neither in areas of the same leaf where chlorosis is not evident nor in its vascular tissues. It seems that there is an initial rapid burst of virus production and that this occurs only in a host fungus that is inside the leaf since no virus is detected in fungi collected and cultivated from the outer side of the affected regions of the same analysed leaves.

It is possible that the virus host, is a fungus that penetrates the leaf with no evidence of external growth, cycling between fallen and new leaves. It is also possible that the virus might infect specific areas of leaves using as a host a fungus that is already present in the leaf, transforming it into a pathogen. Studies will be made to investigate on these alternatives.

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14.4

CYCLIC NUCLEOTIDES LEVELS AND cAMP/cGMP PHOSPHODIESTERASES ACTIVITY EVALUATION IN STEM CELLS OF THE HUMAN PERIODONTAL LIGAMENT (PDL-MSCs)

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Mesenchymal stem cells (MSCs) are multipotent, capable of developing into mesenchymal, bony, cartilaginous, muscular and connective tissues.

Even thus bone marrow represents an excellent source of mesenchymal stem cells, adult stem cells can be harvested in large quantities from several organs and tissues like the umbilical cord, blood, dental pulp, periodontal ligament, amniotic fluid. One interesting and promising source of adult stem cells is the periodontal ligament (PDL).PDL does not only have a role in sustaining the teeth, but it also contributes to their trophism. Diseases which affect the periodontal tissues are the most frequent causes of teeth loss, which is the result of the destruction of the tissues which support the teeth (PDL, cementum, gingiva and alveolar bone)[2]. As a consequence of these phenomena the reconstruction of a healthy periodontium is the basis of periodontal therapy. Given that cyclic nucleotide signaling regulates a wide variety of cellular functions, it is not surprising that cyclic nucleotide phosphodiesterases are represented by a large superfamily of enzymes. PDEs have a modular architecture, with a conserved catalytic domain proximal to the carboxyl terminus and regulatory domains or motifs often near the amino terminus. [1]

The aim of our work was to trace the cAMP and cGMP dependent phosphodiesterases activity and cyclic nucleotides levels in stem cells obtained from PDL during their osteogenic differentiation process. The PDL-MSCs were obtained and cultured in MSCM medium (Cambrex Company, Walkersville MD 21793-0127) according to the manufacturer's directions provided (Trubiani et al. 2005). Cyclic nucleotides levels and the enzymatic reaction for cAMP/cGMP PDE assay were carried out using the method of Spoto et al.2006, with minor variations, by reverse-phase HPLC. The samples were organized into control stem cells (undifferentiated) and three groups of inducted stem cells: after 1 week, 2 weeks and 4 weeks. We achieved an interesting result concerning the cAMP PDE activity which shows a bell-shaped trend graph reaching its maximum at 2 weeks. Meanwhile cAMP level increases rapidly reaching its maximum value at one week and after PDE activity decreases below basal levels. Contrarily to our expectations, no implication of cGMP and cGMP PDE activity were detected during the differentiation process. The work demonstrates that cyclic AMP and cAMP PDE enzymatic activity are crucial steps in the differentiation process of the human stem cell.

cAMP Phosphodiesterase Activity Evaluation in Human Carcinoma of Salivary Glands. Nucleosides Nucleotides Nucleic Acids. (2006) 25: 1113-1117

1. A family of cAMP-binding proteins that directly activate Rap1 *Science* 1998, 2275-2279
2. Regenerative potential of human periodontal ligament derived stem cells on three-dimensional biomaterials: A morphological report

14.5

DIFFERENT EXPRESSION OF NICOTINAMIDE MONONUCLEOTIDE ADENYLYLTRANSFERASE ISOZYMES IN NORMAL AND PATHOLOGICAL TISSUES AND CELL LINES.

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Nicotinamide mononucleotide adenylyltransferase (NMNAT) is the central enzyme of the NAD biosynthetic pathway, catalyzing the transfer of the adenylyl moiety of ATP to NMN and leading to the formation of NAD.

Three human NMNAT isozymes, differing for the oligomeric state, subcellular localization and catalytic properties have been identified and characterized: NMNAT1 has an hexameric organization and is a nuclear protein; NMNAT2 is monomeric and is localized in the Golgi complex; NMNAT3 is a tetrameric mitochondrial protein.

By using a discrimination assay developed in our laboratory, we have been able to assess the individual isozymes contribution to NAD formation and, consequently, to determine their relative amounts in cell lines and tissues[1].

The mRNA and protein expression profiles, determined by RT real-time PCR and Western blotting, respectively, validate our protocol, in that in the cell lines and tissues analyzed the distinctive presence or absence of each isozymes perfectly matched.

The results show that, in all extracts, the three isozymes are not simultaneously present and do not equally contribute to NAD formation. Indeed, NMNAT1 is ubiquitously expressed, while NMNAT2 and NMNAT3 show a tissue-specific expression.

For example, in brain NMNAT3 is not detectable, whereas NMNAT2 predominates. Instead, in addition to the predominance of NMNAT1, NMNAT3 is present in liver tissue and in the hepatic cell line HepG2.

Our discrimination assay applied to red blood cells (RBCs) indicates that, unexpectedly, mitochondrial isozyme 3 largely exceeds the presence of isozyme 1 whereas isozyme 2 is absent. Moreover, NMNAT total activity is higher in younger RBCs, with NMNAT3 exceeding NMNAT1 in all different-aged RBCs.

An interesting correlation between NMNAT isozymes expression and pathological conditions was found in white blood cells (WBCs): total NMNAT activity is significantly higher in lymphocytes B of B-cell chronic lymphocytic leukemia patients than in normal lymphocytes B, and NMNAT3 seems to be exclusively expressed in the tumoral lymphocytes.

The results described in this presentation could be exploited for the identification of NMNAT isozymes as putative tumoral markers.

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14.6

EVALUATION OF BIOMARKERS IN PATIENTS WITH HAND OSTEOARTHRITIS

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Osteoarthritis (OA) is a common chronic joint disease with pain, functional impairment and radiographic changes at joints involved. In post-menopausal women, hand OA is the most common form, with a higher frequency of erosive type. Various clinical and radiographic criteria are used to classify and predict the progression of the disease but a gold standard definition has not yet been achieved. Usually, affected hand joints evolve from space reduction leading to swollen and painful periarticular tissue to formation of osteophytes and nodules with bone erosion in some patients. Objective of this study was to evaluate the performance of some biochemical markers in comparison to the radiographic assessment. MMPs (1, 3 and 13), TIMP-1, cathepsin B and COMP were measured in serum samples (n= 70). OA patients were divided into subgroups as: erosive (n=10) and nodular (n=40), on the basis of radiographs taken at the time of their enrolment. Healthy volunteers were chosen as controls (n= 20). Routine biochemical tests, ESR, CRP and total blood cells count were assayed on blood withdrawn and sera stored at -80°C for further analyses.

Preliminary data obtained by means of zymography, fluorometric and immunoenzymatic techniques show that MMP-1 content is significantly increased in nodular OA; whereas MMP-3 is markedly increased in erosive form. ESR levels were high compared to controls in both forms of OA.

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14.7

ALA147THR SUBSTITUTION IN TSPO IS ASSOCIATED TO ADULT SEPARATION ANXIETY IN PATIENTS WITH DEPRESSION

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Although anxiety disorders are heritable, their genetic and phenotypic complexity has made the identification of susceptibility genes difficult. In animal models, separation distress has been repeatedly shown to induce profound and irreversible alterations in neurosteroid-mediated central mechanisms controlling HPA activity and aberrant behaviours indicating increased anxiety[1].

A well-validated anxiety animal model is represented by rats that have been subjected to chronic maternal separation.

A human condition that might share some of those mechanisms described in animals is adult separation anxiety. We evidenced a reduction of expression levels of Translocator Protein (TSPO), the key protein of the first-limiting step of neurosteoid biosynthesis, in individuals with adult separation anxiety[2]. In this study, we sought to test whether two allelic variants of TSPO gene, 439G>A and 485G>A single nucleotide polymorphisms (SNPs), were associated with the presence of adult separation anxiety in patients with a principal diagnosis of bipolar disorder or major depression.

A case-control study was performed in 182 patients and 190 controls. With regards to 485G>A SNP, analysis did not elicit any association with adult separation anxiety. For 439G>A, a risk to develop adult separation anxiety was evidenced for A allele carriers. This SNP is likely to have a functional effect, because it codes for the aminoacidic change of Ala 147 in Thr. This aminoacidic position is in CRAC domain of TSPO, that is responsible for the uptake and translocation of cholesterol into mitochondria. Substitution of a non-polar aminoacid with a polar aminoacid might determine an alteration of cholesterol translocation that is the first rate-determining step in neurosteroid biosynthesis. A carriers showed significantly higher total scores on the scale for adult separation anxiety than patients with GG genotype. Our findings suggest that the A-allele of 439G>A SNP might be implicated in pathophysiology of adult separation anxiety.

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2. Chelli B, Pini S, Abelli M, Cardini A, Lari L, Muti M et al. (2008) Eur Neuropsychopharmacol 18, 249-254

14.8

A GENE EXPRESSION STUDY FOR B-CELL CHRONIC LYMPHOCYTIC LEUKEMIA

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B-CLL is the most frequent type of leukemia in the Western countries. The disease is most common among the elderly with a peak of incidence over 60 years. B-CLL follows a variable course both overall survival times and symptoms during disease course.

There is evidence that the accumulation of lympocytes in peripheral blood and bone marrow is due to a cell resistance to apoptosis rather than to high proliferative cells.

The analysis of gene expression profiling through microarray technology, in lymphocytes of patients with B-CLL, has revealed a number of biochemical and biomolecular changes. Particularly affected are the mechanisms of apoptosis, membrane signal transduction, the expression of interleukins, the activity of certain purinic metabolism enzymes and those involved in the responses to oxidative stress.

In our study peripheral blood lymphocytes were isolated by gradient density centrifugation and B cells were separated by immunomagnetic procedure. Total RNA was extracted and microarray analysis was performed by Ocimum Hybridization Service (Netherland).

Under the Customer Service was developed a chip consists of 50 genes coding for proteins involved in the processes mentioned above. Moreover, the presence or absence of programmed cell death in leukemic cells was determined by the study of enzyme activity of caspase 3 and protein-tyrosine phosphatase (PTP), the presence of fragmented DNA was determined by gel electrophoresis of agarose.

Preliminary results identified many genes of purine metabolism with different expression from controls. The expression of 17 of 50 genes studied is altered (34% of genes on the chip). Their expression is increased or reduced by at least two times compared to control. Among de novo enzymes, the Gairs-Airs-Gart complex was overexpressed and IMP dehydrogenase seemed underexpressed. Of salvage pathway enzymes, APRT revealed underexpression, but HGPRT was unchanged. Ecto-5'-nucleotidase (CD73) was underexpressed in agreement with the low enzymatic activity, determined during our studies.

Since extracellular adenosine induces apoptosis via A2b receptors, enzymes such as CD73 involved in extracellular adenosine release could play a role in B-CLL cell resistance to apoptosis.

14.9

LEVELS OF HOMOCYSTEINE AND TRANSAMINASE

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Recent epidemiologic studies[1-2] have shown that homocysteine (Hcy), a sulfur-containing amino acid formed during the metabolism of methionine, is a risk factor of atherosclerosis, myocardial infarction, stroke, thrombosis, chronic kidney disease and cognitive impairment in various neurological diseases. Therefore, hyperhomocysteinemia is associated with a lot of diseases, but it has not been clarified exactly which mechanism is responsible for occurence disease.

Between possible negative effects of Hcy can also count taking away of free pyridoxal-phosphate (PLP) or PLP bound with enzymes, alterated the enzymatic activity.

In previous our study[3] we examined the effects of different aminothiols on enzymatic activity of PLP dependent enzymes. These sulfurated substances strongly inhibited enzymatic activity.

The aim of this study is to assess the relationship between level of Hcy and two enzymes: aspartate transaminase (AST 2.6.1.1) and alanine transaminase (ALT 2.6.1.2). These two enzymes are characterized by the presence of PLP and its participation in catalysis. The PLP coenzyme is covalently bound to the ε-amino group of lysine 258. It provides the characteristic absorption spectra with a major absorption peak at 390 nm.

PLP reacts chemically with Hcy to give 2-(3-hydroxy-5-phosphoryloxymethyl-2-methyl-pyridyl-D-tetrahydro-1,3-thiazine-1-carboxylic acid[4]. During reaction the original absorption maxima shift to 330 nm to formation of new substance. In this way we can evaluate adduct formed.

To demonstrate inhibition of AST and ALT by homocysteine, we evaluate enzymatic activity, monitored via decrease in E_{340} in the presence and absence of different concentration of Hcy.

AST mixtures in final volume 1 ml: 12 mM aspartate; 2 mM α-ketoglutarate; 0.1 M potassium phosphate buffer pH 7.3; 0.15 mM NADH; 0.1 U malato dehydrogenase. It was prewarmed to 37 °C in a cuvette for 10 min. For one experiment, to this mixture added 0.05 U AST.

ALT mixtures in final volume 1 ml: 4 mM alanine; 16 mM α-ketoglutarate; 0.1 M potassium phosphate buffer pH 7.3; 0.15 mM NADH; 0.1 U LDH. It was prewarmed to 37 °C in a cuvette for 10 min. For one experiment, to this mixture added 0.05 U ALT.

For inhibition experiment, added different aliquot of Hcy (3.8 - 7.6 - 15.2 µmoles).

Hcy don't inhibit the activity of two enzyme considered, at difference of cysteine and analogous aminothiols[3]. Hcy, in total plasma elevated concentration, can be involved in many interactions, but don't interfere with two important enzymes, the transaminase, frequently assayed in clinical laboratories.

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14.10

IDENTIFICATION OF INOSINE NUCLEOTIDES IN CELLULAR RNA BY AN HPLC-LINKED METHOD: POSSIBLE UTILIZATION IN THE STUDY OF mRNA EDITING

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The presence of IMP in mRNA is due to the action of a specific class of RNA-adenosine deaminases (ADAR), present in different forms indicated as ADAR1, ADAR2 and ADAR3. ADARs share the common characteristic of converting specific mRNA adenosine nucleotides into inosine ones by hydrolysis of the amine group. The mutated nucleotide is recognized as a guanosine nucleotide during translation, thus changing the triplet and often coding for a different aminoacid. This process, indicated as A-to-I RNA editing, leads to mutated proteins often resulting in significant functional consequences. RNA editing represents a finely regulated mechanism changing the readout of a gene within the primary RNA transcript.. ADARs are mainly expressed in nervous tissue and are known to edit mRNA of specific membrane receptors (e.g. glutamate and serotonine) thus modulating signal transduction.
Molecular approach is usually used to detect RNA editing; this study presents a biochemical HPLC-linked method for qualitative and quantitative detection of IMP from RNA.

Up to 1 mg RNA extracted from different biological sources (human blood;human and animal cell lines :HELA, 3T3, ADF, medulloblastoma) was hydrolysed by Nuclease P1 from Penicillum Citrinum (Sigma), releasing 5' phosphate nucleosides. Incubation was conducted in tris-HCl buffer pH 7,5, supplied with $ZnCl_2$ as an enzyme stabilizer, either at 37°C (up to 150 mins) or 50°C (60 mins). Protein-free perchloric extracts of assay mixtures were brought to neutrality and processed by RF-HPLC. Elution was conducted according to a modification of previously described methods [V. Micheli et al., Life Sci, 1999]. Gradient elution using 0.1M KH_2PO_4 containing 6mM TBA, pH 5,5 (eluant A) and methanol (eluant B) allowed the complete separation of released AMP, IMP, GMP, UMP and CMP in 12 minutes. Concentration and percent amount of each nucleotide could be calculated. IMP was only detectable in RNA from one blood sample, but not in other cells; IMP recovery reliability was checked by added internal standard in all samples.

A-to-I RNA editing is known to follow specific events, such as cell differentiation [A.Barbon et al., Mol Brain Res, 2003] and may be altered in pathological processes [S. Kwak, et al., J Mol Med 2005]. The described method was demonstrated to be reliable in isolating and quantifying IMP from RNA, when present. This technique might provide a quick and unexpensive support to molecular investigations, particularly as a screening diagnostic tool. Investigations are in progress concerning disorders in which A-to-I editing is known or suspected to be altered, such as schizofrenia, Alzheimer and Huntington diseases, epilepsy and depression [C.M. Niswender et al., Neuropsychopharmacology, 2001]

14.11

MECHANISTIC STUDIES ON STRUCTURE AND FUNCTION OF CYTOSOLIC 5'-NUCLEOTIDASE (cN-II): EFFECTOR SITES AND SUBUNITS INTERACTION.

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CN-II is a bifunctional enzyme ubiquitously expressed in human tissues, with the highest levels of activity being expressed in several pathological conditions. CN-II shows a complex regulation system and the ability to catalyze both hydrolase and phosphotransferase activities. Both cN-II activities are involved in the regulation of intracellular availability of purine compounds[1] and in the activation/inactivation processes of antiviral and antitumoral purine prodrugs[2]. CN-II is activated by ATP, ADP (high energy charge) and inhibited by phosphate[3]. Hypotheses have been advanced that activators promote subunits aggregation .

Site-directed mutagenesis allowed us to identify the catalytic motif and to assign cN-II as a member of HAD superfamily[4]. Recently, crystal structure of cN-II has been described[5]. Authors indicate the presence of two effector sites based on the interaction with adenosine. In order to unravel the specificity of each site for the several compounds involved in cN-II regulation, we constructed 3 point mutants for putative effector sites and two point mutants for the site of interface between subunits.

Preliminary results indicate that diadenosine tetraphosphate acts through interaction with both sites, while ATP seems to interact only with site 2. Site- directed mutagenesis on residues involved in subunit aggregation strongly affects the conformation of active site as judged by the alteration of K_m for phosphotransferase substrate inosine.

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2. Galmarini CM et al (2003) Leuk Lymphoma 44, 1105-1111.
3. Pesi R. et al (1994) Arch. Biochem. Biophys. 312, 75-80.
4. Allegrini S. et al. (2004) Eur. J. Biochem. 271, 4881-4891.
5. Wallden K et al. (2007) J.B.C. 282 (24) 17828-17836.

14.12

MECHANISTIC STUDIES ON STRUCTURE AND FUNCTION OF CYTOSOLIC 5'-NUCLEOTIDASE: POINT MUTANT Y55G.

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CN-II is a bifunctional enzyme ubiquitously expressed in human tissues, with the highest levels of activity being expressed in several pathological conditions. CN-II shows a complex regulation system and the ability to catalyze both hydrolase and phosphotransferase activities. Both cN-II activities are involved in the regulation of intracellular availability of purine compounds[1] and in the activation, inactivation processes of antiviral and antitumoral purine prodrugs[2]. CN-II is activated by ATP, ADP (high energy charge) and inhibited by phosphate[3].

Site-directed mutagenesis allowed us to identify the catalytic motif and to assign cN-II as a member of HAD superfamily[4]. All members of the HAD superfamily share four highly conserved motifs in their otherwise dissimilar sequences, and similar structural folding with an α/β core domain in which the active site is formed by four loops and a small cap domain for substrate recognition.

Sequence alignment of cN-II with other members of 5'-nucleotidases family suggested that the first conserved motif is involved not only in the formation of a covalent intermediate with phosphate but also in the transfer of the bound phosphate to a nucleoside acceptor. We, therefore, decided to generate a point mutant substituting a tyrosine residue in position 55 with a glycine. In fact, members of the family devoid of phosphotransferase activity possess a glycine in the corresponding position. The ability of the point mutant to operate a phosphate transfer was measured as function of several parameters such as the presence of activators, inhibitors and in combination (energy charge in the presence or absence of phosphate). Our results indicate that the mutant possess a very poor phosphotransferase activity, while every other kinetic characteristics of the enzyme remain unaltered.

1. Ipata PL and Tozzi MG (2006) Purinergic Signalling 2, 669-675.
2. Galmarini CM et al (2003) Leuk Lymphoma 44, 1105-1111.
3. Pesi R. et al (1994) Arch. Biochem. Biophys. 312, 75-80.
4. Allegrini S., et al. (2004) Eur J. Biochem 271, 4881-4891.

14.13

CHARACTERIZATION OF THE TRANSCRIPTIONAL REGULATOR OF THE NRTR FAMILY CONTROLLING NAD SALVAGE IN γ-PROTEOBACTERIA

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By using comparative genomic techniques to analyze conserved NAD biosynthetic operons we have recently identified a new family of Nudix-related transcriptional regulators (NrtR) in a broad range of bacteria[1]. NrtRs contain an N-terminal domain homologous to ADP-ribose (ADPR) pyrophosphatase of the Nudix family and a C-terminal domain which adopts a winged-helix turn helix fold found in many prokaryotic transcription factors[1]. While the Nudix domain is likely responsible for a specific binding of an effector molecule, the C-terminal domain binds to the operator sites of the regulated genes. The NrtR-binding sites were predicted in a variety of bacterial genomes, allowing *in silico* reconstruction of NrtR regulons that primarily include genes involved in various aspects of NAD metabolism[1].

In some γ-proteobacteria, including *Shewanella oneidensis*, NrtR is predicted to control transcription of the *prs-nadV* operon, involved in nicotinamide (Nam) recycling to NAD. The operon codes for the PRPP synthesizing enzyme (Prs) and Nam phosphoribosyltransferase (NadV), which catalyzes the transfer of the phosphoribosyl moiety of PRPP to Nam forming NMN, the direct NAD precursor. Combination of *prs* and *nadV* into one functional unit would allow NMN synthesis from Nam, providing at the same time the phosphoribosyl donor PRPP. In order to functionally characterize *Shewanella oneidensis* NrtR (*so*NrtR), the corresponding gene has been cloned and over-expressed in *E. coli*. The specific binding of the regulator to the predicted DNA sites in the upstream region of the *prs-nadV* operon was demonstrated by both electrophoresis mobility shift assay (EMSA) and a fluorescence based thermal shift assay. The regulator was found to be a repressor, as revealed by assaying its effect on *prs-nadV* operon transcription *in vitro* with σ^{70}-saturated RNA polymerase from *E. coli*, following quantification of the resulting transcript by RT-mediated real-time PCR. The thermal shift assay was also used to analyze the binding of *so*NrtR to various NAD metabolites. The regulator showed the highest binding affinity (17 μM) towards ADPR, phospho-ADPR and NAADP. These molecules were also the most effective in suppressing *in vitro* binding of NrtR to its DNA target sequence and in preventing the repressive effect of NrtR on transcription.

The effect of ADPR on *so*NrtR suggests that the regulator might control NAD biosynthesis by sensing intracellular ADPR levels. Accumulation of ADPR in the cell might be interpreted as a signal to replenish NAD pool, as ADPR is formed together with Nam from the enzymatic hydrolysis of NAD. Binding of ADPR to NrtR would promote dissociation of NrtR-DNA complex, leading to derepression of NAD biosynthetic genes transcription. *In vivo* functional studies are in progress to verify this model.

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14.14

PARTIAL INHIBITION OF *ESCHERICHIA COLI* DIHYDROFOLATE REDUCTASE BY SPERMINE

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Multiple intermediates and conformations are general characteristics of enzyme catalysis and allosteric regulation. Dihidrofolate reductase (EC 1.5.1.3), an enzyme ultimately involved in DNA biosynthesis, is recognized to possess structural features able to guide the substrate (dihydrofolate) and cofactor (NADPH) through the preferred catalytic pathway by specific active site conformational changes, modulated by long range interloop interactions[1]. In this study we examined the modulatory effect of natural polyamines (putrescine, spermidine and spermine) on the enzymatic activity of dihydrofolate reductase overexpressed and purified from *E. coli*. Polyamines are charged molecules essential for cell life and found to be involved in many biological processes like cell growth, regulation of gene expression and cell signalling[2]. Many molecular aspects of polyamines function remain still to be clarified; the aim of this study was to investigate a possible role of polyamines as regulators of cell cycle by directly interacting with the key enzyme of folate metabolism, and directly involved in nucleotide biosynthesis. The results reported by our study shown a partial inhibition effect exerted by spermine on dihydrofolate reductase from *E. coli* at pH 7.3 and 30 °C. Partial inhibition can be interpreted as the conversion of the enzyme from a fast to a slow kinetic form, still able to catalyze the transformation of the substrate to product, even if at lower rate. From the fractional-velocity plots, spermine resulted to behave as a partial non-competitive inhibitor of *E. coli* DHFR with respect to dihydrofolate, with a K_i of 335 μM. Analysis of the temperature dependence of the catalytic constant in presence of spermine confirmed the effect of the polyamine in increasing the activation energy of the reaction catalyzed by dihydrofolate reductase. The structural aspects of the partial inhibition of DHFR by spermine have been analyzed by performing a pH screening of the inhibitory effect. Our data clearly demonstrate that the spermine does modulate the enzymatic activity of dihydrofolate reductase from *E. coli* by stabilizing, through weak interactions, a kinetically slower conformation of the protein. This kind of enzymatic modulatory effect is worth to be investigated in order to understand cellular signalling regulation and to design new multi-target drugs.

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15.1

ARCHAEAL PROTOGLOBIN STRUCTURE SUGGESTS NOVEL LIGAND DIFFUSION PATHS AND HEME-REACTIVITY MODULATION.

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Despite its strict anaerobic nature, *M. acetivorans* genome hosts genes that can be related to O_2 metabolism; among these, an open reading frame encodes for a "protoglobin" (NP_617780; Pgb). Pgbs are single domain heme proteins of ~195 amino acids, related to the N-terminal domain of archaeal and bacterial globin coupled sensor proteins (GCS)[1-3].

Sequence comparisons indicate that Pgbs, despite their 30-35% larger size, are structurally related to the single chain hemoglobins (composed of about 150 amino acids, folded into a 3-on-3 a-helical sandwich,12-16% residue identity to Pgbs), and to the heme-based aerotaxis transducer sensor domain of *Bacillus subtilis* GCS. Pgbs bind O_2, CO, and NO reversibly in vitro. Although functional and evolutionary issues are openly debated[1-3], Pgb may facilitate O_2 detoxification in vivo promoting electron transfer to O_2, or may act as CO sensor/supplier in methanogenesis.

We report here the 1.3 Å crystal structure of oxygenated *M. acetivorans* protoglobin, together with first insight into its ligand binding properties[4]. We show that Pgbs are composed of a single heme-binding domain strongly related in tertiary and quaternary structure to the N-terminal domain of archaeal and bacterial GCSs. Furthermore, contrary to all known globins, protoglobin-specific loops and a N-terminal extension completely bury the heme within the protein matrix. Structural modulation of the globin fold in Pgb translates into entirely new access routes to the heme, which is granted by protoglobin-specific apolar tunnels reaching the heme distal site from locations at the B/G and B/E helix interfaces.

The high experimental resolution of the Pgb structure provides unequivocal evidence of substantial distortion in the porphyrin ring system, which suggests a proactive role of Pgb in modulating heme reactivity (e.g. chemical and photophysical properties, axial ligand affinity, O_2 off-rates, redox potentials) providing a mechanism for differentiating between CO and O_2 binding.

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15.2

MOBILITY OF MEMBRANE PROTEINS EXPLORED BY SINGLE PARTICLE TRACKING (SPT): THE LATERAL DIFFUSION OF THE GLYCINE RECEPTOR DEPENDS ON THE OLIGOMERIZATION STATE OF THE SCAFFOLDING PROTEIN GEPHYRIN.

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Recent studies have revealed the importance of lateral diffusion on the plasmamembrane for the regulation of neurotransmitter receptor number at synapses, and thus for the modulation of synaptic strength. Great attention has been especially paid to factors underlying the confinement and clustering of the receptors, i.e. the cytoplasmic scaffolding proteins present at the postsynaptic terminal. Among these, gephyrin has a major role in anchoring and stabilizing the glycine receptor (GlyR) at inhibitory postsynaptic sites. Isolated N- and C-terminal domains (G and E) of gephyrin can form trimers and dimers, respectively, and thus contribute to the formation of sub-membranous gephyrin clusters found at synapses.

Here, we investigate how the expression of constructs able to modify the oligomerization state of gephyrin can affect GlyR dynamics. These constructs include the isolated E and G domains, and a natural gephyrin splice variant that has a decreased propensity to form trimers. Experiments were carried out in spinal cord neurons and COS7 cells. A reduction of the size and number of gephyrin-GlyR clusters was found in both cell types expressing the various constructs. To determine the dynamic properties of gephyrin and the constructs, fluorescence recovery after photobleaching (FRAP) of mRFP and YFP tagged proteins was performed in spinal cord neurons. Although the different constructs have relatively fast rates of recovery, they do not alter the exchange rate of wild-type gephyrin. This result shows that gephyrin and the constructs are at steady state and the remaining clusters are mainly constituted by full-length gephyrin. The GlyR was immunochemically bound to quantum dots (QDs), very small and photostable fluorescent probes, and real-time SPT was used to analyze the mobility of the receptor. We demonstrate that gephyrin-GlyR complexes are present in the extra-synaptic compartment. The presence of isolated G and E domains can interfere with gephyrin oligomerization and increase the mobility of these complexes. Over-expression of the variant results in a similar increase in the lateral diffusion of GlyR.

We therefore provide direct evidence that the reduction in the amount of synaptic GlyR following expression of the gephyrin variant results from the formation of extra-synaptic gephyrin-GlyR complexes, which diffuse freely in the plasmamembrane. These findings may implicate the variant in the endogenous regulation of GlyR dynamics and trafficking. The oligomerization properties of gephyrin not only contribute to the formation of gephyrin clusters, but also affect the lateral diffusion of the glycine receptor in the extra-synaptic compartment.

15.3

ESPRESSION OF THE RHODANESE-LIKE PROTEIN RhdA IN A. VINELANDII UNDER OXIDATIVE STRESS CONDITIONS.

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Characterized rhodanese-like proteins *in vitro* catalyse the transfer of a sulfur atom from a suitable sulfur donor (e. g. thiosulfate for rhodaneses) to cyanide, with concurrent formation of thiocyanate[1]. Genome sequencing analysis has clustered at least 3000 proteins in the rhodanese domain homology superfamily (Pfam Acc. N.: PF00581). The physiological role of these proteins is still elusive, although *in vitro* studies with rat mercaptopyruvate sulfurtransferase[2], and proteomic analyses[3-4] pointed to a possible relevance of specific rhodanese-like proteins in processes related to oxidative events.

Among the redundant rhodanese-like proteins of *Azotobacter vinelandii*, the tandem domain protein RhdA contains an active-site motif not commonly found in rhodanese-domain proteins. Preliminary phenotypic characterization of an *A. vinelandii* mutant strain in which *rhdA* was deleted, evidenced that RhdA protected Fe-S enzymes that are easy targets for oxidative damage[5].

To explore a possible role of RhdA in maintaining redox homeostasis in *A. vinelandii,* the expression of RhdA was evaluated in cell cultures grown under conditions of induced oxidative stress. The analyses of mRNA for RhdA and immunodetectable RhdA were performed in RNA extracts and in crude extracts of *A. vinelandii* cell cultures grown either in the absence (control samples) or in the presence of the oxidative agent phenazine methosulfate (15 μM). A two-step Reverse Transcription PCR (RT-PCR) was used for semiquantification of *rhdA* mRNA. We found that *rhdA* transcript was more abundant (2-fold) in *A. vinelandii* cells grown under oxidative stress conditions than in control growth conditions. Western blot analyses with anti-RhdA antibody did not revealed an increase of RhdA protein in conditions of induced oxidative stress. A possible modification involving the RhdA Cys_{230} catalytic thiol was suggested by measurements of the thiosulfate:cyanide sulfurtransferase activity in extracts from PMS-treated cells.

Taken together, these results support the hypothesis that RhdA helps *A. vinelandii* in maintaining cellular redox balance, and that events related to sulphur redox chemistry could increase oxidative-induced RhdA turnover.

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15.4

ROLE OF RESIDUES Asp161, Arg164 AND Glu211 IN *MYCOBACTERIUM TUBERCULOSIS* NADPH-FERREDOXIN REDUCTASE

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Mycobacterium tuberculosis NADPH-ferredoxin reductase (NFR or FprA)[1], is a FAD-containing oxidoreductase, homologous to mammalian adrenodoxin reductase (AdR). The bacterial enzyme has been proposed to play a major role in the lipid metabolism of the pathogen, as a source of reduced ferredoxin(s) to sustain the activity of its several cytochrome P450s.

The resolution of the crystal structure[2] of NFR allowed us to identify two ionisable residues (D161 and E211) located in the active site and potentially involved in catalysis of hydride transfer. In order to evaluate the role of these residues, we produced six variants of the enzyme carrying various single point mutations (E211A, E211D, E211Q, D161A, D161E, D161N). All NFR forms were overproduced in *E. coli*

We were able to isolate all E211 mutants in holoenzyme form. Their functional characterization indicated that E211 plays a significant but non-essential role in substrate binding, resulting in a moderate contribution to catalysis. On the contrary, the variants carrying a substitution at position 161 could only be isolated as apoenzymes. Indeed, all D161 variants undergo loss of FAD prosthetic group during purification, making impossible any kinetic characterization. Moreover, attempts to reconstitute the holoenzyme adding exogenous FAD were unsuccessful. These results indicate a critical role for the D161 side chain in the stabilization of the NFR native conformation and in FAD binding.

Since the side chain of D161 forms a salt bridge with R164, it was argued that replacement of the former residue with a neutral one, could induce a reorganization of the orphan Arg side-chain, resulting in destabilization of the native conformation. Thus, two additional NFR variants were produced, carrying either a single mutation (R164A) or a double mutation (D161A/R164A). Both mutants turned out to be highly unstable, suggesting that both member of the ionic couple D161/R164 are critical for the stability of the enzyme. These results indicate that most of the ionisable residues located at active site of NFR are more important for maintaining the native conformation of the enzyme than for its catalytic activity.

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15.5

A PLANT-TYPE ENZYME AS A PUTATIVE TARGET FOR NOVEL ANTIMALARIAL DRUGS: PROPERTIES OF THE *PLASMODIUM FALCIPARUM* FERREDOXIN-NADP$^+$ REDUCTASE.

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Apicomplexan parasites harbor a specific organelle, named apicoplast, which is related to plant non-photosynthetic plastids and displays a plant-like metabolism. The apicoplast has been shown to contain typical vegetal proteins, such as ferredoxin–NADP$^+$ reductase (FNR) and ferredoxin (Fd)[1-2]. Both proteins from *Plasmodium falciparum* (PfFNR and PfFd) have been produced in recombinant form and characterized[3]. The PfFNR/PfFd couple was shown to be catalytically active *in vitro* yielding reducing power to support the activity of LytB[4], the last enzyme of the biosynthetic pathway for isoprenoid precursors, a known site of action of antiplasmodial compounds. On this basis, PfFNR has been proposed as a possible target for new antimalarial drugs[2].

The three-dimensional structure of PfFNR has been determined by X-ray crystallography[3]. Compared to other plastidic-type FNRs, PfFNR displays a significantly lower catalytic efficiency and lower selectivity against NADH. These functional features are probably the consequence of the lack of protein positive charges stabilizing the 2'-phosphate of the bound substrate. NADP(H) binding to PfFNR occurs through an induced-fit mechanism never observed in other FNRs. The conformational changes induced by binding to the enzyme of 2'-P-AMP, a NADP$^+$ analogue, includes the partial unwinding of an α-helix localized in the NADP$^+$-binding domain. Furthermore, the binding of NADP$^+$ triggers the formation of a disulfide-stabilized homodimer resulting in the inactivation of PfFNR. This process, observed *in vitro*, could represent a physiologic mechanism regulating the enzyme activity.

Structure-based design of PfFNR inhibitors is in progress and has already yielded some active compounds, with inhibitory constants in the range of micromolar or lower.

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15.6

SMALL ANGLE X-RAY SCATTERING STUDIES REVEAL IMPORTANT CLUES FOR MEMBRANE BINDING AND ACTIVITY OF FATTY ACID AMIDE HYDROLASE (FAAH)

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Amides, esters, and ethers of long-chain polyunsaturated fatty acids, collectively referred to as “endocannabinoids”, represent a growing family of lipid signaling mediators found in several tissues with a wide variety of biological actions. The main members of this group of molecules are anandamide (N-arachidonoylethanolamine, AEA) and 2-arachidonoylglycerol. Fatty acid amide hydrolase (FAAH) is a typically membrane enzyme that catalyzes the conversion of AEA into arachidonic acid and ethanolamine, thus terminating the signaling of this endocannabinoid[1]. The homodimeric structure of FAAH has been deducted as a biological unit from the crystallographic structure of a mutant rat enzyme (DTM-FAAH), a catalytically active form still able to bind the membranes even if missing the a-helices mainly responsible for this interaction. Until now, direct information on FAAH aggregation state in solution and in the presence of inhibitors, substrate analogues, or lipids is still lacking.

In this study we analyzed the oligomerization state of DTM-FAAH in solution by small angle X-ray scattering (SAXS). We found that, among several effectors, high concentrations of Tris buffer (≥ 0.5 M) are able to stabilize monodisperse oligomers. Data analysis shows that these oligomeric structures have a value of radius of gyration of 129±13 Å, as calculated from the p(r) function (in accordance with the Guinier analysis) and a value of the maximum dimension of the particle (D_{max}) of about 410±10 Å. Superimposing the crystallographic unit of FAAH (pdb entry 1MT5.pdb), which is an octamer of dimers, to the low resolution DAM model of our oligomers, we observed that these last are composed of three octamers revealing an unprecedented oligomerization state of FAAH in solution. Furthermore, we studied the structural effects of different FAAH inhibitors observing an increase of the oligomerization state of the protein in the presence of these molecules. Then we paralleled this structural information with a functional analysis of the enzyme investigating the role of these inhibitors in modulating the membrane association of FAAH using both fluorescence resonance energy transfer measurements (FRET) and Laurdan fluorescence. Taken together, the complementary information collected allowed to understand the molecular mechanisms leading to FAAH function. It also revealed the presence of an unprecedented oligomerization state of FAAH and a key role of inhibitors in favouring the subunit-subunit interactions that may impair the FAAH activity.

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15.7

MODULATION OF THE ENZYMATIC ACTIVITY AND MEMBRANE BINDING PROPERTIES OF SOYBEAN LIPOXYGENASE-1 THROUGH LIMITED PROTEOLYSIS AND METAL SUBSTITUTION

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Lipoxygenases are non-heme, non-sulfur iron containing enzymes that catalyze the dioxygenation of polyunsatured fatty acids containing one or more pentadiene systems to the corresponding hydroperoxy derivatives. Structural studies in solution of the mammalian and plant enzyme revealed that the latter has a more stable and compact conformation[1]. As yet, metal atom extraction, reconstitution and substitution with vicariate metals have not been successfully applied to soybean lipoxygenase-1, because of the highly buried position of the iron atom within the active site. Tryptic digestion of lipoxygenase-1 and the subsequent isolation of the 60 kDa C-terminal region allowed to generate a “mini-lipoxygenase-1 (miniLOX)” that retains the catalytically active iron, but in a more accessible position[2]. In this study, we investigated by near-UV-circular dichroism and fluorescence spectroscopies the structural and functional effects of iron removal, reconstitution and vicariation in miniLOX. Moreover, we report the kinetic analysis and the membrane binding ability of the apo- and metal-substituted forms of miniLOX, using fluorescence resonance energy transfer and monolamellar vesicles. Taken together, these data demonstrate an unprecedented structural role of iron, which is involved not only in the catalytic activity but also in the membrane binding ability of lipoxygenase-1.

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15.8

OXIDATIVE RESPONSES IN DIFFERENT HUMAN GASTRIC NEOPLASMS

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Carcinogenesis is a multistep process associated with accumulated genetic alterations in somatic cells.
Reactive oxygen species (ROS) may participate in the multistage carcinogenesis from initiation to malignant conversion by causing oxidative DNA damage and mutations in proto-oncogenes and tumour suppressor genes, and by activating signal transduction pathways[1].
Since many human neoplasms have shown significant changes in superoxide dismutase (SOD) and catalase (CAT), we have investigated them in healthy gastric tissues respect to adenocarcinoma, *Helicobacter pilory* (HP), autoimmune-atrophic-gastritis (AAG) and chronic gastritis (CG) mucosa.

MnSOD activity significantly increased in adenocarcinoma, CG and HP tissues respect to healthy control. Cu/ZnSOD was significantly lower in adenocarcinoma and HP tissues respect to healthy control. Parallel MnSOD activity, with respect to Cu/ZnSOD, is 3-4 fold higher in HP and tumours samples respectively. Relatively to the expression, MnSOD and Cu/ZnSOD are significantly more expressed in adenocarcinoma and HP tissues and less expressed in CG tissues respect to control group. A significant decrease in CAT activity in adenocarcinoma and HP tissues was observed.

SODs are correlated with the degree of cancer differentiation but their biological significance in gastric cells is unclear[2]. Indeed only MnSOD is associated with poor survival in carcinoma patients suggesting different regulatory mechanisms between SODs isoforms. In conclusion our data suggest a specific condition of stress in HP and cancer and partially in CG tissues due to SOD increase and low levels of CAT. This state establishes an apoptotic intracellular environment characterized by several mutations, cell transformations and cancer, confirming the importance to detect an early signal involved in neoplasms development.

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15.9

IDENTIFYING DISORDERED REGIONS IN PROTEINS BY LIMITED PROTEOLYSIS

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Nowadays there is a strong interest in partly folded or even fully disordered or natively unfolded proteins, since these protein states can have a role in the proper functioning of proteins[1]. However, the analysis of protein structural disorder is quite problematic and, to this aim, physicochemical and computational techniques for identifying and characterizing protein conformational disorder are being explored[2]. In a series of papers from our laboratory we have demonstrated that limited proteolysis experiments can be successfully used to probe conformational features of proteins. This approach relies on the fact that the sites of limited proteolysis along a polypeptide chain are characterised by enhanced backbone flexibility and, therefore, proteolytic probes can pinpoint the sites of local unfolding or protein disorder in a protein chain. The first indication that limited proteolysis correlates with protein locall dynamics was established by us two decades ago using thermolysin as an experimental test[3].

We have successfully used the limited proteolysis technique for the analysis of the molecular features of several partly folded proteins and, in particular, for the identification of disordered regions within otherwise folded globular proteins. A striking correlation was found between sites of limited proteolysis and sites of enhanced chain flexibility of the polypeptide chain, this last evaluated by the crystallographically determined B-factor. In crystal structures of proteins, the B-factor reflects the uncertainty in atom positions in the 3D protein model and often represents the combined effects of thermal vibrations and static disorder. When plotted against residue number, B-factor values provide a graphic image of the degree of mobility existing along the polypeptide chain.

We have now found that limited proteolysis very often occurs at chain regions characterized by missing electron density, thus indicating unfolding of these regions. In general, a clear-cut correlation exists between sites of limited proteolysis and sites of flexibility/disorder in a dozen of proteins for which the crystallographic structure is known. Here, we show only a couple of examples, but the generality of the approach has been substantiated with a dozen of protein systems.

We conclude that limited proteolysis is a very useful and reliable experimental technique that can detect sites of disorder in proteins, thus complementing the results that can be obtained by the use of other physicochemical (X-ray, NMR, hydrogen-deuterium exchange) and computational approaches.

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15.10

L-ASPARTATE OXIDASE AND QUINOLINATE SYNTHASE FROM *B. SUBTILIS*

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NAD is an essential molecule in all living organisms. In many eubacteria, including several pathogens, the first two steps in the *de novo* synthesis of this important cofactor are catalyzed by the so called quinolinate synthase complex made by L-aspartate oxidase (NadB) and quinolinate synthase (NadA). Despite the important role played by these two enzymes in NAD metabolism, many of their biochemical and structural properties are still largely unknown. In the present study we cloned, over-expressed and characterized NadA and NadB from *B. subtilis*, one of the best studied bacteria and a model for low-G-C Gram-positive bacteria including pathogens. Our data add new information regarding the NadA cofactor and the interaction between NadA and NadB. In particular, it is demonstrated that the cofactor for NadA from *B. subtilis* is a [4Fe-4S] cluster and for the first time the cysteines involved in the cluster binding are identified. All together the data suggest that in NadA from *B. subtilis* the [4Fe-4S] cluster is coordinated by three highly conserved cysteine residues (C110, C230, C320) suggesting that NadA presents a new non canonical binding motif that, based on sequence alignment studies, may be common to all the quinolinate synthases from different sources. Moreover, the results show for the first time that the interaction between NadA and NadB is not species-specific and that the integrity of the Fe-S cluster may be important for the binding of iminoaspartate to NadA.

15.11

PROTEOMIC ANALYSIS OF XENOPUS EMBRYOS EXPOSED TO SIMULATED MICROGRAVITY

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Exposure to space-flight environment, notably microgravity and radiations, can induce rapid changes in living systems that are similar to changes occurring during ageing. Indeed ageing and life in space are both associated with the same undesirable effects on normal physiological processes, such as increased stress hormones, altered musculoskeletal system structure and function, altered inflammatory response and mitochondrial function with increased oxidative stress. Because of special requirements, only few animal systems are suitable for space experiments. In particular, we decided to use Xenopus laevis as a model, because it is a vertebrate and the embryos possess some of the advantages of both animal and cells systems. They are easily produced in large number by in vitro fertilisation, can be kept in Petri dishes, develop at room temperature in fresh water and do not need to be fed during the first few days of development.

Xenopus embryos of different ages (3 and 6 days) were exposed to simulated microgravity for different periods; morphology and enzymatic activities were measured after the treatment revealing that the embryos exposed to microgravity have axial malformations and a potentiation of GSH system (see poster by AM Rizzo et al.). To better investigate the proteins involved in this process, a proteomic approach was applied. Two-dimensional protein maps were obtained for head and tails of Xenopus embryos exposed to microgravity (RPM). Two-dimensional map of Xenopus embryos grown in normal condition was kept as a control (CTR).

In details: frozen embryos were suspended in 500 µl of lysis buffer (15 mM Tris-HCl at pH 6.8 plus 150 µg PMSF) on ice. Mechanical lysis was done with a Potter homogenizer, the soluble fraction obtained was diluted with 500 µl of lysis buffer. To delipidate the sample, 1 ml of Freon was added, after vigorous stirring the material was centrifuged at 4°C for 15 min a 15000 rpm. The upper material was recovered, the protein content dosed by BCA-assay and then precipitated with cold acetone on ice. After 30 min of incubation, the precipitate was collected and stored at -80°C until use.

For 2D-PAGE each fraction was resuspended in an appropriate volume of sample buffer (1% DTE, 4% CHAPS, 8 M Urea, 35 mM Tris and a trace of bromophenol blue). For analytical purposes, 200 µg of protein were loaded on each IPG strip (pH 3-10 NL, 18 cm). 1 mg was loaded for semipreparative 2DE. For the first dimension the total voltage applied was 74.65 kV at 9°C; the second dimension was performed at 40 mA per gel at 9°C. The gels obtained were visualized by silver or blue colloidal staining. All the spots that appeared to be different between CTR gels and RPM gels were destained and digested with trypsin at 37°C for mass spectrometry identification.

15.12

INTERACTION OF PROTEASE INHIBITORS FROM MUCUNA PRURIENS SEEDS EXTRACT (MPE) WITH ECHIS CARINATUS VENOM PROTEASES.

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Over the years protease inhibitors (PIs) have been established as an effective natural protective system of plants. Their use as an alternative natural source of drugs for the treatment of some medical conditions is of growing interest.

The aqueous protein extract of Mucuna pruriens seeds has been reported to be active against Echis carinatus snake venom. It is reported that it contain the kunitz-type of serine protease inhibitor[1].

Snake venom is made up of a complex mixture of toxins, amongst which are metalloproteases and serine proteases. These components has been studied over the years.

As a continuation of our efforts to fully elucidate the anti-snake venom properties of Mucuna pruriens, we studied the anti-protease activity of MPE against venom proteases and compare them with standard proteases like trypsin and chymotrypsin. We compared the reduction in enzymatic activity after incubation of these enzymes with the extract. Spectrophotometric assay using chromogenic substrates was used to measure the kinetics and level of inhibition of these proteases.

Protein–Protein interaction of standard proteases (1:1 molar ratio) leading to formation of complexes was studied, after pre-incubation of 30, 60 and 120 minutes with MPE. Elution profiles were monitored based on absorbance at 280nm.

The anti-protease activity of MPE against snake venom proteases was again studied using in vitro incubation assay. In this case, three incubation mixtures were prepared one containing ecarin and prothrombin; the second, P and prothrombin, and the third had a 2 µl of a 1 hr pre-incubated mixture of P and ecarin (equivalent to 0.1 µg P and 0.1 µg ecarin) and of prothrombin. All mixtures were incubation for 1 hr at 37°C. The mixtures were then separated on SDS-PAGE.

Zymography was also employed to study anti-protease activity of MPE against venom proteases after 1 hr pre-incubation of venom protease with MPE.

In conclusion, using different experimental approach we observed that our Mucuna pruriens seed extract contains protease inhibitor that interacts with Echis carinatus venom proteases.

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15.13

PROTEOMIC ANALYSIS AS NEW RESEARCH TO IDENTIFY VULNERABLE PLAQUE

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The non-invasive recognition of a vulnerable plaque at an early stage and before an acute clinical event is very difficult.

In such an early stage markers that play a fundamental role in the formation and progression of a plaque would be appropriate, but for this type of study proteomic analysis of the plaque as a whole is still difficult, due to its heterogeneous cellular composition and abundance of plasma proteins. In a previous study[1], we proposed a new strategy for studying plaque proteomes, which makes it possible to select the proteins exclusive to plaques, by constructing a synthetic reference gel. The aim of this study is to verify the possibility of comparing a sample of interest from a carotid plaque with the synthetic reference gel.

Samples of different types of carotid artery plaque were obtained from 10 patients (4 symptomatic, average age 74years) after carotid endarterectomy. We matched the spots of the synthetic gel of the plaque pool with the spots of the removed carotid plaque pool, in order to only select spots exclusive to plaques from the 2D-electrophoresis of the plaque pool. We selected some spots from among the exclusive ones and identified them by mass spectrometry(MS).

Among the spots exclusive to the plaque pool, as first approach, were identified by MS six proteins: haptoglobin-related protein, superoxide dismutase, AMBP protein, Rho GDP-dissociation inhibitor1, tropomyosin alpha-4 and osteoglycin. Some of these proteins are involved in transport, others take part in elimination of toxic radicals, others are metabolic enzymes or structural proteins.

We proposed a new strategy for studying plaque proteome, which makes it possible to select the proteins exclusive to plaques via the construction of a synthetic reference gel. The results presented are an example of the application of this new approach, demonstrating that the reference gel makes it possible to select only the spots exclusive to plaques in any sample of interest. This new approach could identify novel markers of lesions which could be associated with plaque instability.

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15.14

STUDIES ON *MYCOBACTERIUM TUBERCULOSIS* GLUTAMYL-tRNA SYNTHETASE

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Mycobacterium tuberculosis glutamyl-tRNA synthetase (Mt-GluRS) is an essential enzyme which provides Glu-tRNA^{Glu} for protein biosynthesis and for that of δ-aminolevulinic acid (ALA), the first common precursor of tetrapyrrole biosynthesis.

Soluble recombinant protein was obtained in large amounts and purified to homogeneity. The catalytic properties of Mt-GluRS are being investigated using the well characterized *E. coli* GluRS (Ec-GluRS) as a reference in order to highlight peculiar properties of the *M. tuberculosis* enzyme. The steady-state kinetic parameters of Mt-GluRS reaction were found to be similar to those exhibited by other GluRS except for a higher value of the K_m for L-Glu (2.7 mM instead of 0.1 mM for Ec-GluRS). The enzyme reaction mechanism and the Glu-AMP intermediate formation was studied by using a colorimetric method for pyrophosphate (PP_i) detection, [^{3}H]-labelled ATP and chromatographic separation of reaction components and by monitoring the [^{32}P]-PP_i-ATP exchange reaction. Mt-GluRS was shown to catalyze the formation of Glu-AMP only in the presence of bound tRNA^{Glu} as reported for GluRS from other sources. Among the several GluRS substrate and product analogs tested, only the reaction product PP_i and the Glu-AMP analog glutamol-AMP (GOM) had an effect on GluRS activity. PP_i was a non competitive inhibitor with respect to ATP, in agreement with the observed [^{32}P]-PP_i-ATP exchange. GOM was a competitive inhibitor with respect to both L-Glu and ATP (K_i 3.9 and 1.5 μM, respectively) suggesting random binding of these substrates to the enzyme•tRNA complex. At variance with other GluRS, Mt-GluRS was found to catalyze the hydrolysis of ATP to ADP+P_i in the absence of tRNA and Glu, although at a rate of 5.1 min^{-1}, corresponding to only 4% of the turnover rate of the physiological reaction.

Analysis of *M. tuberculosis* genome revealed the absence of genes encoding Gln-tRNA^{Gln} synthetase indicating that Mt-GluRS belongs to the class of the non discriminating GluRS, which misacylate tRNA^{Gln} with L-Glu forming Glu-tRNA^{Gln}. The misacylation is corrected by the specific amidotransferase generating Gln-tRNA^{Gln}. Genes encoding the amidotransferase have indeed been identified in *M. tuberculosis* genome. However, the overexpression of Mt-GluRS in *E. coli*, which expresses a Gln-tRNA^{Gln} synthetase and lacks the amidotransferase, was not toxic, raising the question of the tRNA specificity of Mt-GluRS.

Genome analyses indicated that in *M. tuberculosis* ALA is formed from Glu-tRNA^{Glu} by glutamyl-tRNA reductase (GluTR), in the presence of NADPH, and glutamate 1-semialdehyde aminomutase in the so-called C5 pathway. Thus, we tested the hypothesis of the possible formation of a complex between GluRS and GluTR, which would commit Glu-tRNA^{Glu} to tetrapyrrole biosynthesis as opposed to protein synthesis. By affinity chromatography with immobilized His-tagged forms of GluRS or of GluTR as the bait to fish out the second enzyme, we obtained data consistent with complex formation.

15.15

THE INTERACTION OF APOMYOGLOBIN WITH OLEIC ACID LEADS TO A PROTEIN COMPLEX THAT DISPLAYS CELLULAR TOXICITY

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Several studies have shown that fatty acids can induce the formation of toxic aggregates in some amyloidogenic proteins involved in neurodegenerative diseases. In particular, it has been demonstrated that oleic acid (OA) can induce the formation of oligomers in proteins such as tau[1], superoxide dismutase[2] and amyloid β-peptide[3]. Here, we have studied the interaction of OA with horse heart apomyoglobin (apoMb), which is not related to any pathology. This single chain and disulfide-free 153-residue protein is extensively used since decades as a model for studies of protein structure, folding, misfolding and aggregation. The interaction of OA with apoMb (1:10 molar ratio) leads to the formation of an apoMb/OA complex given by protein oligomers, as evidenced by protein cross-linking experiments with glutaraldehyde followed by SDS-PAGE. Indeed, cross-linked oligomers of apoMb are formed in the presence of OA, while apoMb alone does not form oligomers. Far-UV circular dichroism spectroscopy measurements indicated that apoMb maintains a highly helical conformation in the presence of OA. We tested the toxicity of the apoMb/OA complex on Jurkat cells and we have found that the protein complex causes cell death by an apoptosis-like mechanism. Conversely, the protein is not active when tested alone and OA shows a much reduced toxicity. Of interest, it has been demonstrated previously that OA can induce cellular toxicity by an apoptosis-like mechanism[4]. Our results suggest that the interaction of OA with apoMb strongly enhance the water solubility of the otherwise insoluble fatty acid, thus leading to a significant enhancement of its intrinsic apoptotic activity[3]. We suggest that the apoMb/OA complex acts as a delivery system of the toxic fatty acid to the cell. This mechanism could explain also the previously reported toxicity of OA complexes with other proteins, including those with human and bovine a-lactalbumin (LA)[5]. We conclude that the toxic effect for tumor cells of the OA/LA complex previously reported[6] is not protein-specific and we suggest that other proteins can display similar toxic effects if combined in an OA complex.

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15.16

DPS PROTEINS AND OXIDATIVE PROCESSES IN THE THERMOPHILIC CYANOBACTERIUM T. ELONGATUS

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Iron is an essential element to all organisms but is both poorly available and potentially toxic in the presence of oxygen. Bacteria have evolved various mechanisms to counter the problems imposed by the chemistry of this metal and thereby to achieve effective iron homeostasis. Highly efficient iron acquisition systems are used to scavenge this metal from the environment under iron-restricted conditions. Bacteria possess iron storage proteins, ferritin and bacterioferritin, that provide intracellular iron reserves to meet the metabolic requirements. Dps proteins (DNA-binding proteins from starved cells) also possess iron storage capacity, but primarily protect bacteria and their macromolecules against iron-induced oxidative damage. Dps proteins are homododecamers belonging to the ferritin superfamily, assembled into hollow spheres. The protection against oxidative damage is achieved by binding Fe(II) at the ferroxidase centers and oxidizing it with H_2O_2. Fe(III) produced is stored in the protein cavity. Some members of the Dps protein family, for example E.coli Dps, are able to physically bind DNA by means the formation of electrostatic interactions[1].

The occurrence of oxygenic photosynthesis in cyanobacteria entails an increased formation of reactive oxygen species as a result of the photosynthetic transport of electrons, such that photosystems I and II are main targets of photodamage. Possibly, in accordance with the increased requirement of antioxidant activity, the thermophilic cyanobacterium Thermosynechococcus elongatus possesses two Dps proteins, Dps-Te and DpsA-Te[2]. The two T. elongates Dps proteins have been cloned in E.coli and characterized in terms of X-ray crystal structure, ferroxidase catalytic properties and thermo-stability.

The Dps-Te and DpsA-Te sequences were compared; the alignment shows that the Dps-Te ferroxidase site is composed by the "canonical" conserved amino acidic residues (2 His, 1 Asp, 1 Glu) whereas the DpsA-Te one presents a unique feature (3 His, 1 Glu). This peculiarity was confirmed by the crystal structures and finds its functional manifestation in the iron oxidation. Typically in Dps proteins oxidation kinetics by H_2O_2 is 100-fold then by O2, but in DpsA-Te the preference for H_2O_2 is ≈ 3-fold. In accordance with their extremophilic bacterial source, Dps-Te and DpsA-Te show an increased thermostability relative to mesophilic Dps proteins by adopting similar strategies.

In perspective, the expression profile of the two T. elongatus proteins under several growth conditions and their localization will be evaluated to elucidate the specific role of the two Dps proteins in the physiology of this thermophilic cyanobacterium.

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15.17

INVESTIGATING THE DRUG-STABILIZING ABILITY OF BETA-LACTOGLOBULIN

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Beta-lactoglobulin (BLG) belongs to the lipocalin superfamily. Its three-dimensional structure is characterized by antiparallel β-strands arranged to form a barrel-shaped cavity in which hydrophobic ligands can be lodged[1]. The protein can bind naturally occurring molecules, as well as other non polar molecules in multiple binding-sites[2]. In view of the resistance of the protein to acid denaturation and to proteolysis, this feature is of great interest from a pharmacological point of view.

The binding of two different molecules, fluvastatin and flurbiprofen, to BLG was assessed by ESI-MS and NMR at different pH values. The stability of the BLG-fluvastatin and BLG-flurbiprofen complexes were compared with that of BLG bound to one of its natural ligands, palmitic acid. The binding of the two drugs or of palmitic acid to BLG did not change the secondary and tertiary structure of the protein, as detected by CD and tryptophan fluorescence, but influenced the sensitivity of BLG towards chemical and physical denaturation. Although thermal stability data could not be obtained for fluvastatin-loaded BLG as a consequence of the thermal instability of the drug, fluvastatin had a protective effect towards urea denaturation of BLG in a similar fashion to the protection exerted by the natural ligand (palmitic acid), that improves stability of BLG towards both thermal and chemical denaturation[3]. On the contrary, BLG-bound flurbiprofen does not protect toward thermal denaturation, suggesting that the binding of this drug involves a different region of the protein. Competition experiments, carried out by ESI-MS at different pH values and order of addition, clearly shows that palmitic acid and fluvastatin compete for the binding site in the central calyx, thus leading to improved structural stability, whereas flurbiprofen binds to a different site, as indicated by the lack of competition with the natural ligand and of stabilizing effects.

Besides pointing out the versatility of this class of proteins as almost-universal and multi-site carriers for hydrophobes, these results are of practical interest as they indicate a possible use of BLG (and, possibly, of related lipocalins) as a "protective agent" for easing the delivery of unstable/insoluble drugs.

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15.18

INFLUENCES OF TERNARY AND QUATERNARY COMPLEXES ON ORNITHINE TRANSCARBAMOYLASE STABILIZATION PROCESS.

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Trehalose is essential for organism survival under extreme stress conditions, so the molecular mechanism characterization of trehalose protection is fundamental for its biotecnological application. A lot of study statement that bioprotective effect of sugar could be due to molecular, physiological and structure complex interaction between bynary (sugar-water) and ternary (sugar-macromolecules-water) complexes. In fact, trehalose influences markedly dynamic and structural water properties, creating a more rigid unit, capable to protect macromolecular structure by an elaborate mechanism of adaptation[1]. To date interesting for hydrophilic protective glass grows as function of their pharmaceutical, technological and industrial utilization. The mechanisms that hide under this type of stabilization are very complicated and involve, for example, protein reaction dynamics and solvent viscosities. In our work we try to analyze and correlate the results obtained in presence of trehalose, glycerol, and trehalose-glycerol solution at different ratio on ornithine transcarbamoylase (OCT), in function of medium viscosity and activity determination. As previously reported in our works[1-2], OCT thermal stabilization process is markedly influenced by the presence of 0.5 M trehalose solution. So we further increase disaccharide concentration (up to 1.0M) and mix with glycerol at the same concentration in different ratio. Kinematic viscosities of 1.0 M trealose plus 0.2 mg/ml OCT solution is higher than that of the same concentration of glycerol one. In the mix of trealose and glycerol solution plus 0.2 mg/ml OCT, the increase in % of the glycerol solution cause a decrease of kinematic viscosities up to 100 % of this solution. Viscosities determinations reveal the first unexpected result when we mix 95% 1.0 M trehalose plus 5% 1.0 M glycerol and 0.2 mg/ml OCT. The kinematic viscosities of solution increase and exceed trehalose one. So we analyzed the influences of these different solution on thermal OCT stabilization at 68°C. 1.0 M trehalose solution shows the high stabilization activity, while all the other solution show little effects, including the mix 95% 1.0 M trehalose plus 5% 1.0M glycerol. The presence of both glycerol and trehalose in solution induced markedly changes in properties solution, without further increase stabilization effects of trehalose alone.

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15.19

THE THREE-FOLD "FERRITIN-LIKE" PORES CONTROL BOTH THE IRON UPTAKE AND RELEASE PROCESSES IN LISTERIA INNOCUA DPS. A STUDY WITH SITE-SPECIFIC MUTANTS.

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Dps (DNA binding proteins from starved cells) proteins are involved in regulating the cellular response to oxidative stress in bacteria. Dps proteins are 9-10 nm shell-like structures composed by twelve identical subunits arranged with 23 symmetry. This in turn gives rise to three different environments along the symmetry axes of the molecule. The two-fold symmetry interface contains residues that form the catalytic centers for the detoxification of iron and hydrogen peroxide. The three-fold "ferritin-like" interface is characterized by negatively charged pores that traverse the protein shell and resemble those formed at the 3-fold channels in ferritins, whereas the "Dps like" interface is unique to Dps proteins and contains smaller channels (Ilari et al., 2000).
In ferritins, the three-fold pores represent the route for iron entry into the protein cavity and for the opposite process, namely the release of iron from the core (Stefanini et al., 1989, Liu et al., 2003). It is not known whether the "ferritin-like" pores have the same role in Dps proteins. On this basis, the present work focusses on the function of "ferritin-like" pores in the Dps protein from the Gram-positive bacterium, *Listeria innocua*. In *L. innocua* Dps (*Li*Dps) the three-fold pores are lined by three aspartate residues, D121, D126 and D130. The comparison of Dps primary sequences shows that D130 is highly conserved (80%) within the protein family, whereas D121 and D126 are poorly conserved. A set of site-specific mutants (D121N, D126N, D130N and D121N-D126N-D130N) has been utilized to study specifically the role of the pore aspartate in the management of iron by *Li*Dps. When the pores are altered by the mutation of aspartate residues, in particular D130, the iron binding, oxidation and mineralization processes are slowed down significantly, less so the iron release process. Thus, the presence of negative charges and of well-defined electrostatic gradients in the pore area is required for a correct protein function.

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15.20

ESI-MS/MS DETERMINATION OF LYSOZYME CONFORMATION CHANGES IN PRESENCE OF TREHALOSE.

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Hen egg white lysozyme (EC 3.2.1.17), is a 129-residue protein stabilized by four disulfide bonds. It has two domains, rich in α and β structure, respectively. Molecular weight of lysozyme is an approximately 14.7 kDa. Lysozyme is widespread in animals and plants. It is found in mammalian secretions and tissues, saliva, tears, milk, cervical mucus, leucocytes, kidneys and in egg white. The residues of glutamic acid 35 (Glu35) and aspartate 52 (Asp52) have been found to be critical to the activity of this enzyme[1]. There has been interest in lysozyme as a "natural" antibiotic and as an aid in the diagnosis of disease. Measurements of a hydrogen-deuterium (H/D) exchange between peptide backbone amides and solvent provide considerable insight into the protein folding and structure. The H/D exchange rate is closely related to its local environment. In general, a protein in a tightly folded conformation has fewer protons available for deuteration, than the same protein in an unfolded state. Since the deuteration increases the molecular mass of a protein electrospray mass spectrometry is a useful technique to monitor the H/D exchange. We attempted to use mass spectrometry to study lysozyme conformation changes by H/D exchange in different condition, in absence and in presence of trehalose. Lysozyme (2 mg/ml) was denatured in 6 M deuterated GuDCl in D_2O for 1 h to permit exchange of all labile peptide and side-chain hydrogens for deuterons in absence or in presence of 0.5 M trehalose. At different time points in the rate study, aliquots were removed to carry out of H/D exchange experiments, the exchange is quenched, and samples analyzed by ESI-MS/MS. Samples were introduced to the ionization source of the mass spectrometer an infusion syringe pump. Hydrogen exchange labeling experiments monitored by ESI MS were carried out to identify more clearly the origin of the effects of added trehalose on the denaturation kinetics of lysozyme. The native positive-ion Esi mass spectrum of lysozyme (scan-range m/z 900-2000) shows a Gaussian-type distribution of multiply charged ions ranging from m/z 1302.3 to 1789.4. In particular, ESI-MS/MS spectrum is characterized by three abundant ions at m/z of 1432.3, 1591.1 and 1789.4 and attributed to the +10, +9 and +8 multicharged ions of native protein. H/D exchange was remarkably influenced by trehalose presence. This results were also supported by UV-visible spectroscopy, which indicate the formation of a more compact protein structure in presence of trehalose.

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15.21

A MODEL FOR THE AGGREGATION OF THE ACYLPHOSPHATASE FROM *SULFOLOBUS SOLFATARICUS* IN ITS NATIVE-LIKE STATE

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Evidence is accumulating that normally folded proteins retain a significant tendency to form amyloid fibrils through a direct assembly of monomers in their native-like conformation[1]. However, the factors promoting such processes are not yet well understood. The acylphosphatase from *Sulfolobus solfataricus* (Sso AcP) aggregates under conditions in which a native-like state is initially populated[2]. Aggregation occurs in two phases. In a first step aggregates form in which the monomers maintain their native-like topology[3]. In a second phase these early aggregates convert into amyloid-like protofibrils[3]. An unstructured N-terminal segment and an edge β-strand were previously shown to play a major role in the process[4]. In this work, using kinetic experiments on a set Sso AcP variants and a set of peptides corresponding to the unstructured N-terminal segment of the protein, we show that the major event of the first step is the establishment of an inter-molecular interaction between the unstructured segment of one Sso AcP molecule and the globular unit of another molecule. Control experiments show that this interaction is determined by the primary sequence of the unstructured segment and not by its physico-chemical properties. Experiments of aggregation carried out with Sso AcP dimers in which two edge β-strands or two unstructured segments are forced to interact are in agreement with an inter-molecular interaction between unstructured segment and β-strand 4. Possible destabilizing effects induced by the N-terminal segment on the globular unit do not seem to play any role in the aggregation process. Moreover, we show that the conversion of the initial aggregates into amyloid-like protofibrils is an intra-molecular process in which the Sso AcP molecules undergo conformational modifications from a native-like fold to an amyloid-like fold. The obtained results allow the formulation of a model for the assembly of Sso AcP into amyloid-like aggregates at a molecular level.

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15.22

FLUORESCENCE STUDIES ON HUMAN SERUM BUTYRYLCHOLINESTERASE: EFFECT OF TEMPERATURE AND PALMITOYLETHANOLAMIDE, AN ENDOGENOUS ANTI-INFLAMMATORY LIPID.

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Two types of cholinesterases are present in vertebrates: Acetylcholinesterase (AChE) and Butyrylcholinesterase (BChE). AChE plays an important role in the synaptic transmission. BChE is found in blood serum, CNS and other tissues, and, together with AChE is one of the most used enzymes to monitor organophosphorous and carbamate environmental pollutants. Moreover, it was suggested its possible involvement in neural function and, as AChE, a possible role in neurodegenerative disorders, such as Alzheimer's disease (AD)[1]. Recent studies indicated increased plasma and RBC activities of BChE and AChE in several low grade systemic inflammatory conditions[2], and it was suggested that these enzymes could be markers of inflammation[2]. Palmitoylethanolamide (PEA) is an endogenous lipid that is thought to be involved in endogenous protective mechanisms of inflammatory response. It is present in a large variety of tissues, included brain and blood, and a significant increase of its levels was found in pathological conditions, such as inflammation[3]. Although the presence of cholinesterases and PEA in the same tissues raises the hypothesis of their possible interaction, no studies have been performed on this field. The current study examines the possible effect of PEA on BChE structural and kinetic features. It was demonstrated that Arrhenius plots of BChE-catalysed hydrolysis of esters presents a break at 18-22°C[4] that was related to the presence of two different BChE active forms on both sides of the break. This break was attributed to a temperature-induced conformational change of the active site gorge in the transition state [ES][4], but is not related to any overall conformation alteration. Human BChE purified in our laboratory presents similar kinetic features, with a break in the range 19.6°-22.2°C. Arrhenius plot of Trp fluorescence intensity measurements, shows a break at similar temperatures (Ea was about 17 kJ/mol, and about 10.5 kJ/mol below and above the break, respectively), indicating that the fluorescence of BChE Trp are sensitive to the conformational change induced by temperature on the active site gorge, and that this conformational modification is not induced by the binding of the substrate. The same measurements performed in the presence of 5 µM PEA show a slight increase of Ea, more evident below the transition temperature, suggesting that PEA can interact with the two different active forms of BChE, but likely with different affinity.

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15.23

OLIGO-BRANCHED PEPTIDES AS MULTIVALENT AND MODULAR TUMOR TARGETING AGENTS

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Specific targeting of tumor-associated antigens that are selectively expressed or over-expressed by tumor cells is the goal of modern cancer therapy aimed at overcoming the non-specific toxicity of most anticancer drugs. Qualitative and quantitative expression of tumor-associated antigens varies among different tumors and patients, resulting in highly variable responses to targeted therapies. For a selective tumor therapy to be effective, tumor diagnosis should provide information on the expression of the targeted antigen in each patient, thus enabling prediction of the possible efficiency of a personalized therapy. In order to translate tumor diagnostic information into reliable prognostic data, the molecules used for tumor tracing –either in vivo or ex vivo- should be as close as possible to those used for therapy.

Antibodies and small molecules have been used as targeting agents, and both have limits and advantages. We demonstrate here that oligo-branched peptides can be efficient tumor-targeting agents that combine the advantages of antibodies and small molecules. They allow multimeric binding, despite a much lower molecular weight compared to antibodies, and can be easily synthesized and chemically modified, like small molecules. Moreover, oligo-branched peptides are extremely resistant to proteases and peptidases[1, 2].

We produced tetra-branched peptides targeting the neurotensin (NT) receptors, which are over-expressed in a number of human tumors. Tetra-branched peptides were used either for spotlighting tumor cells or for killing them, by simply exchanging the functional moiety that can be coupled to a conserved targeting core[3].

Fluorophore-conjugated branched NT peptides allow discrimination between tumor and healthy tissues in human biopsies from colon and pancreas adenocarcinomas and can be used to measure tumor versus healthy peptide binding in each patient, giving an indication of the possible efficiency of an NT-mediated therapy. The same NT branched molecules, when conjugated to drugs, allow selective tumor cell killing and also allow by-passing drug resistance in colon, pancreas and prostate tumor cell lines.

The approach proved promising for the personalized therapy of tumors that over-express NT receptors, such as colon, pancreas and prostate carcinoma, and might be applied to many different tumor targets.

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15.24

STRUCTURALLY DISTINCT PROTOFIBRILS OF HYPF-N SHOW DIFFERENT CYTOTOXIC EFFECTS

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Recent reports indicate that the oligomers that precede the formation of well-defined fibrils play a critical role in the pathogenesis of protein deposition diseases, at least in the case of neuropathic disorders[1]. Nevertheless, the structural elucidation of such species remains elusive, because their dynamic and metastable nature prevents the use of high resolution techniques.

Oligomers by the N-terminal domain of HypF from *E. coli* (HypF-N) were prepared using two distinct protocols, i.e. at low pH and in the presence of 330 mM sodium chloride; and at pH 5.5 and in the presence of 12% (v/v) trifluoroethanol[2,3]. The resulting aggregates consist of spherical oligomers having a height of 3.0 nm, as revealed by atomic force microscopy, an extensive b-sheet structure and the ability to bind the Thioflavin T dye. When the two kinds of aggregates were tested for their possible citotoxic effects on cultured SH-SY5Y cells, only those formed at pH 5.5 and in the presence of trifluoroethanol resulted to be toxic.

The excimer forming fluorescence probe pyrene-maleimide was thus used to monitor the structure of the protofibrils formed under the two sets of conditions. Excimers form when two pyrene moieties stack each other within 4-10 Å of distance and result in a broadened pyrene emission spectrum with a new peak or shoulder in the 450-470 nm region[4]. Thus, by conjugating the dye to a polypeptide chain one can investigate inter-chain interactions. HypF-N variants carrying a single cysteine residue located at different positions of the polypeptide chain were selectively labeled with the probe and incubated for four hours at 25 °C under the two sets of conditions, in order to monitor the pyrene emission spectrum after the formation of protofibrillar aggregates.

Residues located in three regions of the sequence (approximately 25-34, 55-59 and 75-87) have been found to give rise to a significant excimer signal when labeled with the probe, indicating that such regions contribute to the structure of the protofibrillar aggregates and form inter-chain interactions in the protofibrils. These regions correspond to the three major peaks of the hydropathy profile of HypF-N. Nevertheless, the extent of the tendency to form excimers at pH 5.5 with 12% trifluoroethanol is lower, indicating that the aggregates formed under this condition have a lower degree of packing of hydrophobic residues.

Overall, the data provide structural information on two kinds of protofibrils formed by the same protein, and suggest the structural origin of cellular toxicity of non fibrillar aggregates.

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15.25

REGULATION OF AMYLOID PRECURSOR PROTEIN PROTEOLYSIS BY CALMODULIN BINDING

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Abnormal metabolism of the amyloid precursor protein, APP, through the amyloidogenic pathway results in the accumulation of heterogeneous Aβ peptides in the central nervous system, causing the onset of Alzheimer's disease. Aβ and other different proteolytic fragments of APP are present in human plasma, and derive from circulating platelets. Platelets express APP isoforms similar to those found in neurons, and metabolise APP through α- and β-secretases to produce soluble fragments sAPPα, sAPPβ and Aβ [1]. Very little is known about the exact function of APP and of its proteolytic fragments in normal cellular metabolism. However, APP is expressed on the platelet membrane as an intact protein, suggesting that it may serve as a functional receptor. In this study we investigated the metabolism of APP in human platelets.

We identified two different forms of APP expressed in human resting platelets. A 110-120 kDa soluble fragment (sAPPα and/or sAPPβ was stored into platelet α-granules and was released upon platelet activation by several agonists, including thrombin. A 140 kDa full length intact protein, named $APP_{FL,}$ was found to be expressed on the platelet surface, and represented about ten percent of total platelet APP. APP_{FL} underwent proteolysis upon stimulation of platelets with thrombin and other agonists. Agonist-induced proteolysis of APP_{FL} occurred independently of platelet aggregation or secretion, but was inhibited in the presence of EDTA. Interestingly, proteolysis of $APP_{FL,}$ but not secretion of soluble APP fragments, was observed also upon incubation of platelets with the cell permeable calmodulin (CaM) antagonist W7. W7-induced APP_{FL} proteolysis was time and dose-dependent, was not associated to platelet activation, and was prevented by preincubation with the metalloproteinase inhibitor GM6001 or with EDTA. In platelets, shedding of the extracellular domain of several membrane glycoproteins, including GPIbα, GPV, GPVI, and PECAM1, represents a recognized mechanism for receptor down-regulation. In all these cases, shedding is constitutively inhibited by CaM binding to the intracellular regions of the receptors, and occurs upon CaM dissociation with W7[2]. Therefore, we screened APP sequence for CaM binding consensus sequences, and we identified two main potential sites. Co-immunoprecipitation experiments have provided preliminary evidence for an association between APP_{FL} and CaM in intact platelets. Using a GST fusion protein containing the intracellular domain of APP, however, we have been unable to detect any specific association with purified CaM. Further studies are necessary to clarify this issue. Nevertheless, our results indicate a novel, CaM-dependent mechanism for APP proteolysis in human platelets.

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15.26

ENZYMATIC PROPERTIES OF RECOMBINANT PEPSINS FROM THE ANTARCTIC ROCK COD *TREMATOMUS BERNACCHII*.

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Antarctic fish are highly cold adapted and remarkably stenothermal[1], as a consequence of evolution of antifreeze glycoproteins and their higher catalytic efficiency of many enzymes at low temperatures[2]. For this reason, the study of enzymes in these poikilothermic species is of interest, especially in relation to the strategies adopted by these organisms to achieve a normal level of proteolysis at temperatures well below that of homeothermic species. In our studies on aspartic proteinases in notothenioids, a fish group endemic to Antarctica, we have focused our attention on pepsins because of their important nutritional role. Pepsins are a family of aspartic proteinases accomplishing important digestive functions in both invertebrates and vertebrates[3]. Like other aspartic proteinases, pepsin is produced as a zymogen. The primary structure of the zymogen includes a signal peptide (or presequence) and the so-called propart, whose autocatalytic cleavage leads to the formation of the active enzyme[4]. To better understand the molecular mechanisms responsible for adaptation of food digestion at temperatures below 0 °C, we have produced the two *T. bernacchii* fish pepsin variants A1 and A2 by heterologous expression in *E. coli*. The enzymes were purified, and their biochemical properties were studied in comparison to pepsin A from porcine stomach. The properties of these two Antarctic isoenzymes were compared to porcine pepsin and found to be unique in a number of ways. Fish pepsins were found to be more temperature sensitive, generally less active at lower pH and more sensitive to inhibition by pepstatin than the mesophilic counterpart. The specificity of Antarctic fish pepsins was similar but not identical to pig pepsin, likely owing to changes in the sequence of fish enzymes near the active site. Gene duplication of Antarctic rock cod pepsins is the likely mechanism for adaptation to the harsh temperature environment in which these enzymes must function.

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15.27

ON THE MECHANISM OF SELF-REGULATION OF GLUTAMATE SYNTHASE: THE ROLE OF K937 AND M479

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Glutamate synthases (GltS) catalyze the reductive transfer of the glutamine (L-Gln) amide group to the C_2 carbon of 2-oxoglutarate (2-OG) producing two molecules of L-Glu. They form with glutamine synthetase an essential pathway of ammonia assimilation in microorganisms, plants and lower animals. All GltS are complex Fe/S flavoproteins, which share a subunit or domain for the L-Gln-dependent L-Glu synthesis from 2-OG. GltS tightly control and coordinate the partial reactions taking place in the α subunit of the NADPH-dependent GltS (αGltS, 162.4 kDa) or the homologous plant-type ferredoxin-dependent enzyme (FdGltS), namely: i. ammonia release from L-Gln at the glutaminase site in the N-terminal amidotransferase domain; ii. ammonia transfer through the 30 Å-long intramolecular tunnel to the synthase site where (iii) ammonia adds to bound 2-OG forming the 2-iminoglutarate (2-IG) intermediate that is reduced by FMN at this site. The crystal structures revealed which residues may be important for the control of GltS reactions, which has been proposed to occur through conformational changes initiated by 2-OG binding and reduction of the FMN and 3Fe-4S cofactors at the synthase site, which are transmitted across the tunnel to activate glutamine hydrolysis and open the tunnel entry point. Among these residues, K937 of the synthase domain interacts with 2-OG carbonyl oxygen potentially favoring ammonia addition and signaling 2-OG presence to the glutaminase site. M479 belongs to the central domain and is in van der Waals contact with both FMN and the [3Fe-4S] cluster in the synthase domain. Thus, M479 may modulate the redox properties of the cofactors and may signal the enzyme redox state. To determine the role of these residues, we engineered K937A and M479A substitutions in αGltS. The resulting αGltS variants have been co-produced with a C-terminally His(6)-tagged form of βGltS for a rapid purification of the resulting K937A- and M479A-GltSHis. Both GltS variants have been successfully produced in *E. coli* and purified. Both species are indistinguishable from the wild-type enzyme with respect to stability and cofactor content. Steady-state kinetic studies of the K/A variant showed that K937 is essential for the synthesis of L-Glu at the synthase site, presumably by favoring ammonia addition to the carbonyl C of 2-OG. Indeed, sulfite titrations followed by backtitrations with 2-OG ruled out that K937 is important for 2-OG binding, but also revealed the lowering of the K_d for the FMN-sulfite adduct, This property correlates with a 10-fold increase of the NADPH oxidase activity of K937A-GltSHis which has been shown to be quenched by 2-OG and L-Gln. The latter observation is of interest showing for the first time an effect of ligand binding to the glutaminase site on the properties of the synthase site. The M/A substitution led to only a 5-fold decrease of the turnover number with no detectable effect on the apparent affinity for the substrates and only a mild enhancement of the oxidase activity. Experiments are in progress in order to test the effect of the substitutions on the properties of the glutaminase site and on the coupling the glutaminase and synthase reactions.

15.28

HUMAN CYTIDINE DEAMINASE MUTANT ENZYME Y33G: STUDIES OF PROTEIN STABILIZATION MEDIATED BY CHAPERONES.

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Cytidine deaminase (CDA) has been the focus of several studies because it deaminates some cytosine nucleoside analogues used in chemotherapy leading to the loss of their pharmacological activity. Human CDA Y33 is part of a conserved region in most tetrameric CDAs and is probably involved in the contact between subunits and in the active site. By a series of site-directed mutagenesis on human CDA cDNA were obtained the mutants Y33G, Y33F and Y33S[1] but in the prokaryotic expression system used, only Y33G produced a partially soluble protein without enzymatic activity. To correct the protein folding and therefore to increase the solubility and the activity of an enzyme expressed in an heterologous system low molecular weight compounds (osmolytes) that stabilize proteins in their native conformations[2] are useful.

In this work the effect of some osmolytes such as glycerol, DMSO, cyclodextrins, sorbitol (chemical chaperones), and some specific ligands of CDA (pharmacological chaperones), added to the culture medium during induction, was investigated with regard to protein content and specific activity of the CDAs protein mutated in the tyrosine 33 residue. At this purpose, each crude extracts containing 20 µg of total proteins obtained by the titration of Y33G, Y33F and Y33S with each chaperone concentration, was subjected to immunoblot analysis with the polyclonal primary antibody anti-CDA and to enzymatic (CDA) activity determination by spectrophotometric assay[3].

The results indicated that the use of glycerol and DMSO or the presence of substrates (cytidine) or inhibitors (uridine, 5-fluoro-zebularine, 6-[3,5(cytidyl) acryloyl amino] hexanoic acid) of human CDA increased the content and the specific activity of the Y33G mutant enzyme. Other osmolytes such as cyclodextrins and sorbitol had no effect on the protein stability. The presence of glycerol in culture media increased the protein content of the other mutant CDA Y33F but not the specific activity, whereas the chemical and pharmacological chaperones had no effect on Y33S mutant CDA. Finally, on the purified Y33G mutant CDA, a kinetic characterization was performed and the effect of temperature on the protein stability was also investigated.

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15.29

EXPRESSION IN *PICHIA PASTORIS* AND CHARACTERIZATION OF CERATO-POPULIN, A FUNGAL PROTEIN BELONGING TO THE CERATO-PLATANIN FAMILY

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Cerato-populin (PoP) is an about 12.900 Da protein, produced by the Ascomycete *Ceratocystis populicola (Cepop)*, the causal agent of poplar black canker. It belongs to Cerato-platanin Family, a fungal protein family characterized by high sequence similarity, presence of four cysteine residues forming two S-S bridges and involvement in biological recognition phenomena.

The founder member of this protein family is cerato-platanin (CP), a 120 amino acids protein, long since studied in our laboratories, produced by *Ceratocystis platani* (*Cep*) and involved in plane canker stain[1]. CP is located in *Cep* mycelial cell walls and is early released into the medium. It induces over-expression of defence-related genes and reduction in *Cep* growth on plane leaves[2]. This protein has ability to self assemble *in vitro* and CP aggregates seem to be involved in interactions with host.

Furthermore, CP homologous proteins have been identified in other species of *Ceratocystis*, as *C. populicola*, *C. cacaofunesta*, *C. variospora*, *C. fimbriata* clones from *Coffea arabica*, *Mangifera indica*, *Fagus* sp., *Crotolaria juncea* and *Ipomea batatas*. Among these proteins, PoP seems to be particularly interesting since its sequence has a homology of only 62% with that of CP and since *Cepop* attacks the poplar, an industrially and commercially important specie.

PoP has been recently purified from *Cepop* liquid culture in low yield. The secondary structure of PoP, determined by Circular Dicroism (CD), presents some similarities with CP 2D structure but it shows a larger percentage of helix which seems to be organized in a coiled coil structure.

To improve functional and structural characterization, we are cloning *pop* gene in the yeast *Pichia pastoris*, using pPIC9 extracellular expression vector.

At the same time, we are studying if also PoP is able to self-aggregation like CP does. Aggregation studies are carried out incubating 1,3 mM PoP in 10% acetic acid and are monitored by ThioflavinT (ThT) fluorescence, Circular Dicroism and Atomic Force Microscopy. Actually we have found that POP self assembles *in vitro* forming ThT positive aggregates that show a morphology similar to that of CP aggregates.

Recombinant PoP will allow us to deepen the study of PoP aggregation process, the biological activity both of the soluble protein and the aggregates and to compare it with those of CP, in order to understand the mechanism through which CP Family proteins interact with their respective hosts. At this respect the determination of 3D solution structure of CP is in project.

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15.30

MOLECULAR APPROACHES TO STUDY THE MITOCHONDRIAL BEHAVIOUR DURING A MYOGENIC PROGRAM

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Numerous data suggest that mitochondria are involved in the regulation of the skeletal muscle physiology and play a critical role in cell growth, proliferation, differentiation and death. In particular, mitochondrial activity is involved in the regulation of myoblast differentiation through myogenin expression, activity of myogenic factors and by control of c-Myc expression[1,2]. Furthermore, differentiation seems to be a program depending on both mitochondrial function and mitochondrial biogenesis as indicated by a rapid increase in mitochondrial mass/volume, mtDNA copy number, enzyme activities and mitochondrial mRNA levels within the first 48hrs of myoblasts differentiation[3]. We have studied the changes in mitochondrial biogenesis and activity in murine myoblast cultures (C2C12 line) undergoing differentiation: myoblasts were grown, and induced to differentiate. Staining of mitochondria by Mito Tracker Green was used to evaluate their mass/volume by epifluorescence microscope. The Real time RT-PCR was used to study the expression level of PGC-1α, T-fam A and COX II, target genes for mitochondrial biogenesis and activity. The mitochondrial functionality was also tested by their ATP production and cytochrome *c* oxidase activity using HPLC, HPCE and spectrophotometer approaches respectively. These assays show that the mitochondrial biogenesis and activity significantly increase in differentiating myotubes, also compared to undifferentiated and quiescent cells. Intriguingly, using a proteomic approach, changes appear also in mitochondria proteomic pattern during differentiation. Indeed a significant number of new and up-regulated proteins were revealed in mitochondria isolated from myotubes compared to those isolated from myoblasts.

Such data are also supported by a preliminary study where an increase of energy production during the differentiation, is observed. Thus, the mitochondrial biogenesis seems to be correlated to a change in cellular metabolism during myogenesis.

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15.31

PROTEOMIC APPROACH IN C2C12 MYOBLAST DIFFERENTIATION

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Skeletal muscle cell differentiation is a multistage process that has been studied extensively over the years[1-3]. Even if great improvements have been made in defining biological process undergoing skeletal muscle differentiation, many molecular and biochemical mechanisms need still to be clarified.

To provide further evidence in understanding this process, we compared 2-DE gels obtained from differentiating C2C12 cells.

We studied cells at the undifferentiated stage (T_0), at intermediate (T_3 -T_5) and high (T_7) differentiation stages, analyzing, for each condition, morphological and proteomic changes. We also identified the proteins that showed statistically significant changes by a ESI-Q-Tof mass spectrometer. In this way we have identified 26 specific phase proteins, some of which overlooked in skeletal muscle development. Furthermore, the discovery of myogenesis correlated proteins, which are known to be involved in the apoptotic process, suggests a link between apoptosis and differentiation. Intriguingly, many heat shock proteins at high level of expression suggest a relationship between differentiation and cellular stress.

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15.32

PROTEOMIC ANALYSIS OF THE TOXICITY OF OCHRATOXIN A IN MOUSE KIDNEY.

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Ochratoxin A (OTA) is a mycotoxin produced by the fungi *Aspergillus* and *Penicillum*. It is a secondary metabolite found as a contaminant in a variety of very common foods: mostly cereals and grain products. Because of its very prolonged half life in blood the human exposure to this contaminant is of relevant importance, in particular because OTA was found to be cancerogenic, nephrotoxic, teratogenic neurotoxic and immunotoxic in several species of animal experiments[1]. Despite its toxicity, the mechanisms underlying these various toxic effects of OTA have not been still elucidated in detail.

The aim of this study was to gain more insight into the molecular changes that occur after oral exposure to OTA in kidney that is the target organ of the toxin injury.

Kidney protein extracts were obtained from mice fed OTA and analysed by 2D electrophoresis. Comparative image analysis of five replicates highlighted the differentially expressed proteins, which were identified by peptide mass fingerprint on Voyager-DE Pro MALDI-TOF or by MS/MS analysis on ESI-Q-TOF (Q-STAR). The exposure to OTA modified the expression of some cytoskeleton proteins (up-regulation of Vimentin and Tropomyosin and down-regulation of Spectrin 2); moreover some proteins involved in the cellular metabolism (up-regulation of Adenylate Kinase 3, Glyceraldehyde 3-phosphate Dehydrogenase and ET Flavoprotein Dehydrogenase; down-regulation of Bisphosphate 3'-Nucleotidase 1, V-ATPase H^+, Malate Dehydrogenase and Isocitrate Dehydrogenase) and in protein homeostasis (up-regulation of Calreticulin; down-regulation of the Elongation Factor 1-α−1 and Homogentisate 1,2- dioxygenase), as well as some proteins with antioxidant functions (up-regulation of Phosphotriesterase; down-regulation of HSPD1 Protein and Glutathione Synthetase) were affected by the treatment of prolonged (8 weeks) OTA intoxication at sub-acute doses (0.5 mg/kg body weight).

The results show that the OTA toxicity is exploited by altering protein synthesis, cellular metabolism and cell cycle; by a general rearrangement of the cytoskeleton proteins and by depleting some of the proteins involved in the protection of the cell from oxidative damage.

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15.33

INSIGHTS INTO THE CATALYTIC MECHANISM OF THE BCP FAMILY: FUNCTIONAL AND STRUCTURAL ANALYSIS OF BCP1 FROM SULFOLOBUS SULFATARICUS

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Bcps constitute a group of antioxidant enzymes, belonging to the Prx family, that are widely distributed in bacteria, plants and fungi. These proteins can contain two conserved cysteines within the CXXXXC motif. Recent studies demonstrated that while the role of the first cysteine is well defined, being the catalytic peroxidatic cysteine in all the members of this protein family, data on the function of the second cysteine are controversial and require further investigation.[1]

Here we report on the functional and structural characterization of Bcp1, an archaeal Bcp isolated from *Sulfolobus solfataricus*, which presents two conserved cysteine residues at positions 45 and 50. Functional studies revealed that this enzyme carries out the catalytic reaction using an atypical 2-Cys mechanism, where Cys45 is the peroxidatic and Cys50 is the resolving cysteine. The X-ray structure of the double mutant C45S/C50S, representative of the fully reduced enzyme state, was determined at a resolution of 2.15 Å, showing a Trx fold similar to that of other Prx family members. Superposition with a structural homologue in the oxidized state provided for the first time a detailed description of the structural rearrangement necessary for a member of the Bcp family to perform the catalytic reaction. From this structural analysis it emerges that a significant conformational change from a fully folded to a locally unfolded form is required to form the intramolecular disulfide bond upon oxidation, according to the proposed reaction mechanism. Two residues, namely Arg53 and Asp54, which play a key role in this rearrangement, were also identified.

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15.34

IDENTIFICATION OF MOLECULAR PARTNERS OF MLL-AF4 ONCOPROTEINS.

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The mixed-lineage leukemia (*MLL*) gene, located on chromosome 11q23, is involved in numerous chromosomal aberrations associated with human leukemia. At least 50% of cases of infant acute lymphoblastic leukemia (ALL) harbor the balanced translocation t(4;11)(q21;q23), which fuses the genomic sequences encoding the N-terminal portion of *MLL* to sequences encoding the C-terminus of *AF4,* on chromosome 4q21.3-5. This translocation is the hallmark of a high-risk ALL that has a very poor prognosis. *MLL* translocation breakpoints cluster between exon 8 and 12; the *AF4* breakpoint usually lies within exon 4. The resulting fusion gene encodes an in-frame MLL–AF4 fusion protein *(usual MLL-AF4* chimera) that exerts oncogenic activity. We identified a different MLL-AF4 fusion protein *(novel MLL-AF4* chimera) in a 5-month-old child affected by ALL[1]. The translocation junction of the novel fusion gene falls between *MLL* exon 9 and *AF4* exon 11, and the resulting novel MLL-AF4 chimera lacks part of the putative AF4 transcriptional activating domain. In an attempt to identify MLL-AF4 molecular partners, we cloned cDNAs encoding the *usual* and *novel* chimeras to produce Flag-tagged recombinant proteins. Using functional proteomics, we identified several proteins that interact with human AF4. These include MED7, MED23, CDK9 and YWHAQ. The first three are directly involved in RNA pol II transcription activation mechanisms. YWHAQs are ubiquitous proteins that exert a remarkably widespread effect on cellular functions, including cell-cycle control and apoptosis[2]. To evaluate whether MLL-AF4 oncoproteins could bind these four AF4 interactors, we transiently expressed recombinant flagged chimeras in HEK-293 cells and affinity-immunoprecipitated them using anti-Flag monoclonal antibody. We found that both chimeras bind YWHAQ, MED7 and CDK9, the latter being a negative regulator of the transcriptional activator function of AF4[3]. Only the most frequent fusion protein binds MED23. Therefore, chimeras gain the ability to bind specific AF4 partners that may influence gene expression at transcriptional level. These preliminary data suggest that a gain-of-function pathomechanism might explain the oncogenic properties of MLL-AF4 chimeras.

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15.35

THE Ses i 2 MAJOR ALLERGEN FROM SESAMUM INDICUM: PURIFICATION, STRUCTURE, STABILITY AND ANTI-THROMBIN ACTIVITY

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The world incidence of food allergy is estimated to be as high as 6% in children and approximately 3-4% in adults. The majority of plant food allergens can be grouped into four protein superfamilies: prolamins (comprising the 2S albumin seed storage proteins and nonspecific lipid transfer proteins), cupins, profilins, and proteins related to the major birch pollen allergen Bet v 1. Seed storage proteins are synthesized in developing plant embryos during seed germination and play an important biological role as a source of nitrogen and sulfur for germination.

Ses i 2 is the major allergen from white sesame seeds and belongs to the 2S albumin family. In this work, we have purified Ses i 2 in sufficiently large amounts (10-30 mg) for subsequent chemical, conformational and stability characterization.

Ses i 2 was purified to homogeneity (> 98%) in two consecutive chromatographic steps, involving size-exclusion chromatography on a sephacryl HR-100 column and semi-preparative RP-HPLC on a C18 coulmn. Reduction and carboxamidomethylation of Cys-residues allowed us to establish that mature Ses i 2 is composed of a light (LC) and heavy (HC) chain held together by three disulfides, while two extra disulfides are present in the HC. Peptide mass fingerprint analysis of LC and HC with *S. aureus* V8 protease, combined with a MASCOT search, allowed us to unambiguously identify all the peptide masses of the proteolytic fragments as originating from the precursor protein of the *Ses i 2* allergen from *Sesamum indicum* (Swiss-Prot entry code: Q9XHP1). Notably, the abundance of Cys (10.6%), Arg (13.8%), Met (16%), and Gln (17%) in the mature Ses i 2 (containing 94 amino acids) is much higher than that normally observed in natural proteins. The structural model of Ses i 2, obtained by homology on the NMR structure of napin from Brassica napus (PDB code 1sm7), revealed that Ses i 2 is arranged in a four-helix bundle topology.

Ses i 2 possess an extraordinarily high stability to heat, Gnd-HCl, low pH, and proteases. In particular, at both pH 2.0 and 7.0, Ses i 2 structure exhibits only a partial unfolding even at high temperatures (e.g., 90°C). In the presence of Gnd-HCl, Ses i 2 reversibly unfolds with a $[Gnd\text{-}HCl]_{1/2}$ of 6.8 M. Strikingly, Ses i 2 is fully resistant to the combined action of low pH and pepsin in simulated gastric fluid, while in simulated intestinal fluid it is only partially trimmed at the N- and C-termini by trypsin and chymotrypsin, leaving a compact and folded protein structure. Quite surprisingly, Ses i 2 inhibits the amidolytic activity of thrombin, a key enzyme in blood coagulation, with an IC_{50} value of 1.60±0.05 μM.

Altogether, our results indicate that Ses i 2 may preserve its structure from the degradation in the gastrointestinal tract and this behaviour may be crucial to sensitise the mucosal immune system in the gut.

15.36

MOLECULAR INSIGHTS INTO THE INTERACTION BETWEEN ALPHA-SYNUCLEIN AND PUFAS

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alpha-Synuclein is a 140-residue protein, of unknown function, involved in several neurodegenerative disorders, such as Parkinson disease. Recently, a wide interest has risen around the putative interaction between alpha-synuclein and polyunsaturated fatty acids. Indeed, lipids are able to trigger multimerization of the protein *in vitro* and in cultured cells and alpha-synuclein is implicated in fatty acid uptake and metabolism. Docosahexaenoic acid is one of the main fatty acids in cerebral grey matter; it is dynamically released following phospholipid hydrolysis and it has been found in high levels in brains areas with alpha-synuclein inclusions from patients affected by Parkinson disease. Debated and unresolved questions regard the nature of the molecular interaction between alpha-synuclein and docosahexaenoic acid and the effect of the protein on the fatty acid aggregated state. We report a detailed characterization of the complex formed by alpha-synuclein and docosahexaenoic acid, analyzing the physical state of the lipid and the conformation adopted by the protein upon binding to the fatty acid by biophysical and biochemical methods. Docosahexaenoic acid is prone to assemble in vesicles with a large size distribution. Turbidity measurements, fluorescence studies and transmission electron microscopy have shown that alpha-synuclein disrupts the vesicles, stabilizing micellar forms of the fatty acid. Upon binding to docosahexaenoic acid micelles, alpha-synuclein acquires alpha-helical conformation in a simple two-state transition. Furthermore, proteolysis experiments by proteinase K and trypsin have allowed to define that only the first 70 amino acid residues of the polypeptide chain are strictly involved. In the presence of docosahexaenoic acid micelles, alpha-synuclein is highly prone to aggregate and form amyloid fibril. A thorough understanding of the role of lipids in protein structural transition and aggregation process should provide insights into the implication of lipids in amyloid formation *in vivo*.

15.37

SEMI-SYNTHESIS OF CONSENSUS TETRATRICOPEPTIDE REPEAT PROTEINS FOR FOLDING STUDIES

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The *tetratricopeptide repeat* (TPR) is a 34-amino acid α-helical motif that occurs in a great number of proteins from all kingdoms of life. In this type of proteins there are three or more TPR motifs in tandem arrays that function to mediate protein-protein interaction in a myriad of biological processes[1]. The repetitive and elongated nature of TPR proteins causes them to differ radically in their folding from normal globular proteins. As matter of fact, TPR proteins fold by a 1-D Ising model and are dominated by short-range interactions, whereas globular proteins fold by the typical two-state mechanism and exhibit complex topologies that frequently have numerous long-range interactions[2,3]. Based on a statistical analysis of TPR sequences, Regan and co-workers designed a idealized, consensus TPR modules which can be combined to create TPR proteins, named *Consensus TPR number of repeats* (e.g. CTPR2, CTPR3)[4]. We want to investigate the folding mechanism of repeat proteins using the consensus TPR comprised of different number of tandem repeats by ensemble and single molecule FRET. These experiments require homogenously labelled proteins with fluorescent dyes. In order to obtain CTPR variants in which fluorescence donor and acceptor pairs are incorporated at different, specific positions, we use a semi-synthetic strategy which combines solid phase peptide synthesis and protein expression techniques[5].

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15.38

PROTEIN ENGINEERING OF REPRESSOR OF PRIMER (ROP): CONSTRUCTION OF MOLECULAR SCAFFOLDS FOR THE INTRODUCTION OF NEW FUNCTIONS.

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Rop (repressor of primer) is a small dimeric protein of *E. coli* with a stable four helix bundle structure.

Protein engineering of Rop has been used: 1- to introduce a heme binding site into the four helix bundle scaffold; 2- to create a new three helix bundle molecular scaffold.

1- Heme ligands were introduced in the hydrophobic core of an engineered monomeric rop (rop-S55) in two different layers of the heptad repeat. Mutants rop-L63M/F121H (layer 1) and rop-L56H/L113H (layer 3) were found to bind heme with a K_D of 1.1±0.2 and 0.47±0.07 μM respectively. The unfolding of heme-bound and –free mutants in the presence of guanidine hydrochloride was monitored by both circular dichroism and fluorescence spectroscopy and the total free energy change resulted 0.5 kcal/mol higher in the case of heme bound rop-L56H/L113H respect to rop-L63M/F121H. Heme binding resulted also to increase ΔG by 1.4 and 1.8 kcal/mol in rop-L63M/F121H and rop-L56H/L113H respectively.

Spectroelectrochemical titrations allowed to calculate redox potentials of-154±2 mV in rop-56H/113H and –87.5±1.2 mV in rop-L63M/F121H.

The mutant designed to bind heme in a more hydrophobic environment (layer 3) resulted to have a tighter binding, a higher stability and a more negative redox potential than the mutant designed to bind heme in layer 1 demonstrating the possibility to control the properties of heme binding of rop by protein engineering.

2- The last helix of the monomeric ROP protein was removed by PCR and the resulting protein was purified.

The far-UV circular dichroism spectrum showed a high helical content. Thermal and chemical unfolding were monitored and the stability of the mutant was found to be very similar to a synthetic three helix bundle peptide. Analysis in gel filtration and native electrophoresis showed a dimeric behaviour of the protein due to the presence of a disulphide bridge. Molecular modeling was used to predict the structure of the protein according to th experimental data.

The results suggest that it is possible by protein engineering to turn Rop into a redox protein with controlled properties and to create new molecular scaffolds for the introduction of new functions.

15.39

EXPRESSION OF EXTRACELLULAR DOMAINS OF VEGF RECEPTORS AND THEIR USE IN NMR DRUG DISCOVERY STUDIES.

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Angiogenesis is a remodeling process characterized by the sprouting of new blood vessels from pre-existing ones. It occurs during embryogenesis and to a limited extent in the adult, for example in the female reproductive system, in physiological wound healing and in pathological disease processes such as cancer[1]. Vascular endothelial growth factor (VEGF) is a homodimeric protein and has been characterized as a prime regulator of angiogenesis and vasculogenesis; when cells lose the ability to control the synthesis of VEGF, angiogenic disease ensues[2]. In vitro studies show that VEGF is a potent and specific angiogenic factor involved in the development of the vascular system and in the differentiation of endothelial cells[3]. VEGF biological function is mediated through binding to two receptor tyrosine kinases: the kinase domain receptor (KDR) and the Fms-like tyrosine kinase (Flt-1), which are localized on the cell surface of various endothelial cell types. This binding activates signal transduction and can regulate both physiological and pathological angiogenesis[4]. VEGF and its receptors are in fact overexpressed in pathological angiogenesis, making this system a potential target for therapeutic and diagnostic applications[5, 6].

The extracellular portion of VEGF receptors is comprised of 7 immunoglobulin-like domains that is a common feature of membrane-anchored proteins; deletion studies have shown that the ligand binding function resides within the first three domains of Flt-1 and in domains 2 and 3 of KDR. Both VEGF receptors contain several putative N-glycosylation sites and apparent molecular weights of the mature proteins suggest that both receptors are extensively glycosylated. Anyway, glycosylation is not a prerequisite of high affinity binding of VEGF to its receptors[7]. Actually, no structural data are known on the extracellular portion of these receptors except for the second domain of Flt-1[8]. So, our aim is the cloning and the expression of part of extracellular domains of both VEGF receptors for structural characterization and to be used in interaction studies with peptide ligands or small organic molecules.

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15.40

THE THREE-DIMENSIONAL STRUCTURE OF THE GLOBIN DOMAIN OF GLOBIN-COUPLED SENSOR FROM GEOBACTER SULFURREDUCENS

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The recently discovered globin-coupled sensors (GCS) are heme-containing two domain transducers, that mediate the cellular responses to metabolic and environmental stimuli, such as NO, CO and O_2. In particular, the GCSs identified in the strict anaerobic □-Proteobacteria *Geobacter sulfurreducens* and *Geobacter metalloreducens* may be involved in sulfate/sulfur reduction. They are composed of 300 amino acid residues and characterised by a N-terminal globin domain (162 amino acids) and by a bundle of four transmembrane helices at C-terminal[1].

Here, we present the X-ray three-dimensional structure of the globin domain of GCS from *Geobacter sulfurreducens* (Gs-GCS162), at 1.5 Å resolution. The crystal structure of ferric Gs-GCS162 was solved by MAD techniques (data collected at the synchrotron beamline ID23-1, ESRF, Grenoble, France), based on the heme Fe atom anomalous scattering.

The Gs-GCS162 fold is a variant of the classical globin fold of myoglobin and hemoglobin, spanning helices A through H, with an additional Z helix at the N-terminus. The Gs-GCS162 displays a six-coordinated heme and is one of the first examples of bishistidyl hexacoordinated GCS. Strikingly, the distal heme-coordination is provided by a His residue located at the E11 topological site, an unprecedented feature within globins.

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15.41

THE Na+-BOUND AND Na+-FREE CONFORMATION OF HUMAN Alpha-THROMBIN PROBED BY SOLUTION STUDIES

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Thrombin exerts either procoagulant and anticoagulant functions in hemostasis. The procoagulant role entails conversion of fibrinogen into fibrin and platelets activation, while anticoagulant role regards the activation of protein C. The most effective modulator of thrombin function in solution is Na^+, that triggers the transition of the enzyme from an anticoagulant (slow) form to a procoagulant (fast) form. The Na^+-bound (fast) form displays procoagulant properties, since it cleaves more specifically fibrinogen and protease-activated receptor 1, whereas the Na^+-free (slow) form is anticoagulant because it retains the normal activity toward protein C, but is unable to promote acceptable hydrolysis of procoagulant substrates. Several crystal structures of the enzyme with and without Na^+ bound have been recently reported, but none of these seem to fully account for the different biochemical properties of the two forms.

Conformational studies were conducted by far- and near-UV circular dichroism (CD) and quenching of fluorescence; stability studies were carried out by recording the fluorescence changes of thrombin upon urea-induced-unfolding; the conformational flexibility of thrombin allosteric forms were probed by limited proteolysis with chymotrypsin; the accessibility of the binding sites of the enzyme in the fast or slow form was probed by using synthetic analogs of the N-terminal domain of hirudin.

In this work, the effect of Na+ binding on the conformational, stability, and molecular recognition properties of thrombin in solution was investigated. The binding of Na+ reduces the CD signal in the far-UV region, while increasing the intensity of the near-UV CD and fluorescence spectra. These spectroscopic changes have been assigned to perturbations in the environment of aromatic residues at the level of the S2 and S3 sites. The Na+-bound form is more stable to urea-denaturation than the sodium-free form. Notably, the effects of cation binding on thrombin conformation and stability are specific for Na+ and parallel the affinity order of monovalent cations for the enzyme. The Na+-bound form is even more resistant to limited proteolysis by subtilisin, at the level of the 148-loop, suggestive of the more rigid conformation this segment assumes in the fast form. Finally, we have used hirudin fragment 1-47 as a molecular probe of the conformation of thrombin recognition sites in the fast and slow form. From the effects of amino acid substitutions on the affinity of fragment 1-47 for the enzyme allosteric forms, we conclude that the specificity sites of thrombin in the Na+-bound form are in a more open and permissible conformation, compared to the more closed structure they adopt in the slow form. Our results indicate that the binding of sodium to thrombin serves to stabilize the enzyme into a more open and rigid conformation.

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15.42

ALLOSTERIC AND BINDING PROPERTIES OF A TRUNCATED RECOMBINANT HUMAN SERUM ALBUMIN – AN OPTICAL AND NMR SPECTROSCOPIC INVESTIGATION

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Human serum albumin (HSA), the most prominent protein in plasma, is best known for its exceptional ligand binding capacity, the most strongly bound compounds being hydrophobic organic anions of medium size, long chain fatty acids, heme and bilirubin. Moreover, HSA abundance (its concentration being 45 mg/mL, in the serum of human adults) makes it an important determinant of the pharmacokinetic behavior of many drugs. The conformational adaptability of HSA involves more than the immediate vicinity of the binding site(s), affecting both the structure and the ligand binding properties of the whole HSA molecule that displays ligand-dependent allosteric conformational transition(s)[1].

Here, we report the heme binding properties of a truncated form of HSA, encompassing domains I and II. The truncated sequence fully contains the primary binding sites for heme and warfarin, and the secondary ibuprofen binding site.

Results obtained demonstrate that the truncated form of HSA include the heme binding site and shows magnetic and optical properties quite similar to the full length form of HSA.

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15.43

NON STEROIDAL ANTI-INFLAMMATORY DRUGS MODULATE MATRIX METALLOPROTEINASES EXPRESSION IN HUMAN RHEUMATOID SYNOVIAL CELLS

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Non steroidal antiinflammatory drug (NSAIDs) because of their analgesic and anti-inflammatory properties are used to treat both rheumatoid arthritis (RA) and osteoarthritis (OA). Their mechanism of action is generally attributed to the inhibition of cyclooxygenases (COXs) (Vane, 1971). Recent evidences suggest that NSAIDs could play other important roles such as chemopreventive activity for colon cancer, apoptotic action and other functions mediated through COX-independent mechanism (Tegeder, 2001). The aim of the present study is to investigate if and how NSAIDs could play a role in the modulation of the expression and activity of matrix metalloproteinases (MMPs) produced by rheumatoid synovial cells. It is well known that MMPs act on the cartilage and extracellular matrix leading to the progressive joint destruction occurring in RA and OA. Synovial cells were obtained from patients with RA and characterised for CD90 content. Cultures of confluent cells were treated with IL1α and/or test drugs (5-25 uM) for 24-48h. Expression of MMP-1, 2, 3, 9 and TIMP-1 was monitored by zymography, western blotting and enzymatic analysis using fluorogenic synthetic peptides as substrate. Semiquantitative analysis of mRNA expression was also performed. All tested NSAIDs showed , with different extent the ability to down-regulate the expression of proMMPs.TIMP-1 production remained in each case unaffected, At the same time the ability of proMMP-2 to convert in its active form was inhibited in a concentrantion dependent manner. These COX-independent mechanisms suggest to further investigate new potential role of different NSAIDs as therapeutic agents, in the light of their good tolerability on long term and reduced side-effects.

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15.44

HUMAN alpha−-THROMBIN EXPRESSED IN *E. COLI*: PRODUCTION AND CHEMICAL, CONFORMATIONAL, AND FUNCTIONAL CHARACTERISATION

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Similarly to other clotting factors, *in vivo* thrombin is mainly present as a zymogen, prothrombin. Activation of prothrombin occurs via proteolytic cleavage by factor Xa, generating prethrombin-2 (PreT-2), the smallest single-chain intermediate precursor of alpha-thrombin, which is subsequently cleaved to produce the mature enzyme. Among several different expression systems developed so far, production of thrombin in *E. coli* has demonstrated as a convenient method to produce active human thrombin in reasonably high yields. Human thrombin (hThb) contains four disulfide bridges and is glycosylated at Asn60g. Recombinant alpha-thrombin obtained in *E. coli* (rThb) lacks glycosylation and it has being widely used by numerous research groups for structural and functional studies. However, no detailed characterisation of wild-type rPreT-2 and rThb has been reported in the literature.

With the aim to fill this gap, we have carried out a thorough chemical, conformational, stability, and functional characterisation of rPreT-2 and rThb expressed in *E. coli* and compared the mature recombinant thrombin with the natural enzyme. PreT-2 cDNA was expressed in *E. coli* under the control of T7 polymerase promoter. rPreT-2 formed intracellular inclusion bodies from which the protein was refolded in a reverse-dilution renaturation process. After centrifugation, soluble rPreT-2 was purified to homogeneity by heparin-sepharose affinity chromatography, with a final yield as high as 12%. Highly pure (> 95%) rThb was obtained after activation of rPreT-2 with *Echis carinatus* snake venom and subsequent purification by affinity chromatography. The major results obtained in this work can be summarised as follows: 1) the disulfide bond topology of rThb, established by peptide mass fingerprint analysis, is the same as that of the natural hThb; 2) rThb and hThb share a common 3D structure, as deduced from far- and near-UV CD and fluorescence spectroscopy; 3) the kinetic constants (Km and kcat) for the hydrolysis of the chromogenic substrate D-Phe-Pro-Arg-pNA are identical for both rThb and hThb; 4) both rThb and hThb display identical affinities for Na^+ ion (an allosteric modulator of thrombin), full-length hirudin (the most potent and selective thrombin inhibitor known so far) and its N- and C-terminal fragments Hir(1-47) and Hir(48-64); 4) rThb ($[urea]_{1/2}$ = 2.6±0.1 M) is less stable than hThb ($[urea]_{1/2}$ = 3.2±0.1 M) to urea-induced unfolding; 5) rPreT-2 and rThb display significant differences in the far-UV CD and fluorescence spectra; 6) active rThb is more stable than rPreT-2 ($[urea]_{1/2}$ = 2.1±0.1 M); 7) rPreT-2 binds full-length hirudin with an affinity $7x10^6$ fold lower than rThb.

Our results indicate that the lack of glycosylation in rThb selectively affects the stability of the recombinant enzyme, while keeping the functional and ligand-binding properties of the natural enzyme unchanged.

15.45

REDOX PROTEOMICS OF 3'-NITRO-TYROSINE-CONTAINING PROTEINS IN UREMIC PLASMA

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Oxidative and nitrosative stress may cause post translational changes in plasma proteins that have been described to accumulate in inflammatory and degenerative conditions. 3'-Nitro-Tyrosine (3NTyr) has been so far suggested to represent one of the main protein changes occurring by the sustained exposure to nitric oxide derived species formed during inflammatory cell activation.

However, current analysis methods applied to human plasma have missed the exact identification of proteins containing this post-translational change, thereby providing incomplete and unverified information, and ultimately limited clinical value.

In this study we used a LC-MS/MS analysis method recently developed by some of us[1] that combined with an immuno-purification step[2], allows the direct identification of 3NT-containing proteins in human plasma. A clinical application of this analysis was performed in chronic renal failure patients on regular hemodialysis (HD) therapy that are notoriously affected by chronic inflammation and were tentatively identified to accumulate protein nitration hallmarks[3].

The specificity of the LC-MS/MS analysis method proposed in this study is based on the chemoselective labelling with DNS of 3'-amino-Tyr-containing peptides produced after trypsin digestion by the reduction with $Na_2S_2O_4$ and the immunopurification step was demonstrated to be a pre-requisite to achieve sensitivity in this redox-proteomics analysis of human plasma proteins.

This method was successfully applied to the qualitative analysis of 3NT-containing proteins with different relative abundance and molecular mass, in plasma and dialysis fluids of HD patients. This analysis method can be used also to identify the number and position of Tyr residues affected by this post-translational change in individual proteins. Semi-quantitative data obtained with this procedure clearly show that healthy control plasma contains much less nitrated peptides, and these findings were in agreement with lower levels of biochemical indices of oxidative stress and inflammation. Further studies are in progress to apply this assay procedure to a more extensive identification of 3NT-containing proteins in plasma of HD patients and also in other chronic diseases associated with oxidative stress and inflammation.

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15.46

RNase A OLIGOMERIZATION THROUGH 3D DOMAIN SWAPPING IS AFFECTED BY A RESIDUE LOCATED FARAWAY FROM THE SWAPPING DOMAINS

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Bovine pancreatic ribonuclease A is monomeric in its native state, but forms three-dimensional (3D) domain-swapped dimers, trimers, tetramers, and larger oligomers by lyophilization from 40% acetic acid solutions[1], according to the method of Crestfield, Stein and Moore[2]. The same oligomers form if the protein is subjected to a thermally-induced oligomerization procedure lacking the lyophilization step[3]. Several studies have been performed to determine which are the structural determinants that promote RNase A oligomerization; these studies have been accompanied by others performed on BS-RNase, a member of the RNase superfamily that displays more than 80% of sequence identity with RNase A, and is found in bull semen. The seminal variant is dimeric in nature for the presence of two intersubunit antiparallel disulphide bonds, and about 70% of its native molecules swap their N-terminal ends [4]. It has been recently found that the R80S-BS-RNase mutant lowers its swapping propensity to about 30% if the residue 80 mutation is accompanied by the substitution of the 16-22 loop (connecting the N-terminal domain with the core of the protein) of the seminal enzyme with the loop present in RNase A [5].

Considering that in RNase A the residue present in 80 is serine, we tried the opposite mutation in the pancreatic enzyme to see if this could induce a significant increase in the aggregation propensity of the pancreatic enzyme through 3D domain swapping. The S80R-RNase A mutant was produced, sequenced, purified and analyzed. No significant loss of stability, structural features or enzymatic activity were detected. The aggregation propensity of the mutant was then studied, in parallel with that of the wild type, using either the 'classical' acetic acid lyophilization method or the thermally-induced oligomerization. All results obtained to date indicate that the aggregation propensity of the S80R-RNase A mutant is higher than the w.t., and that that of the species formed through the N-terminus swapping significantly increase their yield in the mutant with respect to the w.t. enzyme. Invesitgations on the stability of the dimeric species produced by the mutant with respect to the stability of the w.t. are presently under study, while the future goal will be to see if the more basic oligomers of the S80R RNase A mutant may show increased biological activities with respect to the ones displayed by the omologous oligomers formed by the wild type[1].

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15.47

ACTIVITY OF PROTEASES FROM ECHIS CARINATUS VENOM ON HUMAN PLASMA PROTEINS

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Snake venom on evenomation has many toxicological effects that eventually leads to death in human, however these toxins contained in venoms are undoubtely proven to be useful to human.

Venom from *Echis Carinatus* has been reported to be active on plasma proteins. It is reported that it contain proteases.

These proteases affect various physiological functions: platelet aggregation, blood coagulation, fibrinolysis and complement system. In particular the ecarin is active as prothrombin activators[1].

We studied and identified the properties of *Echis Carinatus* proteases in comparison with other snake venom proteases that are active on plasma.

In addition we carried out an experiment to compare ecarin activity with thrombin activity on depleted plasma.

An appropriate ratio concentration between *Echis Carinatus* and plasma was used for this assay in SDS-PAGE.

We observed venom activity on plasma, in particular changes which occurred in the bands that we analysed with Total Lab. This we studied using whole plasma and depleted plasma.

Plasmatic changes that occurred was studied after pre-incubation with venom in a different times of 0', 10', 20',30',40',50' and 60'.

The 2D Image of the various spots detected were then analysed using Image Master Platinum to evaluated the changes that occurred in the plasma proteins

In conclusion using a proteomic approach we observed that *Echis Carinatus* venom contains proteases that are active on plasma proteins.

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15.48

LACTOFERRIN INHIBITS PAPAIN-LIKE CYSTEINE PROTEASES

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Transferrins (Tfs) belongs to a family of iron-binding glycoproteins. The different proteins of the Tf family have been subdivided on the basis of their amino acid sequence, biological function and location. Lactoferrin (Lf), is expressed and secreted from glandular epithelial cells and from mature neutrophiles of mammalian, and it is an important component of the aspecific host defence or natural immunity[1]. Serum transferrin (sTf) is synthesized by the liver of mammals and secreted into the blood; its primary function is iron transport. Ovotransferrin (Otrf), instead, is synthesized by avians and displays both iron transport and protective functions[2]. A 17-mer sequence Y679–K695 present near the C-terminus of human (hLf) and bovine (bLf) lactoferrin shows 90% homology and 60% identity with the sequence of the reactive site of the cystatins, which are competitive inhibitors of papain-like cysteine proteases[3]. The same sequence is present, though with lower homology, in Otrf and, with even lower homology, in sTf. Parasites synthesize papain-like cysteine proteases that are relevant for the virulence and pathogenicity of parasites, being involved in several aspects of the parasite life cycle[4], it is therefore possible that the antiparasitic activity of Lf could be due to parasitic papain-like cysteine protease inhibition.

In this study we have investigated the kinetic parameters of Tfs inhibition of the parasitic papain-like cysteine protease type I from *Leishmania infantum* and *Trypanosoma cruzii*. bLf, hLf and Otrf inhibit papain and the parasitic proteases. The inhibition of such cysteine proteases by these proteins conforms to a competitive mechanism. K_I values for bLf and hLf inhibition of *L. infantum* protease and *T. cruzi* (K_I = 3.1 nM) are lower than those for papain (K_I = 24 nM). The inhibition of cysteine proteases by Otrf is still present, although much lower than that displayed by the mammalian proteins (K_I = 6.0 μM. On the contrary, sTf does not display any inhibition, according to its different role in mammals. Moreover, SDS-PAGE shows that bLf, hLf and Otrf are easily degraded by papain during the assay incubation time and it is likely that a cystatin-like peptide is generated from protease hydrolysis to competitively inhibit the protease itself. The unequivocal identification of this peptide is presently under investigation by HPLC-Mass Spectroscopy technique.

These data strongly support the hypothesis that Lf antiparasitic activity could be due, at least partially, to the inhibition of parasitic proteases.

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15.49

ISOFORM SPECIFIC PHOSPHORYLATION OF THE N-TERMINAL DOMAIN OF p53 BY PROTEIN KINASE CK1 - AN IN VITRO ANALYSIS

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The tumor suppressor p53 and the cellular oncogene mdm2 have been identified as key signal integrator molecules. Alterations in their phosphorylation status can abolish their function resulting in uncontrolled cell growth. Several CK1 isoforms have been shown to phosphorylate p53. Whereas CK1α is able to phosphorylate p53 only *in vitro*, CK1δ and ε phosphorylate p53 *in vitro* and in cultured cells. CK1δ/ε-mediated phosphorylation has been reported to occur at serines 4, 6 and 9 of murine p53 (Knippschild, 1997), whereas phosphorylation of human p53 at serine 9 and threonine 18 by CK1δ/ε seems to require prior phosphorylation by other kinases of serine 6 and 15, respectively (Sakaguchi, 2000).

These somewhat conflicting observations prompted us to assay a series of peptides encompassing residues 1-28 of human p53 protein as substrates for different CK1 isoforms. Phosphorylation assays with radiolabelled ATP show that only the CK1 delta phosphorylates p53 peptides, albeit with low efficiency (Km≈4mM). In the case of full length human p53 GST-fusion protein, both CK1 alpha and delta are able to phosphorylate the protein with Km in the low nM range.

In the peptide model, mutation of S15 and S20 to alanines results in a very low radioactivity incorporation, suggesting a major implication of these residues as the main target of CK1 delta. Phospho-aminoacid analysis, moreover, rules out the involvement of threonine 18 in CK1δ-mediated phosphorylation. To further analyze the residues phosphorylated in the p53 peptide, we have performed solid-phase Edman degradation: S15 alone accounts for about 80% of the whole radioactivity incorporated; a much lower but significant phosphorylation of S9 as well as traces of radioactivity in correspondence of S6 and S9 are found.

At present we are working on the full length p53 protein to compare its phosphorylation pattern to that of the peptides and to gain information about remote docking sites responsible for the dramatic increase in Km observed with the protein as compare to the peptides.

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15.50

PRODUCTION AND CHARACTERISATION OF THERAPEUTIC PROTEINS IN *NICOTIANA BENTHAMIANA*.

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In the last decades, bacterial, yeast, and animal cells have been exploited as factories for the production of therapeutic proteins. More recently, molecular farming has rapidly pushed towards plants for producing recombinant proteins. Expression of therapeutic proteins in plants is safe and extremely cost-effective. Unlike microbial fermentation, plants are indeed capable of introducing post-translational modifications and, unlike production systems based on mammalian cell cultures, they are devoid of human infective viruses and prions.

In this study, *Nicotiana benthamiana* was used as a novel expression system for the production of human interleukin-6 (hIL-6) and human granulocyte-macrophage colony-stimulating factor (hGM-CSF), two protein drugs of outstanding pharmacological interest. DNA coding sequence for mature hIL6 protein was fused with an extensin target peptide sequence at the 5'-position and a 6xHis tag at the 3'-position. Construct with a double 35S promoter and a 35S terminator was inserted in pBin32 vector. Plants of *Nicotiana benthamiana* were transiently transformed by *Agrobacterium tumefaciens* and green leaves were collected 6 days after transformation. Immunoblotting analysis showed that the recombinant proteins were efficiently accumulated in transgenic plants, with expression levels of about 0.1-0.2%, calculated on the total soluble proteins in leaves. The presence of multiple species of hIL-6 and hGM-CSF at different molecular weights is the result of heterogeneous N- and O-linked *in vivo* glycosylation. After extraction, the recombinant proteins were purified to homogeneity by combining several chromatographic steps and the purity of the protein samples was ascertained by RP-HPLC and IEF/SDS 2D gel-electrophoresis.

By using peptide mass fingerprint analysis on Coomassie-stained electrophoretic bands, we isolated several tryptic peptides covering 26 and 48% of the entire sequence of hGM-CSF and hIL-6, respectively. This allowed us to unambiguously identify the two recombinant proteins. *In vitro* functional analysis were performed on a cell proliferation assay using a human growth factor-dependent TF-1 cell line suggesting that the recombinant proteins expressed in *N. benthamiana* are biologically active, with a ED_{50} value of 0.02-0.05 ng/mL for hGM-CSF and 0.10-0.25 ng/mL for hIL6.

Our results demonstrate that *Nicotiana benthamiana* is a suitable eukaryotic expression system for producing in reasonable yields correctly glycosylated proteins of therapeutic interest.

15.51

THE HUMAN KERATOEPITHELIN FAS1-4 DOMAIN AS A MODEL FOR THE STUDY OF PROTEIN MISFOLDING AND AMYLOIDOGENESIS: RECOMBINANT EXPRESSION AND PRELIMINARY CHARACTERISATION.

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Keratoepithelin (KE), also known as Big-h3, TGF-β-induced protein h3 or RGD_CAP, is an extracellular matrix protein highly conserved among all the vertebrate species so far analyzed. It is encoded by the gene BIGH3 (also known as TGFBI) that was discovered in a search for genes induced by transforming growth factor β (TGF-β). KE is expressed in various tissues and organ systems, such as cornea, skin, bone, tendon and kidney, where it interconnects the extracellular matrix proteins to resident cells binding collagens, fibronectin and integrins. In a recent work we have also detected KE, by immunofluorescence experiments, in the perimysium and endomysium of skeletal muscle fibers[1]. KE is formed by four domains in close succession, discovered in the insect cell adhesion molecule fasciclin I and thus named FAS1 domains. This kind of domains has been identified within Eukarya in Plantae, Fungi and Animalia. Many studies have shown a role of KE in 5q31-linked human autosomal dominant corneal dystrophies, such as granular corneal dystrophy (GCD) and lattice corneal dystrophy (LCD) characterised by the formation of amyloid-like or granular deposits of KE within the corneal stroma[2]. It has been proposed that some missense mutations found into the BIGH3 gene may trigger KE misfolding, eventually resulting in the deposition and/or fibrillation of KE mutant protein[3]. To verify this hypothesis, we have cloned and recombinantly expressed domain FAS1-4 of human KE, and analysed its structural properties and stability by circular dichroism and fluorescence spectroscopy. We have introduced into this domain a point-mutation found in patients affected by lattice corneal dystrophy (LCD) type I, namely L518R, and we have compared the biochemical properties of wild-type and mutant FAS1-4 domains. Amyloid protein aggregation is a prominent feature of many neurodegenerative diseases, such as Alzheimer's, Huntington's and Parkinson's diseases as well as spongiform encephalopathies. KE and the study of its amyloidogenicity may represent an attractive model for the analysis of protein misfolding and aggregation phenomena.

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15.52

IDENTIFICATION OF THE MMP-9 CLEAVAGE SITE ON THE RECOMBINANT BETA-DYSTROGLYCAN ECTODOMAIN.

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Dystroglycan (DG) is a membrane receptor belonging to the complex of glycoproteins associated to dystrophin. DG is formed by two subunits, alpha-DG, an extracellular matrix protein highly glycosylated, and beta-DG, a transmembrane protein resulting from a post-translational cleavage, which are held together by non-covalent interactions. The association between alpha-DG and beta-DG results in a contact between the extracellular matrix and the cytoskeleton, and it is crucial to maintain the integrity of the plasma membrane. In inherited neuromuscular diseases such as Duchenne and Becker muscular dystrophies, disruption or even a weakening of the interaction between the two DG subunits, due to a reduction or a complete loss of one or both DG subunits, causes sarcolemmal instability and necrosis of muscular fibers. Interestingly, Western blot experiments carried out on tissue extracts from patients affected by such diseases revealed the proteolytic fragment of beta-DG missing its extra-cellular domain and showing an apparent molecular weight of 30 kDa that was originally discovered in breast cancer cell lines[1]. It was demonstrated that the 30 kDa beta-DG fragment is produced by a matrix metalloproteinase activity resulting in the shedding of all or part of the beta-DG ectodomain[2]. The 30 kDa beta-DG fragment was also detected in other pathological conditions such as Fukuyama-type congenital dystrophy, sarcoglycanopathy, ischemic injury, osteoarthritis, as well as in healthy tissues such as peripheral nerve, bladder, lung, kidney and skin. Although many studies have been focused on the 30 kDa beta-DG fragment produced by matrix metalloproteinase activity, the molecular mechanism of the enzymatic digestion of beta-DG extracellular domain is still elusive; for this reason we have carried out an in vitro enzymatic digestion of the recombinant extracellular domain of beta-DG, beta-DG(654-750) with the metalloproteinase MMP-9, and characterised the reaction both kinetically and thermodynamically. Moreover, we have analysed the reaction products using a combined approach based on SDS-PAGE and HPLC-ESI-IT mass spectrometry. Thus we were able to identify the exact beta-DG peptide bond primarily cleaved by MMP-9, localized between the amino acidic positions 716-717.

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15.53

AMYLOID CONFORMATIONS OF HUMAN LYSOZYME

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Among the variety of proteins found to be able to form amyloid fibrils in vivo and in vitro, human lysozyme is a very interesting case[1]. Some lysozyme variants, in fact, have been proved to be responsible for abnormal fibril deposition in familial non-neuropathic systemic amyloidosis. Moreover, wild type lysozyme itself has been demonstrated to form in vitro amyloid fibrils highly similar to the pathological ones. In order to understand the mechanism of aggregation of both variant and wild type lysozyme, experimental conditions have been tuned to allow the in vitro formation of lysozyme amyloid fibrils. Unfortunately, due to the extreme stability of all lysozyme variants (midpoint of thermal denaturation is between 55 and 80 C under physiological conditions), fibrils have been formed under mild and high denaturant conditions, including high temperature, low pH, moderate concentration of denaturant or high pressure. Except for an overall fibrillar morphology, lysozyme fibrils formed under different conditions have never been shown to share the same amyloid structure. Here, human lysozyme fibrils have been formed under two sets of conditions, at low and neutral pH. Their amyloid nature has been confirmed by thioflavin T assay, transmission electron microscopy and X-ray diffraction. Interestingly, FTIR analysis revealed a significant difference in the fibrils structure and fibrils dissociation experiments confirmed a different conformational stability for the two kinds of fibrils. This study clearly indicates that aggregation conditions promote the formation of amyloid fibrils with different structure and stability due to different rearrangements of lysozyme polypeptide chain into the core of amyloid fibrils. Such differences in amyloid conformations could arise from the properties of the amino acid sequence of lysozyme in each condition or could be driven by kinetic factors. Moreover, the structure of the precursor protein may also lead to different folds of the lysozyme polypeptide chain in the beta-sheet core of fibrils.

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15.54

NOVEL ANTIBACTERIAL PEPTIDES ISOLATED FROM THE EUROPEAN PAPER WASP, *POLISTES DOMINULUS* (HYMENOPTERA, VESPOIDEA)

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Insects have very efficient defense mechanisms against pathogens and parasites. Their systemic antimicrobial response is mainly based on the synthesis of antimicrobial peptides (AMPs) by the fat body and certain blood cells and on the release of these factors into the hemolymph after bacterial challenge. AMPs can also be produced as a local response in the epithelia such as tracheal epithelium, gut lining and salivary glands. On the basis of their structural features, AMPs are classified into three classes (1): (i) linear peptides, devoid of cysteines and forming α-helices, such as cecropins; (ii) peptides with an over-representation in proline and/or glycine residues; (iii) peptides stabilized by intramolecular disulfide bonds, such as defensins. Here we report the isolation of two constitutive antibacterial peptides from the larval saliva of the social wasp *Polistes dominulus*, a very common specie in Europe. This secretion has an alimentary function but it is also important for the infection prevention in the social insect community. Microbiological tests proved that larval saliva of *Polistes dominulus* has an antibiotic activity against *Bacillus subtilis*, Gram +, and *Escherichia coli*, Gram - (2). The antimicrobial peptides were purified by reversed-phase HPLC and characterized by peptide sequencing and mass spectrometry. The first one has a molecular weight of 2279.50 Da, is formed from 21 aminoacids, it is rich in proline and its sequence has been completely determined. Unfortunately the synthetic form of this molecule had no antibacterial activity and it was probably not correctly structured: we think that treatment with proline *cis-trans*-isomerase inducing the isomerization of the proline peptide bond(s) can allow the activation of the synthetic peptide. The second peptide, with a mass of 3700,54 Da, has been only partially sequenced and belongs to the group of defensins, because of the presence of six cysteine residues. It shows some similarities with a putative defensin of the dipter *Culicoides sonorensis*, but a definitive characterization of this molecule awaits further work.

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15.55

STRUCTURAL INSIGHTS OF HEPARIN-BINDING HAEMAGGLUTININ FROM *M. TUBERCULOSIS*

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Mycobacterium tuberculosis (MTB) is one of the most devastating human microbial pathogens. It invades and multiplies in both phagocytes and epithelial cells. In MTB, adherence to epithelial cells is mediated by the heparin-binding haemagglutinin adhesin, HBHA[1]. This protein binds to heparan sulphate proteoglycans on the surface of epithelial cells and is, therefore, responsible for extra-pulmonary dissemination of tuberculosis[2]. Binding to target epithelial cells involves the C-terminal lysine-rich domain of the protein, which is exposed at the mycobacterial cell surface[2]. However, despite the interest of HBHA both as a potential antigen against tuberculosis and as a diagnostic tool, no structural data are so far available, nor detailed information on the protein oligomerization state have yet been provided. We here present a biophysical characterization and the low resolution structure by Small Angle X-ray Scattering (SAXS) of both the full length HBHA and a truncated form, here denoted as HBHADC, which lacks the C-terminal heparin-binding domain (residues 161-199)[4]. We have cloned, expressed and purified both HBHA and HBHADC to study their molecular properties and oligomerisation, a key event for bacterial haemagglutination[5]. Circular dichroism studies have provided an experimental evidence that HBHA presents a coiled coil domain, like predicted by sequence analysis[6]. To validate this result, we performed chemical denaturation, using urea as a denaturating agent, of HBHADC. The urea denaturation profile of HBHADC is characteristic of a two-state helix-coil transition with a peculiarly low urea Cm, a characteristic which is typical of coiled coil systems. By performing cross-linking experiments using both glutaraldehyde and bis-sulfosuccinnimidyl-suberate (BS3) as cross-linking agents, we evidenced that HBHA is dimeric in solution. Small Angle X-ray Scattering (SAXS) experiments on both HBHA and HBHADC confirmed the dimeric nature of these proteins[5]. The experimental R_g and D_{max} values showed that HBHA exhibits an elongated shape. The increase of both R_g and D_{max} of the full-length protein compared to HBHADC suggests peripheral location of the C-terminal of HBHA. This is consistent with the role attributed to HBHA C-termini in epithelial cell adhesion. Furthermore, our data suggest that, like for other known systems, the coiled-coil nature of the N-terminal region of HBHA is responsible for dimerization, which may be regarded as a process that leads to enhanced affinity to epithelial cells and improves cell adhesion.

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15.56

STRUCTURAL AND FUNCTIONAL CHARACTERIZATION OF TWO RIBONUCLEOSIDE HYDROLASES FROM THE ARCHAEON *SULFOLOBUS SOLFATARICUS*.

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Nucleoside hydrolases (NHs) comprise a superfamily of structurally related metalloproteins with a unique β-sheet topology[1]. Functionally, NHs are glicosidases that hydrolyse the N-glycosidic bond of β-ribonucleosides, forming the free purine/pyrimidine base and ribose. All characterised members impose a stringent specificity for the ribose moiety, but exhibit variability in their preference for the nature of the nucleic base[1]. Sequence alignments highlight a recurring N-terminal DXDXXXDD motif as a hallmark of NH activity.

To elucidate the mechanisms by which hyperthermophilic enzymes acquire their unusual thermostability and to increase our knowledge on the structure of NH we have chosen as experimental model system NH from *Sulfolobus solfataricus,* (SsNH) an hyperthermophilic *archaeon* optimally growing at 87°C. The analysis of the complete sequenced genome of *S. solfataricus* revealed two open reading frames (SSO0505 and SSO2243) encoding two proteins, the citosine-uridine preferring nucleoside hydrolase (SsCU-NH) and the adenosine-inosine-guanosine preferring nucleoside hydrolase (SsIAG-NH). The genes were amplified by PCR, ligated into the plasmid pET-22b(+) and expressed in *E. coli* BL21. The purified recombinant proteins were enzymatically active, thermophilic and thermostable. The enzymes are characterized by a subunit of 35 kDa. SsCU-NH is a homotetramer of 140 kDa that recognizes only pyrimidine nucleosides as substrates. On the other hand, SsIAG-NH is a homodimer of 70 kDa specific for purine nucleosides. To obtain information about the flexible regions of the protein exposed to the solvent, SsCU-NH and SsIAG-NH were subjected to limited proteolysis. Finally, the structures of the two enzymes provided insight into substrate specificity and proteolytic analyses as well as into mechanisms of thermal stability. Molecular model analysis reveals that the overall structure and the position of catalytically important residues in the calcium and ribose binding are conserved while the base-binding pocket reflects the difference in substrate specificity between SsCU-NH and SsIAG-NH strongly supporting the hypothesis that SsIAG-NH and SsCU-NH are the same enzyme whose active site was partially modified to specifically recognize purine or pyrimidine. The evaluation of the models allowed to state that a higher compactness of the structure and the presence of clusters of hydrophobic residues such as Ile and Leu at the subunit interfaces, enhance protein thermostability of both enzymes whereas the presence of an intersubunit disulfide bridge in SsIAG-NH appears to be more relevant to obtain higher enzyme stability. These are the first example of nucleoside hydrolases reported in *Archaea.*

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15.57

CATALYTIC FIBRILS, A NANOSCALE BIOMATERIAL GENERATED BY AMYLOID FIBRILS FUNCTIONALIZED WITH AN ENZYME

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Amyloid fibrils are ordered protein structures generated by molecular self-assembly ("molecular Lego"). Being resistant to proteases, detergents and mechanical stress and stable under a variety of conditions, amyloid fibrils are optimal tools for the construction of novel nanoscale biomaterials.

We used amyloid fibrils as a scaffold for the production of enzymatically active polymers, named "catalytic fibrils", generated by functionalizing a fibrillogenic polypeptide with an enzyme. This was achieved by genetically fusing glutathione S-transferase (GST), an enzyme widely used for biotechnological applications, to the Apolipoprotein A-I (ApoA-I) fibrillogenic domain, to generate a bifunctional chimeric protein consisting of a fibrillogenic and a catalytic moiety.

The fibrillogenic domain of ApoA-I (90-100 residue long) corresponds to the N-terminal region of the protein. In ApoA-I variants associated to amyloidosis, this domain is released and generates amyloid fibrils in various organs and tissues. Recently, we were able to produce a recombinant version of the 93-residue N-terminal polypeptide, named [1-93]ApoA-I[1], previously extracted from cardiac amyloid fibrils of patients affected by amyloidosis[2]. It was found to be a natively unfolded polypeptide[1] with high propensity to generate fibrils as its natural counterpart.

This polypeptide was fused to GST. The fusion protein GST-[1-93]ApoA-I was expressed, isolated and characterized. It was found to fully retain GST catalytic activity and to have high propensity to aggregate in fibrillar structures, as indicated by tests for protein aggregation in amyloid-like structures. Upon incubation for 3 weeks at pH 6.4, almost all the protein molecules generated insoluble aggregates. AFM analysis showed the presence of straight fibrils and fibril networks.

Fibrils were loaded on a polypropilene-based filter and tested for enzymatic activity by injecting through the fibrillar matrix a reaction mixture containing GST substrates. Spectrophotometric analyses of the flow-through demonstrated that fibrils were enzymatically active. The enzymatic activity of catalytic fibrils was found to be time-dependent and stable over time.

Our findings show that a fibrillar matrix, generated by self-assembly of a bifunctional chimeric protein, is catalytically active and reusable over time. It may represent a novel nanoscale catalytically active biomaterial.

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15.58

IN VITRO AND IN VIVO ANTIMICROBIAL ACTIVITY OF A BRANCHED PEPTIDE SPECIFIC FOR GRAM-NEGATIVE BACTERIA

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The growing emergency of multi-drug resistant bacteria is a global concern: a number Gram-positive and Gram-negative bacteria have developed resistance against most traditional, as well as new generation antibiotics. Therefore, the demand for new antibiotics urges researchers and pharmaceutical companies to consider new antimicrobial agents. Among these, antimicrobial peptides turned out to be particularly interesting, in consideration of their peculiar mechanism of action, which is specifically targeted to bacterial membrane. Nonetheless, pharmaceutical companies have shown a general reluctance to the development of peptide drugs, which can be explained by a number of problems related to development of peptide as drugs, including their short half life produced by rapid proteolysis.

We recently identified a non-natural peptide sequence, showing a strong antimicrobial activity[1]. This peptide (QKKIRVRLSA; called M6) was synthesized in the branched Multiple Antigen Peptide form, that we had previously demonstrated to induce general peptide resistance to proteolysis, making this kind of molecules very suitable for in vivo use[2-3]. The branched peptide M6 was characterized for its activity against a number of bacteria showing a strong specificity for Gram negative species. The peptide resulted particularly active against *Escherichia coli*, *Pseudomonas Aeruginosa*, *Klebsiella pneumoniae* and some other enterobacteriacee, including multi-drug resistant clinical isolates. We also demonstrated that M6 showed a poor toxicity for eukaryotic cells, it bound LPS and DNA and it did not produce appreciable haemolysis even upon prolonged incubation. Moreover, we evaluated M6 acute toxicity (LD50 around 125 mg/Kg for i.p. administration) and we also demonstrated that it is not immunogenic upon repeated injections in animals[4].

We report here on M6 in vivo activity in models of sepsis induced in mice by E. coli and P. aeruginosa. These experiments showed that the peptide can prevent animal death and can neutralize sepsis symptoms when used in doses comparable to traditional antibiotics and compatible with a clinical use.

These results make the branched M6 peptide a strong candidate for the development of a new antibacterial drug.

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15.59

ON THE OXYGEN REACTIVITY OF FLAVOOXIDASES

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Cholesterol oxidase (CO, EC 1.1.3.6) is a bifunctional FAD-containing flavoenzyme catalyzing the oxidation and isomerization of cholesterol to cholest-4-en-3-one[1]. CO from *Brevibacterium sterolicum* (BCO) possesses the cofactor covalently linked to the apoprotein moiety; its 3-D structure shows a putative "oxygen-tunnel" extending from the surface of the molecule to the buried active site cavity. This tunnel is blocked from direct access by the side chain of R477, which adopts two alternative structural conformations, corresponding to an "open tunnel" and to a "closed tunnel" forms: E311 and E475 seem to modulate the conformational change. The residues A204, G309 and I423 also participate to the tunnel formation. To assess the role of these residues on the oxygen accessibility/reactivity of BCO, a number of mutants were prepared by SDM, over-expressed in *E. coli* and characterized.

Mutants involving substitutions of residues E311 and R477 show the most significant modifications of the kinetic properties. The conservative substitutions E311Q and R477K resulted in a 50−fold decrease in the k_{cat} value for the dehydrogenation reaction with respect to the wild−type BCO; the elimination of the charge (E311L and R477A BCO mutants) quite completely abolished the enzymatic activity. Substitution of E475 affected mainly the isomerization reaction, whereas mutations at positions 204, 309 and 423 marginally modified the enzymatic activitiy. Furthermore, the midpoint redox potential of BCO mutants were modified to a limited extent (≤40 mV) by the substitutions introduced.

Concerning the oxidative half-reaction, wild−type BCO shows a saturation behaviour of the observed re-oxidation rate constant of the reduced form with increasing oxygen concentration[2]. This behaviour was proposed to arise from an "interconversion" between the two conformations of the residues gating the putative oxygen channel in the reduced enzyme form[2]. Interestingly, the BCO mutants at position E311 show a linear dependence of the rate of reoxidation with O_2 concentration, pointing to the elimination of the "interconversion" process. Furthermore, the rate of reoxidation is significantly decreased for E311Q and R477K BCO mutants.

In conclusion, our results suggest that reactivity of BCO with regard to dioxygen is finely tuned by the interaction of E311 with the E475−R477 couple. The present findings on a flavooxidase support the notion that the presence of specific channels is a common theme in enzymes that act on dioxygen.

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15.60

CHARACTERIZATION OF OLIGOMERIC SPECIES ON THE AGGREGATION PATHWAY OF HUMAN LYSOZYME

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The aggregation process of wild type human lysozyme at pH 3.0 and 60 °C has been analyzed by characterizing a series of distinct species formed on the aggregation pathway, specifically the amyloidogenic monomeric precursor protein, the oligomeric soluble prefibrillar aggregates and the polimeric mature fibrils. Particular attention was focused on the analysis of the structural properties of prefibrillar aggregates, since recent studies have shown that the oligomers formed by lysozyme prior to the appearance of mature amyloid fibrils are toxic to living cells. Here, soluble oligomers of human lysozyme have been purified, and then analyzed by a range of techniques including binding to fluorescent probes such as Thioflavin T and ANS, Fourier-transform infrared spectroscopy and controlled proteolysis. Oligomers were isolated after 5 days of incubation of the protein: they appear as spherical particles with a diameter of 8-17 nm when observed by transmission electron microscopy. Unlike the monomeric protein, oligomers have solvent-exposed hydrophobic patches able to bind the fluorescent probe ANS. FTIR spectra are indicative of highly misfolded species when compared with monomeric lysozyme, with a prevalence of random structure, but with significant element of the β-sheet structure that is characteristic of the mature fibrils. Moreover, the oligomeric lysozyme aggregates are more susceptible to proteolysis with pepsin than both the monomeric protein and the mature fibrils, indicating further the lack of organized structure. In summary, this study shows that the soluble lysozyme oligomers are structurally flexible species, present at low concentration even during the initial phases of aggregation. Their dynamic and non-native conformational features are likely to confer on them the ability to interact inappropriately with a variety of cellular targets including cellular membranes generating the toxicity that is observed experimentally.

15.61

BETA-2 GLYCOPROTEIN-I INHIBITS THROMBIN-MEDIATED PLATELET AGGREGATION AND PAR-1 HYDROLYSIS

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$Beta_2$-glycoprotein I (β2-GpI) is the principal target of autoantibodies in the antiphospholipid syndrome (APS) and it is abundant in human plasma (0.1-0.2 mg/ml). Although the exact physiological function has not been fully elucidated, earlier studies suggest that β2-GpI may function as a mild anticoagulant by binding the phospholipids surface on platelets and thus inhibiting the initiation of the contact pathway[1]. More recent data provide evidence that β2-GpI exerts both anticoagulant and procoagulant activities through binding to factor XI, von Willebrand factor, and alpha-thrombin[2,3].

With the aim to better understand the role of Beta2-GpI in haemostasis, we have investigated the effect of physiological concentrations of this protein on thrombin-mediated hydrolysis of protease-activated receptors PAR-1 and PAR-4, and platelet aggregation.

Gel-filtered platelets (220000/µl) were activated in the presence of human alpha-thrombin (1 nM) and the change in transmittance at 350 nm was recorded in the presence of increasing concentrations of β2-GpI (0-2 µM). β2-GPI inhibited thrombin-induced platelet activation up to 60%, with an IC_{50} value of 0.34±0.03 µM. Cytofluorometric determination of uncleaved PAR-1, carried out with the anti-PAR1 MoAb Span-12 incubated with thrombin activated platelets, yielded an IC_{50} value of 0.35±0.05 µM, indicating that inhibition of platelet aggregation by Beta2-GpI is caused by the inhibitory activity that this protein exerts on thrombin-mediated PAR-1 hydrolysis in intact platelets.

Next, we tested the effect of Beta2-GpI on the hydrolysis of the synthetic peptides PAR-1(38-60) and PAR-4(44-66) by thrombin. Our data indicate that Beta2-GpI inhibits PAR-1(38-60) cleavage by thrombin in a dose-dependent manner, with a IC_{50} value of 0.35±0.04 µM. Conversely, the efficiency of PAR-4(44-66) hydrolysis by thrombin was not affected by β2-GpI. Finally, we have also shown that β2-GpI inhibits the hydrolysis by thrombin of the chromogenic substrate (D)-Phe-Pro-Arg-pNA, with an IC_{50} of 0.43±0.02 µM, whereas it is uneffective on the cleavage of (D)-Val-Leu-Arg-pNA, suggesting that binding of β2-GpI induce allosterically some modifications in the active site of thrombin.

Altogether, our results demonstrate that the proposed anticoagulant function of β2-GpI in haemostasis unfolds by inhibiting PAR-1-dependent platelet aggregation.

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15.62

STRUCTURAL EFFECTS OF MUTATING THE CATALYTIC ASP102 IN HUMAN ALPHA-THROMBIN

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Serine proteases play a pivotal role in many different, important biological processes, including digestion, inflammation, cell differentiation and blood coagulation. The results of protein engineering studies accumulated so far indicate that mutation of the amino acids at the active site of some of these enzymes dramatically decrease or even abolish their catalytic function, while the 3D structure of the resulting mutants remains essentially unchanged. Relevant examples in this field are the mutation of the catalytic Asp102 with Asn in trypsin and that of the catalytic Ser195 with Ala in human alpha-thrombin.

Recently, the Asp102Asn mutant (D102N) of human alpha-thrombin has been produced and its crystallographic structure solved at high resolution[1]. As expected, the hydrolytic activity is decreased by $16x10^3$ fold, compared to that of the wild-type enzyme. Quite surprisingly, the 3D structure is significantly compromised[1], especially in the Na+-binding site and in the primary specificity site, assuming a self-inhibited conformation, with the active site occluded. Importantly, it was proposed that the structural features of the D102N represent genuine properties of the inactive slow form (S*), captured during the crystallization process. According to a widely accepted model: $S^* \Leftrightarrow S \Leftrightarrow F$, thrombin exists in equilibrium between a low populated (< 1%) and inactive slow form (S*) and a highly populated and active slow form (S). The binding of Na+ converts the S form into the fully active fast form (F).

To discriminate as to whether the unique structure of the D102N mutant pertains to the inactive slow form (S*) of the wild type thrombin or it is the results of the mutation Asp102Asn, the overall conformational properties of the mutant in solution were investigated by using several different ligands/inhibitors mapping different regions of the enzyme. In particular, the specificity sites were probed by full-length hirudin, its N-terminal domain Hir(1-47) and p-aminobenzamidine, while exosite-1 was probed by the C-terminal hirudin tail Hir(48-64). Our results show that D102N binds Na+ with an affinity six-fold lower than that of the wild type thrombin and that D102N assumes a more closed conformation, with the recognition sites not readily accessible for ligand binding, in agreement with the crystal structure of D102N. However, D102N is not a dead enzyme, devoid of any ligand binding properties. In the presence of sub-saturating concentrations of Na+ and/or Hir(48-64) it can assume a more open conformation, resembling that of the wild-type enzyme. Our findings highlight the extraordinary structural plasticity of thrombin molecule and demonstrate that even a single isosteric amino acid replacement, like D102N, can cause dramatic and unpredictable changes in the resulting mutant structure.

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15.63

UNRAVELLING AF4 CROSS-TALK MAY GIVE HINTS INTO THE MOLECULAR PATHOGENESIS OF ACUTE LYMPHOBLASTIC LEUKAEMIA

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The AF4 gene is one of the most common MLL fusion partner in the chromosomal translocations that often causes acute lymphoblastic leukaemia (ALL) [1]. The AF4 gene is expressed in many types of tissues and mainly in the haematopoietic cells[2]. The AF4 protein is an ALF protein. It contains three regions that are conserved in the ALF family[1,3]: the N-terminal homology domain, the ALF domain, and the C-terminal homology domain. AF4 also contains a transactivation domain[4] and a nuclear localization signal[3]. The ALF homology domain seems to promote AF4 proteasomal degradation through its interaction with SIAH ubiquitin ligases (1). Although AF4 has been studied for over a decade, little is known about its function. A murine AF4 knockout model demonstrated that AF4 is required for normal lymphocyte development[5]. In a previous study of the functional role of AF4 in eukaryotic cells, we identified, using a proteomic strategy, about 60 proteins that interact with human AF4. The network of interactions strongly suggested that AF4 is a transcriptional activator. We verified, by co-immunoprecipitation and western blot, the molecular interaction between AF4 and four of the proteins we identified, namely, CDK9, MED7, MED23, and 14-3-3 θ. The first three are directly involved in RNA pol II transcription activation mechanisms. In particular, murine AF4 positively regulates CDK9 kinase, which directs Pol II transcriptional elongation[1]. CDK9 also phosphorylates and down-regulates AF4 activity[1]. The 14-3-3s are ubiquitous proteins that control the cellular localization of phosphorylated target proteins. 14-3-3 θ could be involved in the regulation of AF4 activity perhaps via nucleo-cytoplasmic shuttling. To evaluate how these interactions might influence the AF4 transactivation potential, we restricted the AF4 regions responsible for interaction with CDK9 and with 14-3-3 θ. We found that the 14-3-3 θ binding domain is located in an AF4 region usually conserved in MLL-AF4 chimeras. The CDK9 binding region is in the AF4 N-terminal domain, which is absent from the MLL-AF4 oncoprotein, thus depriving the latter of a potential downregulation site. Oncogenic MLL-AF4 chimeras usually lack this domain that could be involved in the down-regulation of the AF4 transactivation potential. Therefore, a gain-of-function pathogenic mechanism might explain the leukaemia phenotypes associated to the t(4;11)(q21;q23) translocations.

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15.64

A PROTEOMIC AND RT-QPCR INTEGRATED APPROACH FOR THE IDENTIFICATION AND CHARACTERIZATION OF DEFENSE PROTEINS EXPRESSED IN GERMINATING LUPIN SEEDS.

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Although the general mechanisms of seed germination have extensively been studied, some aspects are still obscure. For example, it has not been elucidated yet how the growing seedling contrasts possible pathogenic attacks. During the first steps of germination (*i.e.* 0 – 24 hours) *Lupinus albus*, a legume seed, releases various kinds of proteins outside the seeds. Aims of the present work were to characterize the polypeptides accumulated into the germinating medium by 2D electrophoresis and mass spectrometry and to study the expression levels of mRNAs coding for the most abundant proteins released from the seed during time course, carried out by RT-qPCR.

The full set of results obtained indicate that the release of polypeptides in the germination medium is not caused by a passive leakage from the seed but rather it is driven by a specific mechanism. The 2D electrophoretic patterns appeared relatively simple in term of composition, being formed by only about 60 spots. The majority of them (66%) have been identified by mass spectrometry, whereas for the remaining polypeptide chains no attribution has been possible. The characterized proteins all belong to protein functional classes directly or indirectly involved in defense mechanisms. The most abundant protein found in the germinating medium was chitinase (about 50% of all the proteins loaded on the electrophoretic gel). The other main proteins were conglutin γ (Cγ), a tetrameric glycoprotein of 200 kDa sharing a high similarity with tomato xylanase inhibitor and a number of peptides ascribable to vicilin, a seed storage protein, whose peptides generated from its proteolysis during germinations have been proved to have antimicrobial activity[1]. Subsequently, the expression levels of vicilin, chitinase and conglutin γ genes were analyzed by a newly set-up RT-qPCR using seed germinating in different conditions, including the use of elicitor substances known to be involved in the pathogenic-related protein biosynthesis. As result, vicilin was not expressed at germination, whereas chitinase gene was strongly activated in all conditions. Conglutin genes were also expressed, but under different stimuli.

By and large, this work demonstrates that a legume seed is able to actively defend itself with preexisting and newly synthesized proteins from pathogen attacks during the early step of germination.

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15.65

CACO-2 CELL MONOLAYERS TO STUDY THE TRANSPORT OF LUPIN CONGLUTIN γ ACROSS HUMAN INTESTINAL EPITHELIUM.

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Conglutin γ is a glycoprotein which constitutes about 6 % of lupin seed proteins. It shows insulin-binding capacity with a measured K_d of about 10^{-5} M [1]. Moreover, the protein was found to decrease plasma glucose concentration in glucose overload experiments on rats: it is not known whether the two events are related. Moreover, conglutin γ can be responsible of allergic reactions in sensitive subjects[2]. Therefore, it is crucial to investigate the metabolic fate of this dietary protein, including its resistance to proteolysis and intestinal absorption, as already shown for other orally-administered proteins, especially allergens and protein inhibitors, which were found intact in *sera* or tissues[3,4]. To this purpose, we used Caco-2 cell monolayers which are suitable *in vitro* models to mimic certain aspects of human intestinal epithelium.

Absorption experiments were carried out 21 days after initial cell seeding, using a well consolidated protocol[5]. The undigested and pepsin-trypsin-digested protein aliquots were added to the apical chamber and basolateral and apical *media* were collected after 2 hours. Cell monolayer integrity was monitored measuring the trans-epithelial electrical resistance during the whole experiment. The samples from apical and basolateral sides were analysed by SDS-PAGE and Western-Blot, using chemiluminescence enhancement.

These experiments showed that undigested conglutin γ can pass through the Caco-2 cell monolayer, being present both in the apical and also in the basolateral side. The digested protein could not be revealed, owing to its hydrolysis and loss of immunoreactivity. Parallel experiments on conglutin γ susceptibility to proteolysis with various enzymes showed that the protein is susceptible to hydrolysis uniquely upon destabilisation of its native conformation at very low pH values.

These results confirm the possibility of absorption of conglutin γ through Caco-2 cells in intact form, opening the way to further studies on the bioactivity of this protein.

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15.66

STRUCTURAL FEATURES OF TREHALOSE-MALTOSE BINDING PROTEIN FROM THERMUS THERMOPHILUS: STRATEGIES OF THERMAL ADAPTATION AT HIGH TEMPERATURES.

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The trehalose/maltose-binding protein (MalE1) is one component of trehalose and maltose uptake system in the thermophilic organism *T. thermophilus*. MalE1 is a monomeric 48 kDa protein belonging to the class of sugar-binding proteins. Proteins isolated from thermophilic microorganisms are particularly heat-resistant. By spectroscopic techniques is possible to follow the thermal denaturation process[1] when these proteins unfold at temperatures below or close to 100°C, but in the case they have a T_m above 100°C is necessary to study the unfolding process in the presence of high pressure or destabilizing agents. MalE1 belongs to the latter case, its great thermostability was compromised only in the presence of SDS. In this work we used Fourier-infrared (FT-IR) spectroscopy and *in-silico* methodologies for investigating the structural stability properties of MalE1. The protein was studied at neutral p^2H in the absence and in the presence of maltose or SDS as well as at p^2H 9.8. Infrared spectra analysis indicated that MalE1 is a protein predominantly in α-helix conformation with a minor content of β-structure. This finding is confirmed by the model obtained by homology modelling method. At neutral p^2H the protein is extremely thermostable. In particular, the protein preserves its α-helical conformation even when maintained for prolonged time at 99.5°C. The high thermostability of the α-helices is maintained also at p^2H 9.8 and it is even higher at p^2H 7.5 in the presence of maltose. On the other hand, the data showed that at high temperatures β-sheet undergo some structural rearrangement, phenomenon that resulted reversible when the temperature was lowered. The presence of SDS modified slightly the secondary structure of MalE1 and decreased the protein thermostability. In the presence of the detergent a temperature-dependent loss of α-helices and β-sheet took place but β-sheet rearrangement was not observed. The infrared data, corroborated by the inspection of the 3D structure of MalE1, suggest that hydrophobic interactions may play an important role in the high thermostability of MalE1.

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15.67

THE DEGREE OF STRUCTURAL PROTECTION AT THE EDGE β-STRANDS DETERMINES THE PATHWAY OF AMYLOID FORMATION IN GLOBULAR PROTEINS.

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The assembly of proteins into highly organized fibrillar aggregates is a key process in biology[1], biotechnology[2] and human disease[3]. It has been shown that proteins retain a small, yet significant propensity to aggregate when they are folded into compact globular structures and this may be physiologically relevant, particularly when considering that proteins spend most of their lifespan into such compact states. Proteins from the acylphosphatase-like structural family have been shown to aggregate via different mechanisms, with some members forming native-like aggregates as a first step of their aggregation process and others requiring unfolding as a first necessary step. The acylphosphatase from the archeobacterium *Sulfolobus solfataricus* (Sso AcP) has been demonstrated to aggregate *via* native-like oligomers[4]. These aggregates retain enzymatic activity and do not show typical β-amyloid features, such as extensive β-sheet structure, thioflavin T or Congo Red binding. The enzymatic activity is subsequently lost when the aggregates undergo a structural reorganization that convert them into amyloid-like protofibrils[4]. Here we show that assembly of folded Sso AcP molecules into native-like aggregates is prevented by single-point mutations that introduce structural protections within one of the most flexible region of the protein, the peripheral edge β-strand-4. The resulting mutants do not form native-like aggregates, but can still form thioflavin T-binding and β-structured protofibrils, albeit more slowly than the wild-type protein. The kinetic data of folding, unfolding and aggregation show that formation of the protofibrils proceeds via an alternative mechanism that is independent of the transient formation of native-like aggregates.

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15.68

PROTEOMIC APPROACH TO ANALYSE PLASMA PROTEIN CHANGES EXERCISE-INDUCED IN HORSES: A PRELIMINARY STUDY.

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The purpose of this study was to investigate the possible utilisation of classic proteomic approach for studying the plasma protein changes exercise-induced in race horses. It is well known that physical exercise can cause qualitative and quantitative modifications of plasma protein content due to hormonal changes, muscle damage, immune system activation, oxidative stress and metabolic adaptation.

The proteomic approach enables both the large-scale identification of proteins and the comparison of protein levels and could be an important tool to investigate the physiological and pathological processes induced by physical stress. Plasma is a rich collection of information about factors occurring at the same time and it represents the most convenient sample to collect and investigate in vivo exercising athletes. Numerous biomedical studies have demonstrated that plasma protein levels can be used for diagnosis and prognosis of diseases and can help to define biochemical mechanisms not yet completely understood[1].

For this study we used seven horses which had taken part in endurance race. Plasma samples were collected at rest, immediately after race, 24 hours after ad 48 hours after and stored at -80 °C in anti-protease cocktail until analysis.

To find out the more convenient procedure, plasma were analysed in triplicate both individually (from each horse at each time) and by pooling plasma from all horses at each time.

15 µl of plasma were treated in 2% Chaps, 2,3% DTE at 95°C for 5 min and then diluited in rehydratation buffer (8 M urea, 2 M thiourea, 2% CHAPS, 30 mM Tris, 100 mM DTT and 0,8% anfolyte). IPG strip, 17 cm long, pH 3–10 and pH 4-7 (Bio-Rad, Hercules, CA, USA) were rehydrated overnight at 50 V, and focused in Protean IEF Cell (Bio-Rad). The Second dimension was on a 9/16% poly-acrylamide gradient after reduction (5 mM TBP) and alkylation (2.5% IAA) in rehydratation buffer (375mM TRIS-HCl pH 8.8, 6 M urea, 2% SDS, 20% glicerol).

Gels were stained with colloidal Coomassie blue G-250 and to imaging using the Bio-Rad GS-800™ calibrated densitometer. The comparative analysis was performed by with PD-QUEST (BioRad, Hercules, CA).

2D obtained from plasma pooled were analysed and matched in the same matchset. While a different matchset were performed to analyse gels from individually sample. With both the procedures we found significant differences of spot intensity among all times tested. At the moment we are not able to discuss the mining of these variations because MS identification of the spots are still in progress. This preliminary result suggest the possible use of 2D electrophoresis to identify modification of plasma protein pattern induced by physical exercise, despite of the complessity of biological matrix used

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15.69

ANALYSIS OF HORSE SYNOVIAL FLUID BY TWO-DIMENSIONAL GEL ELECTROPHORESIS: A PILOT STUDY.

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Synovial fluid (SF) reflects the patho-physiological condition affecting joints because it represents a dynamic reservoir for protein originating from synovial tissue and cartilage. We focused our attention on equine Osteochondrosis (OC), a pathology with multifactorial ethiology[1,2]. The specific alteration of chondrocyte metabolism as well as ultrastructural modification of extra-cellular matrix that can cause OC have not been yet completely described. The aim of this study was to analyse, by using the proteomic approach, SF collected from normal and OC affected joints of horses in order to define both the protein reference map of horse synovial fluid and to characterise the differential protein expression during disease. Proteomic analysis of SF may provide a minimally invasive opportunity to analyse disease-related proteins and might become a valuable tool in diagnosing and monitoring different articular diseases.

For this pilot study we used horses admitted to the Veterinary Teaching Hospital of Perugia. SF samples were obtained from hock and fetlock joints with OC after clinical evaluation. Synovial proteins diluited in rehydratation buffer (8 M urea, 2 M thiourea, 2-4% CHAPS, 40 mM Tris, 100 mM DTT and 0,8% anfolyte) were focused on IPG strips, 17cm long, pH 3–10 and pH 4-7, in Protean IEF Cell. The Second dimension was performed on 9-16% poly-acrylamide gradient gel in Dalt-six system (Amersham Pharmacia Biotech) after reduction with 5 mM TBP and alkylation with 2.5% Iodoacetamide in rehydratation buffer (375 mM TRIS-HCl pH 8.8, 6 M urea, 2% SDS, 20% glicerol). Gels were stained with colloidal Coomassie blue G-250 and to imaging using the Bio-Rad GS-800™ calibrated densitometer. The comparative analysis was performed by with PD QUEST (BioRad, Hercules, CA).

At first, we optimised 2D protocols in order to obtain the better resolution of protein spots by varying experimental conditions including protein loading, pH range, % chaps, and precipitation steps. The pI of most proteins ranges between pH 4 and pH 7. As a consequence, this range was chosen for comparative analysis between maps obtained from OC and control joint. Not significant differences were found by comparing 2D protein profile of hock and fetlock. However, by comparing 2-D gel obtained from OC and control SF we found differentially expressed proteins that are at the moment under MS investigation. Although, results are still in progress they could improve the diagnosis and the understanding of osteoarticular diseases.

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15.70

STRUCTURAL AND FUNCTIONAL PROPERTIES OF "ZERO-LENGTH" RIBONUCLEASE A OLIGOMERS

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Many ribonucleases were found to be endowed with remarkable biological activities, among which particularly interesting is an *in vitro* and *in vivo* cytotoxic action against tumor cells[1].

This is not the case for native monomeric RNase A, which is devoid of any biological action. On the contrary, RNase A aggregated oligomers obtained through 3D domain swapping after lyophilization of 40% acetic acid solutions of the enzyme, showed to be able to degrade double-stranded RNA and to be cytotoxic[2].

The most important limit for the practical use of the domain swapped RNase A oligomeric species is their metastability.

To avoid this limitation the new strategy proposed by Simons *et al.* [3] was used to oligomerize the enzyme: RNase A, dissolved in ddH_2O and lyophilized, was then maintained under high vacuum at 85 °C for four-six days. This treatment allows the protein to form a new inter-chain peptide bond through an acidic and a basic residue, with the loss of a water molecule.

In this way dimers, but also trimers and higher-order oligomers can be formed. They result to be covalently linked, but without the use of crosslinkers, and therefore without eterologous spacer(s) present in the reaction products. For this reason these species can be called 'zero-length' RNase A oligomers.

The species produced maintain their catalytic activity against ssRNA and acquire a significant activity against dsRNA, similar to the activity shown in the past by the omologous 3D domain swapped RNase A oligomers[2].

The various 'zero-length' oligomers have been chromatographically purified using different techniques and each species showed to be composed by not a single, but different isoforms, contrarily to what was asserted by Simons *et al.* [3]. This result was confirmed by several methods: electrophoresis under non denaturing conditions, measurements of the amino-groups remaining in the dimer(s) after the reaction, and site-directed mutagenesis.

Investigations on the biological (antitumor) activity that the 'zero-length' RNase A oligomers may have acquired are presently under study.

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15.71

ON THE MECHANISM OF L-LACTATE OXIDATION CATALYZED BY FLAVOCYTOCHROME B2: A COMBINED FIRST PRINCIPLE MOLECULAR DYNAMICS AND SITE DIRECTED MUTAGENESIS STUDY.

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Flavocytochrome b_2 (Fcb2) catalyzes the oxidation of L-lactate (Lac) to pyruvate (Pyr) in its C-terminal flavodehydrogenase (FDH) domain (residues 100-486). The N-terminal heme b_2 domain (residues 1-99) mediates electron transfer from FMN bound to the FDH domain to Cyt c. The FDH domain, a $(\beta/\alpha)_8$ barrel, is the prototype of a family of α-hydroxy acid dehydrogenases/ oxidases. In spite of decades of work by several top laboratories world-wide, for none of these enzymes the mechanism of α-hydroxy acid oxidation has been clarified. To contribute to this issue, we have carried out first principles quantum mechanical molecular dynamics simulations on models of the Fcb2 active site. A stable active site-Lac model complex was only obtained with Lac bound as per a hydride transfer (HT) mechanism, with Lac αH pointing toward FMN N(5) position and the αOH hydrogen H-bonded to H373 N_ε. The subsequent simulation of the reaction complemented and extended the available information on the Fcb2-catalyzed reaction yielding an atomistic level description of the process. The simulations also revealed an unexpected role of a crystallographic water (Wat609) and of a third shell residue (S371), both conserved in the Fcb2 family of enzymes. Wat609 forms a S371-Wat609-D282-H373 H-bonded chain and appeared to modulate the acid-base properties of H373. This residue initiates substrate oxidation by abstracting the Lac αOH proton and shuttles between the neutral and the positively charged forms in the oxidized and reduced enzyme, respectively. To test the hypothesis, we produced the S371A variants of the full-length Fcb2 and of its FDH domain. S371A-FDH was produced in *E. coli* as the non reconstitutable apoprotein, while S371A-Fcb2 incorporated only 0.3 mol FMN per mol indicating an unexpected structural role of S371. The S371A substitution caused a 20-fold decrease of both V and V/K_{lac} entirely attributable to a decrease of the rate of the enzyme reductive half reaction (k_{red}), as shown by the analysis of the primary kinetic isotope effects on Lac oxidation. It had no effect on the pH dependence of V and V/K_{Lac}, but it caused a 10-fold increase of the K_d of the FMN-N(5)/sulfite adduct. The data support the hypothesis that the S371A substitution prevents the positioning of Wat609, thus lowering the proton affinity of H373. As a result the rate of Lac oxidation decreases due to: (i) the lower tendency of H373 to abstract the substrate proton and initiate the reaction, and (ii) the lower stabilization of the reduced anionic FMN cofactor by the protonated H373. A positively charged H373 is also required to stabilize the FMN-N(5)-sulfite complex, mimicking the reduced form, explaining the increased K_d of the sulfite complex in S371A-Fcb2. Beside providing information on the Fcb2 class of enzymes, this study shows the power of combining computational and experimental approaches to gain insight on how enzymes work.

15.72

FUNCTIONAL CHARACTERIZATION OF A PSYCHROPHILIC THIOREDOXIN SYSTEM

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The preservation of the redox state of intracellular proteins is mainly regulated by the thioredoxin system which is composed by two proteins, named thioredoxin (Trx) and thioredoxin reductase (TrxR), respectively. Indeed, the reduction of oxidised protein targets by the thioredoxin system involves the reversible and alternate oxidation/reduction of a conserved disulphide bridge present in both components of this enzymatic system. In this abstract we report the purification procedure and the biochemical characterization of Trx and TrxR isolated from the Antarctic psychrophilic eubacterium *Pseudoalteromonas haloplanktis* (*Ph*).

*Ph*TrxR and *Ph*Trx were isolated from *P. haloplanktis* cells collected after 3 days of growth in LB-broth at 4°C; the two proteins were purified from the cell homogenate by conventional chromatographic steps. The *Ph*TrxR activity was determined by the DTNB reduction method in the presence of NADPH as electrons donor, whereas the *Ph*Trx was identified by a nephelometric assay, using DTT as electrons donor and human insulin as protein substrate.

The flavo-enzyme *Ph*TrxR is organised as a homodimer, the molecular mass of the subunit being 35 kDa; vice versa, *Ph*Trx is a monomer with a molecular mass of 12 kDa.

The functional characterisation of the psychrophilic thioredoxin system included the study of the effect of temperature on the activity and stability of both proteins. In particular, *Ph*TrxR showed the maximum value of k_{cat} at 30°C; beyond this temperature, the enzymic activity decreased. In the 5-30°C temperature interval the energy of activation (E_a) value of this reaction was calculated as 31 kJ/mol. A thermal denaturation profile of *Ph*TrxR obtained to fluorescence melting measurements revealed a T_m of 55°C, a value quite high considering the psychrophilic origin of this enzyme. The effect of temperature on the *Ph*Trx activity was maximum at 25°C; in the 0-25°C interval the E_a of the reaction was calculated as 53 kJ/mol. Surprisingly, this protein was found very resistant to heat inactivation, the $t_{1/2}$ at 95°C being 263 min. These overall results support the hypothesis that the structures of *Ph*TrxR and *Ph*Trx are characterised by a localized rather than a global flexibility, a typical property of the psychrophilic enzymes. An *in vitro* reconstituted *Ph*Trx-*Ph*TrxR system showed a lower E_a of the redox reaction catalysed by *Ph*TrxR, hence making thermodynamically more favourable electrons transfer.

The effect of increasing concentration of different monovalent cations on *Ph*TrxR activity has also been investigated. Among the considered cations, the presence of 0.5 M Na^+ provoked a 6 fold increase of the activity with respect the control. This apparent halophilicity of *Ph*TrxR is common to other enzymes isolated from psychrophilic sources.

16.1

RETINOIC ACID BIOSYNTHESIS IS INHIBITED BY ESTROGEN

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The striking influence of retinoic acid on differentiation of epithelial cells and its efficacy to suppress cell proliferation[1,2] prompted studies aimed to clarify its molecular mechanisms and its homeostasis in normal and malignant cells. Several dehydrogenases are involved in the conversion of retinol to retinoic acid. Furthermore, retinoic acid deficiency has been demonstrated in tumor epithelial cells and ascribed to the lack of cellular retinol binding protein (CRBP) and/or to the inactivity of retinol/retinaldehyde dehydrogenase enzyme system[3,4].

Our studies on the biosynthesis of retinoic acid in normal mammary epithelial cells (HMEC) have revealed the crucial role of the xanthine dehydrogenase (XDH) enzyme in the conversion of retinol/retinaldehyde to retinoic acid and the necessary participation of CRBP in this process. In MCF7 and MDA-MB231 cells, both lacking CRBP, retinol oxidation to retinoic acid by XDH could not be observed. However, XDH catalyzes retinaldehyde conversion to retinoic acid, albeit less actively than in HMEC [5].

Estrogens affected both XDH expression and activity in both normal and tumor mammary epithelial cells. Estradiol, added to MCF7 and MDA-MB231 cell cultures at concentrations varying from 10 pM to 100 nM, exerted in fact a marked inhibitory effect on the XDH-driven retinaldehyde oxidation, with the enzyme activity dropping to 14-66% of that observed in control cell cultures. The XDH protein content was also reduced to 40% by 100 nM estradiol.

This evidence suggests that estrogens have a profound negative on retinol-retinoic acid homeostasis and consequently are primary regulators of cell differentiation.

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16.2

REDOX REGULATION OF ANOIKIS: REACTIVE OXYGEN SPECIES AS ESSENTIAL MEDIATORS OF CELL SURVIVAL

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Proper attachment to the extracellular matrix (ECM) is essential for ensuring survival of adherent non-trasformed cells. The loss of integrin-mediated cell-ECM contact results in an apoptotic process termed *anoikis*. However, the underlying mechanisms involved in regulation of cell survival are not fully understood. We demonstrate that in epithelial cells reactive oxygen species (ROS), produced through the involvement of the small GTPase Rac-1 upon integrin engagement, exert a mandatory role in transducing a pro-survival signal that ensures cells to escape from *anoikis*. In particular, we show that ROS are responsible for the redox-mediated activation of the Src kinase that *trans*-phosphorylates EGFR in a ligand-independent manner, culminating in the ERK- and Akt-induced degradation of the pro-apoptotic protein Bim. These evidence underline a fundamental role of ROS-mediated Src regulation in ensuring *anoikis* protection. Furthermore, we investigated the possible involvement of ROS in anchorage-independent growth and in anoikis resistance of metastatic cancer cells. By the comparison between untransformed prostate epithelial cells (RWPE-1) and metastatic prostate carcinoma cells (PC3), we observed that PC3, which exhibit higher amount of intracellular ROS with respect to untrasformed cells, are resistant to anoikis and lack suicidal pathways. As a consequence of ROS increase, Src is constitutively oxidized/activated in metastatic cells, granting for a constitutive Src-dependent *trans*-phosphorylation of EGFR and activation of pro-survival pathways. Antioxidant treatment of PC3 cells or transfection with the dominant negative form of Rac-1 or with redox-insensitive mutants of Src, completely abolish the *trans*-phosphorylation of EGFR and the resistance to anoikis, thus restoring the apoptogenic stimuli. Hence, our data highlight the crucial role of the Rac-dependent redox signalling in ensuring tumour cells resistance to anoikis, proposing the ROS-mediated Src activation as an essential step to promote cancer cell survival.

16.3

S1P1 RECEPTOR NEGATIVELY REGULATES PDGF-DEPENDENT PROLIFERATION OF C2C12 MYOBLASTS.

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It is becoming increasingly apparent that numerous growth factors acting via receptor tyrosine kinases require regulation of sphingosine 1-phosphate (S1P) metabolism and signalling to accomplish their biological response[1]. Actually S1P was initially described as a bioactive sphingolipid generated intracellularly in response to PDGF, implicated in its mitogenic signalling[2]. More recently, S1P1 receptor has been found critically involved in the chemotactic response elicited in vascular smooth muscle cells as well as in endothelial cells[3]. However, although PDGF is known to stimulate myoblast proliferation little information is presently available on its molecular mechanism of action. To fill at least in part this gap, in this study the role of S1P1 in the mitogenic action of PDGF in C2C12 cells has been examined.

Unexpectedly, when S1P1, but not S1P2 or S1P3, expression was down-regulated by antisense oligonucleotide administration the stimulation of radioactive thymidine incorporation induced by 30 ng/ml PDGF was enhanced, suggesting that in these cells the cross-talk between PDGF and S1P1 attenuates the mitogenic signalling downstream of PDGF receptor. This hypothesis was supported by the potentiation of PDGF mitogenic response detected after the specific silencing of S1P1, and by the selective inhibition of S1P1 with (R)-3-Amino-(3-hexylphenylamino)-4-oxobutylphosphonic acid. In addition, in line with these findings, the overexpression of S1P1 was capable of strongly attenuating the PDGF-stimulated DNA synthesis.

Since the cross-talk between PDGF receptors and S1P1 not necessarily implicates the growth factor-directed S1P formation, we also examined the effect of PDGF on the activity of sphingosine kinase (SK), which catalyzes the synthesis of S1P. Interestingly, the enzymatic activity was found to be rapidly enhanced by PDGF. Moreover, selective inhibition of SK by 2-(p-hydroxyanilino)-4-(p-chlorophenyl)thiazole enhanced the mitogenic action exerted by PDGF. These results are in favour of a unique inhibitory role of SK/S1P1 axis in the mitogenic response to PDGF.

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16.4

TARGETING THE X-LINKED INHIBITOR OF APOPTOSIS PROTEIN (XIAP) THROUGH 4-SUBSTITUTED AZABICYCLO[5.3.0]ALKANE SMAC-MIMETICS. STRUCTURE, ACTIVITY AND RECOGNITION PRINCIPLES.

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The X-linked Inhibitor of Apoptosis Protein (XIAP) is overexpressed in several malignant cells, where it prevents apoptosis by binding to and blocking the activation of caspases-3, -7, and -9[1]. Human XIAP (479 residues) is composed of three tandem-repeated BIR domains (BIR1-3), and by a C-terminal RING domain[1]. Smac-DIABLO, the natural antagonist of XIAP, binds through its N-terminal sequence AVPI to the same surface groove, in the BIR domains, that binds caspases[3,4]. Synthetic compounds, mimicking such tetrapeptide motif, effectively block the interaction between IAP and active caspases, thus triggering apoptosis[2]. Peptidomimetics based on an azabicyclo[x.y.0]alkane scaffolds, have been shown to bind the BIR3 domain of XIAP with μM to nM affinities, thus presenting interesting features for drug lead optimization[3]. Here we report a study on three newly synthesized Smac-mimetics, which have been characterized in their complexes with XIAP BIR3 domain through X-ray crystallography and molecular modelling/docking simulations. Based on analysis of the crystal structures, we show that specific substitutions at the 4-position of the azabicyclo[5.3.0]alkane scaffold results in sizeable effects on the peptidomimetic/BIR3 domain affinity. By means of functional, biophysical and simulative approaches we also propose that the same Smac-mimetics can bind XIAP BIR2 domain at a location structurally related to the BIR3 domain AVPI binding groove. Details of the XIAP/Smac-mimetic recognition principles highlighted by this study are discussed at the light of the drug-like profile of the three (potentially pro-apoptotic) compounds developed, that show improved performance in ADMET tests.

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16.5

TRANSFORMING GROWTH FACTOR-β ELICITS MYOFIBROBLASTIC DIFFERENTIATION OF MYOBLASTS VIA SPHINGOSINE KINASE/ SPHINGOSINE 1-PHOSPHATE AXIS.

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Transforming growth factor-β (TGFβ) is a cytokine endowed with multiple biological effects. In skeletal myoblasts, TGFβ inhibits myogenic differentiation[1] while it promotes their transdifferentiation into myofibroblasts[2], thereby favouring muscle fibrogenesis. The molecular mechanisms by which TGFβ evokes its biological response are quite complex. It has been demonstrated that the canonical SMAD-dependent signalling pathway is implicated in the negative regulation of myogenic transcription factors such as MyoD and MEF2 [1], however the signalling downstream of TGFβ responsible for differentiation of myogenic cells into myofibroblasts is largely unknown. In the last ten years the bioactive sphingolipid sphingosine 1-phosphate (S1P) has received increasing attention owing to its multiple biological effects, the complexity of its signalling, mediated by at least five GPCR and the discovery that the regulation of its metabolism is exploited by a number of growth factors and cytokines.

Our previous studies performed in C2C12 mouse myoblasts have established that S1P accelerates myogenic differentiation[3]. Moreover, sphingosine kinase-1 (SphK1), which catalyzes S1P biosynthesis, is required for myogenic differentiation triggered by the shift of confluent cells to low-serum medium[4], indicating that SphK/S1P axis plays a key role in the regulation of biological response of these cells.

Recent studies have established the occurrence of a bidirectional cross-talk between the TGFβ-S1P regulated signalling pathways).

To gain insights into the mechanism of action of TGFβ in skeletal muscle cells here the effect of TGFβ on SphK has been examined together with its possible implication in the biological action of the cytokine.

Our results show that TGFβ exerts a biphasic effect on SphK activity in C2C12 cells, inhibiting the enzymatic activity within the first 4 h of incubation but enhancing it at more prolonged times of incubation (18-72 h). TGFβ up-regulates mRNA and protein content of SphK1, without influencing SphK subcellular localization. Moreover, TGFβ-dependent regulation of SphK1 appears to be implicated in the pro-fibrotic effect of the cytokine, since pharmacological inhibition of SphK1 or its down-regulation by specific siRNA, attenuated the biological response to TGFβ. Finally, the pro-fibrotic effect of TGFβ relied on S1P3 receptor engagement, being it mimicked by FTY720P, which does not activate S1P2 and blunted by siRNA specific for S1P3.

This study demonstrates that SphK/S1P axis is exploited by TGFβ to transform skeletal C2C12 myoblasts into myofibroblasts.

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16.6

PRO-APOPTOTIC AND CYTOSTATIC ACTIVITY OF NATURALLY OCCURRING CARDENOLIDES.

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Cardenolides are steroid glycosides used clinically for their cardiotonic effects that act by binding to the Na^+/K^+-ATPase. Na^+/K^+ ATPase has been shown to possess an interesting role as signal transducer, demonstrating its involvement also in normal and cancer cell proliferation and other biological processes[1]. Thus, besides their cardiotonic property, cardiac glycosides have been shown to exert potential antitumor activity[2]. Here, we evaluated the anti-tumor potential of four cardiotonic steroids, isolated from *Periploca graeca* L.[3]. Two are 17β-cardenolides (compounds 4 and 5), one is a 17α-cardenolide (6), and another one is the aglycon of a 17β-cardenolide (2). All tested cardenolides inhibited in a nanomolar range of doses U937 (monocytic leukaemia) and PC3 (prostate adeno carcinoma) cell growth. The antiproliferative potency ascending order was 4> 5> 6> 2, compound 4 being more effective than ouabain, the cardenolide used as a positive control. The antiproliferative potency of test compounds paralleled, at least in part, their capability to inhibit porcine cerebral cortex Na^+/K^+-ATPase. The differences in the kinetics of inhibition of U937 and PC3 cell growth, suggested that cardenolides evoked different responses in these two cell lines. Accordingly, 24h following treatment, PC3 cells underwent apoptosis (30% hypodiploid cells vs 3% control cells), whereas U937 cells arrested in G2/M. Apoptotic cell death was detectable in U937 cells only after 72 h. The apoptotic mode of cell death in PC3 was confirmed by the occurrence of phosphatidylserine externalization, a hallmark of early apoptosis, loss of mitochondrial potential, and cytochrome *c* release. Apoptosis was caspase-dependent as the pan-caspase inhibitor, Z-VAD fmk, reverted almost completely cardenolide-induced PC3 cell death. Moreover, proteolytic cleavage of caspase 3 (assessed WB analysis) also occurred.

Tumor necrosis factor (TNF)–related apoptosis-inducing ligand (TRAIL) is a promising therapeutic agent that induces apoptosis selectively in cancer cells[4]. Unfortunately, several cancer cells, including U937, are resistant to TRAIL. Interestingly, we found that compound 4 sensitized U937 cells to TRAIL-induced apoptosis. The effect is largely mediated by up-regulation of the death receptor 5 (DR5) at both RNA and protein levels. However, the possibility that other mechanisms might also contribute to the observed cardenolide-induced sensitization to TRAIL should be considered. In particular, as we found that compound 4 induced changes of membrane fluidity, cardenolides might facilitate DR5 clustering and subsequent death-inducing signaling complex formation.

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16.7

CAMPTOTHECIN INDUCES S-PHASE ARREST AND APOPTOSIS IN MG63 OSTEOSARCOMA CELLS

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Osteosarcoma is an extremely aggressive neoplasm that commonly occurs in children and adolescents. Most of osteosarcoma tumors display a broad range of genetic and molecular alterations involving disregulation of the major signaling pathways. Mutations in tumor suppressor genes as p53 and/or RB, are usually detected in the majority of osteosarcoma cells and further molecular alterations are frequently discovered. In particular, MG63 osteosarcoma cell line is characterized by loss of Tp53 expression [1] and by the presence of hyperphosphorylated/inactive pRb form [2], which is responsible for both the loss of the G1/S checkpoint and the altered cell proliferation. Here, we evaluated the effects of camptothecin (CPT) in MG63 human osteosarcoma cells with the aim of contributing to the understanding of the basic biology of osteosarcoma and to devise more targeted therapeutic strategies. CPT is a genototoxic drug that directly inhibits topoisomerase I by inducing double strand breaks in chromosomal DNA, with the maximum toxicity during the S phase of the cell cycle. Here, we have shown by flow cytometric analysis that 200 nM CPT induced a progressive cell cycle arrest in S phase. This effect appeared 6 h after the treatment and lasted approximately 24 h. No entry in G2-M phase was observed as confirmed by the inhibition of BrdU incorporation into DNA. Moreover, after 48 h of treatment, a significant percentage of cells accumulated in subG0/G1 phase suggesting apoptotic events. Apoptotic cell death was confirmed by flow cytometric analysis of annexin V/ PI staining. Western blotting analysis of cyclins A and E and of pRb phosphorylation status showed a strong increase of these proteins after 6 h treatment, followed by the progressive lowering to their basal levels after 24-48 h of treatment. At the same time, we observed a potent increase in both E2F-1 level and its phosphorylated form, accompanied by high levels of p73. These results suggest a scenario where DNA damage induced by CPT in MG63 cells, blocks DNA replication fork progression, thus activating S-phase checkpoint and thereafter inducing apoptosis. Although the exact mechanism responsible for this S phase checkpoint activation followed by apoptosis has to be clearly defined, we hypothesize that the checkpoint kinases (CHK1-2; ATM/ATR) could be primarily involved and that they could act by activating p73 and E2F-1 phosphorylation.

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16.8

PROLONGED TREATMENT OF HUMAN OSTEOSARCOMA MG63 CELLS WITH 3AB INDUCES THE PRODUCTION OF TUMOR INITIATING CELLS

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Recent evidences suggest that a number of human malignancies contain an organized cell hierarchy where a minor population of Tumor Initiating Cells (TICs) is responsible for tumor growth[1]. These TICs are endowed with high self-renewing capacity, propensity to differentiate into actively proliferating tumor cells and high resistance to chemotherapy. Ours previous studies have demonstrated that prolonged exposure of human osteosarcoma MG63 cells to 3AB, a canonical PARP inhibitor, induces the appearence of a cell population, characterized by both the $CD133^+$ expression and a marked reduction in cell volume, containing a potentially stem-like phenotype.

In this study we indicate these cells as TICs and we show that, respect to the wild type MG63 cells, TICs possess a greater ability of self-renewing and a higher proliferative potential.

To compare the ability of MG63 cells and TICs to generate spherical clones and to self-renew, both cell types were grown in the 1% methylcellulose medium in ultra low attachment plates and after 6 and 12 days of culturing, the colonies were quantitated by inverted phase contrast microscopy. We have demonstrated that both cell types were capable of forming spheres, but those formed by TICs were more numerous and larger than those formed by MG63 cells. Growth curves have shown that TICs possess a higher proliferative output than MG63. Moreover, analysis of cell cycle regulators by western-blotting assay have shown that, respect to MG63 cells, TICs are characterized by a greater level of inactive/hyperphosphorylated pRb form, a higher level of E2F1, cyclins D1, E, A, and B1 and a much higher activity of CDC2 kinase. In conclusion, we have demonstrated that TICs possess some characteristics of stem cells, which include the ability for self-renewal and high proliferative potential.

1. Liu J.C. et al (2007) *Cancer Res.* 67, 8671-81.

16.9

THE MECHANISM BY WHICH HISTONE DEACETYLASE INHIBITORS SENSITIZE HEPATOMA AND COLON CANCER CELLS TO TRAIL-INDUCED APOPTOSIS

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Introduction: Histone deacetylase inhibitors (HDACIs) represent a new and promising class of anti-tumor drugs that influence gene expression by enhancing acetylation of histones in specific chromatin domains and induce apoptosis in several tumor cell types.

It has been recently shown that HDACIs are capable of sensitizing tumor cells that display resistance to TRAIL-mediated apoptosis. However, the molecular mechanisms that account for this sensitization have not been fully elucidated.

Our study provides evidence that human hepatoma and colon cancer cells can be potently sensitized by HDACIs to TRAIL-induced apoptosis through increasing the expression of the death receptor DR5 together with downregulation of some antiapoptotic factors such as c-Flip, NF-kB and Akt.

Materials and methods: Apoptosis was assessed either by flow cytometry or AO/EB staining. DISC precipitation was performed using biotin-tagged recombinant TRAIL. Knockdown of c-Flip was obtained using a specific siRNA. Protein levels were evaluated by western blot analysis. The DNA binding of NF-kB subunits was assessed using the Trans-AM NF-kB ELISA assay.

Results: Our study shows that both human hepatoma HepG2 and colon cancer HT-29 cells are resistant to the effect of soluble recombinant TRAIL. Low doses of different types of HDACIs (1 μM SAHA, 0.2 μM ITF2357 and 10 nM TSA) potently sensitized both the cell lines to TRAIL-induced apoptosis resulting in a marked synergistic effect.

The sensitization determined by HDACIs, in particular by 1μM SAHA, seems to be mainly correlated with the increasing effect in the expression of TRAIL receptor DR5 and down-regulation of c-Flip, an inhibitor of caspase-8 activity.

Analysis of the TRAIL death-inducing signalling complex (DISC) confirmed that SAHA provokes up-regulation of DR5 together with the recruitment and activation of caspase-8.

Treatment with combinations SAHA/TRAIL decreased the level of NF-kB. Consequently, also the levels of a number of antiapoptotic factors, such as IAP-2, XIAP, survivin and Bcl-X_L, declined because their expression is stimulated by NF-kB.

Combined treatment also caused downregulation of Akt and inhibition of Bad phosphorylation.

These effects led to activation of both caspase-9 and caspase-3 with the consequent cleavage of NF-kB and Akt and induction of cell death.

Taken together, our data suggest that combination HDACIs/TRAIL might represent a novel strategy to target defensive systems in tumor cells and open new perspectives in tumor therapy.

16.10

THE SYNTHETIC CANNABINOID WIN55,212-2 SYNERGIZES WITH THE DEATH RECEPTOR LIGAND TRAIL TO INDUCE APOPTOTIC EFFECTS IN HEPG2 HEPATOMA CELLS.

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TNF-related apoptosis-inducing ligand (TRAIL), a member of the TNF superfamily, is of special interest for cancer therapy since this factor has been shown to predominantly kill tumor cells, while it seems to be almost ineffective in normal cells. However, many cancer cells fail to undergo apoptosis in response to TRAIL treatment, thus combination therapies have been developed for tumor-cell sensitization towards TRAIL.

Cannabinoids, the active constituents of *Cannabis sativa,* are known to exert a wide range of central and peripheral effects. In recent years, numerous studies have evidenced the role of cannabinoids in the regulation of cell death and survival, focusing their antiproliferative effects in various tumors. Recently, we have demonstrated that WIN, a synthetic cannabinoid receptor agonist, induces a clear apoptotic effect in hepatoma HepG2 cells which is accompanied by up-regulation of Bcl-2 family proapoptotic members and down-regulation of survival factors. In this study we examined whether sub-optimal doses of WIN sensitized HepG2 cells to TRAIL and whether combinations of the two drugs induced synergistic cytotoxic effects in these cells. Furthermore, the underlying mechanisms were investigated.

Western blotting analysis demonstrated that WIN up-regulated the expression of death receptor DR5 and decreased that of decoy receptor DcR2, which is correlated with TRAIL resistance. Following the variations in the level of these proteins, HepG2 cells, which are resistant to TRAIL death pathway, became sensitive to this compounds. In fact, treatment of HepG2 cells with 10 mM WIN and 50 ng/ml TRAIL for 24 h resulted in a reduction of cell viability of about 80%, assessed with both MTT assay and cytofluorimetric analysis while the two compounds, when employed alone, did not show any cytotoxicity.

The addition of TRAIL to WIN-treated HepG2 cells also induced a marked decrease in the level of $FLIP_L$ with the consequent activation of caspase-8. Moreover, pAKT, whose level decreased after WIN treatment, became no more detectable when the cells were co-treated with the two compounds. The consequence of all these events was cell destruction through the activation of executive caspases.

In our opinion, a relevant role in the apoptotic pathway induced by the combination of WIN and TRAIL is played by the transcription factor PPAR-g, whose activation is an early event in WIN-induced apoptosis and that seems to be responsible for up-regulation of DR5 receptor.

16.11

REVERSINE SELECTIVELY INDUCES CELL DEATH IN TUMOR CELLS

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Reversine is a synthetic 2,6-distribuited purine[1], which has been shown to increase cell plasticity with the ability to reprogram lineage-committed cells to a more primitive multipotent state. In particular, reversine-treated dermal fibroblasts could be then induced to differentiate to skeletal muscle both in vitro and in vivo[2]. Nevertheless, reversine mechanism of action is still not fully demonstrated, although it has been shown that the molecule inhibits MEK1, nonmuscle myosin II heavy chain[3], and aurora kinases. Although it is still unclear if these effects are strictly related to de-differentiation, it is now quite clear that the molecule inhibits cell growth. Thus, it has been already reported that reversine inhibits cell growth and colony formation in tumor cells. Surprisingly and remarkably, in our hands, treatment of several types of cancer cells (including neuroblastoma, fibrosarcoma, and glioblastoma cancer stem cells) with reversine at micromolar concentration, not only inhibited tumor cells growth, but extensively induced cell death. On the other hand, we did not observe the same lethal effects on normal cells (including human and murine primary dermal fibroblast, murine myoblasts, rat and human mesenchymal stem cells, and murine mesangioblasts), but only a reversible cell growth arrest. Moreover, we found that tumor cells seem to undergo different death pathways. In fact, while neuroblastoma SK-N-BE cells revealed all the canonical signs of apoptosis (chromatin condensation, caspase 3 activation), we could not find the same evidences in fibrosarcoma cells HT1080. Nevertheless, cell cycle analysis and morphological features, after reversine treatment, seem to point out that cell death may be due to mitotic catastrophe, which may be caspase dependent or independent. In fact, reversine treatment leads to the formation of large cells with several micronuclei, possibly due to the aberrant chromosome segregation and the inhibition of cytokinesis. Reversine inhibition of aurora kinase and nonmuscle myosin II heavy chain, which hold crucial roles in the correct mitotic division, may result in the observed effects. Cell cycle analysis shows that reversine induces endoreplication, but while normal cells can control this effect and block cells in G1 phase, tumor cells, where G1 checkpoint is de-regulated, keep cycling to a point where micronucleated polyploid populations start to die.

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2. L. Anastasia et al. Cell Death Differ 12 (2006) 2042-51.
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16.12

UPSTREAM STIMULATING FACTOR 1 (USF1) IS INVOLVED IN THE TRANSCRIPTIONAL ACTIVATION OF THE HUMAN ALDOLASE C GENE IN PC12 CELLS DURING NEURONAL DIFFERENTIATION INDUCED BY NGF

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Aldolase C is the brain-specific isoform of fructose 1-6 bisphosphate aldolase. We previously identified two distinct regions in the human aldolase C gene promoter. The proximal region (-164/+1), located just upstream from the main transcriptional start site, that drives low levels of the brain-specific expression of aldolase C messenger; and the distal region (-1190/-164) that contains various binding sites for transcriptional activators. In the distal region, we identified a functional binding site for the neuronal transcriptional activator NGFI-B, which is involved in the c-AMP-induced activations of transcription of the human aldolase C gene[1,2]. Here we report the functional characterization of a novel transcriptional activator that is involved in transcription of the aldolase C gene during neuronal differentiation. The expression of endogenous aldolase C messenger increased by about 4-fold in PC12 cells treated with 100 ng/ml nerve growth factor (NGF) for 1 day, and reached maximal levels (9-fold) after 14 days of treatment. Using different constructs containing complete and deleted regions of the human aldolase C gene promoter, we carried out transient transfection experiments in PC12 cells untreated and treated for 3 days with 100 ng/ml NGF. These experiments revealed a novel *cis*-acting element, "element E", in the distal promoter region (-241/-235), that drives aldolase C gene up-regulation during NGF treatment. We demonstrate that the transcriptional activator USF1 binds to element E. NGF treatment for 3 days induced translocation of USF1 from the cytoplasm to the nucleus of PC12 cells, and activated aldolase C gene expression. Further experiments are in progress to elucidate if USF1 phosphorylation is involved transcriptional activation of aldolase C.

1. *Buono P et al, (1997) Biochem. Journal, 323,245-250*
2. *Buono P et al, (2002) Gene, 291,115-121*

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16.13

ABNORMAL PROLIFERATION AND DIFFERENTIATION POTENTIALITY OF MESENCHYMAL STEM CELLS IN A MURINE MODEL OF OSTEOGENESIS IMPERFECTA

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Introduction. Classical Osteogenesis Imperfecta (OI) is a bone dysplasia caused by mutations in the COL1A1 or COL1A2 genes coding for the a chains of type I collagen. Several years ago, we generated the only knock in murine model for the dominant non-lethal form of this disorder, the BrtlIV mouse, reproducing a typical glycine substitution in the a1 chain of type I collagen (G349C) and the moderately severe outcome of Type IV OI. To further investigate the molecular and cellular basis of this adaptation we evaluated *in vitro* the proliferation and differentiation potential of mesenchymal stem cells (MSCs) from WT and BrtI mice towards osteoblasts and adipocytes.

Methods. Femora, tibiae and column from 2 months old WT and BrtlIV mice were dissected and cleaned from soft tissues. MSCs collected from marrow flushing and bone crushing were isolated by plastic adhesion and used at passage 0 and 1. Cellular proliferation was evaluate after 6 days of culture both counting and using the CellTiter 96 AQueous One Solution Reagent kit (Promega). Colony forming unit-Fibroblasts (CFU-F) number was evaluated by means of limiting dilution assay and staining with Giemsa. Differentiation into adipocytes was induced by 10^{-8} M dexamethasone, 10 mg/mL insulin, 0.5 mM isobutylmethylxanthine, 125 mM indomethacin; while differentiation into osteoblasts was induced by 10^{-8} M dexamethasone, 10 mM b-glycerol phosphate, 0.2 mM ascorbic acid 2-phosphate. Culture dishes were then stained with Oil Red O and Von Kossa, respectively for adipocytes and osteoblasts identification.

Results. The proliferation of mesenchymal stem cells from BrtlIV mice, determined as Cell doubling number, was statistically higher than that of WT MSCs after 6 days of culture ($p<0.05$). No difference was detected in the CFU-F number between mutant and WT MSCs both from tibia/femora and column. The number of adipocytes colonies detected in the MSCs from mutant mice were statistically higher than from WT animals ($p<0.05$). The ability of WT and mutant MSCs to differentiate into osteoblasts was assayed by quantify the area of dark brown mineralized extracellular matrix. MSCs from both genotypes deposited mineral, but the mineralized area in mutant cells was statistically lower than in WT ($p<0.05$).

Conclusion. Our data show that at 2 months (the age at which the phenotype is most severe) the Brtl bone shows an increased MSCs proliferation strongly directed to adipogenic differentiation and a decrease in the osteoblasts differentiation. These results let us to hypothesize an alteration in the cellular differentiation signal pathway for our murine model.

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16.14

ROLE OF INTRACELLULAR CALCIUM IN CELL DEATH INDUCED BY A MIXTURE OF ISOTHIAZOLINONES IN HL60 CELLS

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Previously we reported that brief exposure of HL60 cells to a mixture of 5-chloro-2-methyl-4-isothiazolin-3-one (CMI) and 2-methyl-4-isothiazolin-3-one (MI) shifts the cells into a state of oxidative stress that induces apoptosis and necrosis[1]. The aim of this study was to evaluate the participation of Ca^{2+} in apoptosis or necrosis induced by CMI/MI treatment in HL60 cells. We used the intracellular calcium chelator 1,2-bis-(2-aminophenoxy)-ethane-N,N,N',N'-tetraacetic acid acetoxymethyl ester (BAPTA-AM) to investigate the relationships among intracellular calcium, changes $\Delta\psi m$, and induction of apoptosis or necrosis in HL60 cells exposed to CMI/MI.
HL60 cells were exposed to both apoptotic and necrotic doses of CMI/MI (0.001-0.1%) for 10 min. Using time-course (0-3 h), we performed all the analyses using flow cytometry: cytoplamic calcium levels, mitochondrial calcium levels, changes in mitochondrial transmembrane potential ($\delta\Psi m$) were assessed by FuraRed AM, Rhod-2 and Rhodamine 123 (Rhod123) respectively. Apoptosis and necrosis were evaluated as previously described[1]. The addition of CMI/MI to Fura Red-AM-loaded HL60 cells in the absence of extracellular Ca^{2+} induced a rapid and sustained increase in intracellular Ca^{2+} in a dose-dependent manner. In CMI/MI-treated cells the subsequent addition of thapsigargin, an inhibitor of ER Ca^{2+}-ATPase that discharges intracellular Ca^{2+} stores, did not cause a further increase of Ca^{2+} levels, indicating that the ER had already released its Ca^{2+}. A slight increase in fluorescence was observed in the presence of external calcium, suggesting that the primary source of Ca^{2+} is most likely the ER, the primary site of Ca^{2+} storage in eukaryotic cells. Using Rhod-2-AM, we showed that the rapid increase in cytosolic calcium induced by CMI/MI was followed by mitochondrial calcium increase. BAPTA-AM reduced necrosis and secondary necrosis and the loss of $\delta\Psi m$ induced by necrotic doses of CMI/MI, by switching from necrosis to apoptosis, but did not protect against CMI/MI-induced apoptosis, mitochondrial calcium uptake and mitochondrial hyperpolarization. Our results show that CMI/MI induces early perturbation of calcium homeostasis, increasing cytosolic and mitochondrial calcium and depleting the intracellular endoplasmic reticulum (ER) stores. These findings suggest that increased cytoplasmic calcium does not have a causal role in the induction of apoptosis, while cross-talk between the ER and mitochondria could be responsible for the induction of apoptosis, while necrosis is associated with cytoplasmic calcium overload.

1. Di Stefano A et al, (2006) Biochim. Biophys. Acta 1763: 214-225

16.15

GRAFTING MESENCHYMAL STEM CELLS IN CARDIAC INFARCTED AREA USING A HYALURONAN-BASED SCAFFOLD IMPROVES CARDIAC FUNCTION AND SCAR REVASCULARIZATION

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Stem cell based repletion of scarred myocardial tissue and regeneration of cardiomyocytes have been proposed as a potential treatment of ventricular dysfunction. Tissue-engineering is a new strategy to regenerate the infarcted myocardium. This new discipline combines isolated functioning cells and biodegradable 3-dimensional polymeric scaffolds. The scaffold temporary provides the biomechanical support for the cells until they produce their own extracellular matrix. Because tissue-engineering constructs contain living cells, they may have the potential for growth and cellular self repair and remodeling. In the present study, using a heterotopic heart transplantation animal model, we examined whether the implantation of a hyaluronan-based non-woven scaffold with bone-marrow mesenchymal stem cells (MSCs) would result in the formation of alternative cardiac tissue that could replace the scar and improve cardiac function after myocardial infarction in rat heart. Rat hearts were explanted after cardiac arrest by cold cardioplegic solution infusion and left pneumonectomy and left coronary descending artery ligation were performed on the bench. The heart-lung block was transplanted into the abdomen of a receiving syngenic rat performing an end-to-side aorta-aorta anastomosis. After two weeks we measured the cardiac function of the heterotopic rat heart by M-Mode echocardiography . Immediately after, a pocket of 3 mm^2 was made in the thickness of the ventricular wall at the level of the post-infarction scar. The scaffold, previously engineered for 3 weeks with rat bone-marrow MSCs, was introduced into the pocket and the myocardial edges sutured with few stitches. Two weeks later we evaluated cardiac function of the transplanted heart and we sacrificed the rats for histological analysis. Our results showed that after two weeks the scaffold fibres had not been substantially degraded. MSCs delivered through the scaffold were mostly migrated to the surrounding infarcted and found close to small sized vessels. Some of them were still close to the scaffold fibres and expressed cardiac-troponin-I. Scar tissue was moderated in the engrafted region and capillary density was found increased in the scar area of treated hearts compared to non-treated one. Also, the left ventricular shortening fraction was slightly improved when compared to that measured just before construct implantation. Therefore, this study suggests that post-infarction myocardial remodelling can be favourably affected by the grafting of MSCs delivered through a hyaluronan-based non-woven scaffold. *This research was founded by a grant of Compagnia di San Paolo, Turin, Italy*

16.16

REACTIVE OXIGEN SPECIES MEDIATE HIF-1α – INDUCED SURVIVAL IN METASTATIC MELANOMA

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Hypoxia, a common feature of solid tumors, is a major obstacle in the development of effective cancer chemotherapy often decreasing the efficacy of chemotherapic drugs in several solid tumors. The hypoxic environment is able to maintain an anti-apoptotic potential through the activation of critical genes associated with drug resistance and the overexpression of prosurvival proteins as Bcl-2. Hypoxia inducible factor1α (HIF1α) is the master regulator of hypoxic response and its overexpression and enhanced transcriptional activity are linked to tumour initiation and progression by inducing expression of genes mediating angiogenesis and motility, as well as tumor metabolism and survival. Increased level of HIF-1α are associated with resistance to therapy in head and neck, ovarian, esophageal and prostate cancer, thus suggesting that HIF-1α is a key contributor involved in drug resistance acquired by hypoxia. Beside inducing a glycolytic shift of tumour cells, hypoxia also paradoxically increases the intracellular level of reactive oxygen species (ROS) such as superoxide and hydrogen peroxide. Mitochondria appear to be their main source, although hypoxia may affect the intracellular redox state through NADPH oxidase engagement by autocrine growth factor production.

We observed that 1% hypoxia increases the resistance of human Hs29-4T metastatic melanoma to conventional chemotherapy with etoposide, mediating a signalling leading to tumor cells survival. The aim of our work is to identify the role of the redox component of this pro-survival spur in hypoxia signalling. In melanoma cells hypoxia leads to a strong and sustained increase of intracellular ROS, indicating mitochondria as the main source. Mitochondria-derived ROS are both necessary and sufficient to stabilize and activate HIF-1α both in normoxic and hypoxic conditions. In addition, inhibition of HIF-1α with specific siRNA, as well as inhibition of ROS production impairing mitochondrial source (rotenone) or NADPH oxidase (DPI), rescue the hypoxic protection from etoposide-induced apoptosis. Moreover VEGF and VEGF receptor neutralizing antibodies fully abrogate hypoxia-induced survival, thus suggesting a participation of VEGF signalling to sustain survival. Again, removal of mitochondria-derived ROS abrogates VEGF downstream signalling and survival. We therefore propose hypoxia-derived ROS as key modulators of the HIF-1α and VEGF-mediated resistance to chemotherapy of metastatic melanoma.

16.17

THE ACTIVITY OF CYTOSOLIC NADH/CYTO-C ELECTRON TRANSPORT SYSTEM IN APOPTOTIC HeLa CELLS

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The endogenous pathway of programmed cell death is characterized by the release from mitochondria of apoptotic reducing factors included the cytochrome c (cyto-c). The presence of cyto-c into the cytosol is required for the formation of apoptosome, responsible for the activation of caspases cascade. However, the release of cyto-c from mitochondria promotes an impairement of the respiratory chain which entity is directly linked to the amount of cyto-c released. Some Authors indicate that almost all the cyto-c is released with a drastic drop in the energy content of the cell due to impairement or the absence of respiration. This makes the problem of the source of energy required for the correct execution of apoptosis. Indeed, in the early stages of apoptosis, mitochondria continue to maintains a membrane potential. In the absence of energy apoptosis is converted in necrosis. We have shown the existence in liver cells of a rotenone and myxothiazol insensitive cytosolic NADH/cyto-c electron transport pathway. This system is activated only in the presence of catalytic amounts of cyto-c outside mitochondria, promotes the oxidation of exogenous substrates present in the cytosol and generates in mitochondria an electrochemical proton gradient useful for ATP synthesis. In 1998 we proposed that in apoptosis, cyto-c molecules released into the cytosol other than to partecipate to the formation of the apoptosome, activate the cytosolic NADH/cyto-c system with the generation of a mitochondrial membrane potential which contributes to the synthesis of ATP utilized by the apoptotic program. In this report we show that in homogenate of HeLa cells, induced to apoptosis with staurosporin, the oxidation of both NADH and cyto-c is greatly increased in support to the activation of NADH/cyto-c system. Mitochondria from apoptotic HeLa cells oxidize cyto-c and generate a membrane potential at higher rate than those from control cells. Relevant is the finding that the cytosol of both control and staurosporin treated cells promotes the reduction of added cyto-c and the reduction rate is further increased in the presence of oxidized NAD. This indicates that in apoptotic cells but also in control cells the presence of catalytic amounts of cyto-c outside the mitochondria may activate the NADH/cyto-c system to promote the oxidation of cytosolic substrates. The results obtained in reconstituted system made of cytosol and isolated mitochondria are consistent with this view. Patho-physiological implications of the activity of the NADH/cyto-c electron transport pathway will be discussed.

Abstracts poster
and selected oral communications
(SCI-DCSB)

SCI.1

EXOGENOUS DELIVERY AND MOLECULAR EVOLUTION: PEPTIDES BASED ON C^{α}-METHYLATED α-AMINO ACIDS AS ASYMMETRIC CATALYSTS IN THE SYNTHESES OF SIMPLE SUGARS

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It is known that chiral amino acids, as well as their dipeptides, may catalyze the asymmetric condensation of glycolaldehyde in water. On the basis of the particularly large erythrose enantiomeric excesses (*ee*) obtained when utilizing the chiral L-Val-L-Val catalyst and given the possibility of an abundant delivery of other types of amino acids to the early Earth, we have studied the catalytic effect on this synthesis of the peptides based on C^{α}-methylated α-amino acids, such as Iva (isovaline or C^{α}-methyl, C^{α}-aminobutyric acid) and C^{α}-methylvaline, (αMe)Val, that are abundant in meteorites.

Results of the catalysis experiments showed the all C^{α}-methylated peptides to the tetramer level exhibit significant chiral influence on the synthesis of tetroses and mimic the effect of the L-Val-L-Val catalyst in having a larger erythrose *ee* than threose *ee*, as well as in their configuration relationship with the sugars (the product erythrose acquires *ee* of configuration opposite to that of the catalyst in case of peptides, while it is the same for amino acids). Interestingly, the largest *ee* (45% for erythrose) was obtained with the Iva homo-tetrapeptide under mild conditions. The homo-dipeptides of both Iva and (αMe)Val also produced a significant *ee* (41% for erythrose) that appears to increase with time.

Because C^{α}-methylated amino acids are non-racemic in meteorites, do not racemize in aqueous environments, and are known to be (3_{10})-helix formers in peptides with as few as four residues, these results suggest that meteoritic, C^{α}-methylated, α-amino acids may have contributed to molecular evolution upon delivery to the early Earth by catalytically transferring their asymmetry to other prebiotic molecules.

SCI.2

BACKBONE NMR ASSIGNMENT AND PRELIMINARY STRUCTURAL ANALYSIS OF *T. brucei* GRX3

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Glutaredoxins (Grxs) were first discovered as glutathione-dependent reductants for ribonucleotide reductase[1] which is essential for DNA synthesis in all aerobic organisms. Today, Grxs are recognized as versatile regulatory proteins with multiple functions in health and disease[2]. The so called monothiol Grxs belong to a recently discovered class with a CGFS consensus, in contrast to the 'classical' dithiol motif with a CPYC active site. Monothiol Grxs are found either as single domain proteins or in multidomain proteins together with thioredoxin and/or dithiol glutaredoxin entities[3]. Until now, neither the physiological reductant(s) nor substrates of monothiol Grxs are definitely proved, and their functional role is largely unknown. Nevertheless, the extent of conservation of these proteins amongst prokaryotes and eukaryotes, and the poor viability of some knock-outs, suggest a decisive importance in central processes within the cells, and a role which is not redundant with dithiol glutaredoxins.

Trypanosoma brucei is the causative agent of African sleeping sickness. African trypanosomes have a unique thiol metabolism based on the dithiol trypanothione [bis(glutathionyl)spermidine]. Enzymes of this parasite-specific redox metabolism are therefore attractive antiparasitic drug targets[4]. The genome of *T. brucei* encodes three genes for monothiol Grxs. Grx1 and Grx2 are single domain monothiol glutaredoxins, while monothiol *T. brucei* Grx3 contains an additional N-terminal thioredoxin-like domain. We determined the first and as yet only high resolution structure for a single domain Grx[5], and we have now chosen the two-domain *T. brucei* Grx3 as our next target for structure determination. We obtained the full backbone assignment of the 24 kDa protein and a preliminary structural analysis, based on chemical shift data, will be presented here. Furthermore, we show that the cysteines in the two putative active sites behave differently with respect to pH changes and oxidation, which could be relevant for the function of the protein.

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SCI.3

REACTIVE OXYGEN SPECIES HIF-1α INDUCED SURVIVAL IN METASTATIC MELANOMA

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Hypoxia, a common feature of solid tumors, is a major obstacle in the development of effective cancer chemotherapy often decreasing the efficacy of chemotherapic drugs in several solid tumors. The hypoxic environment is able to maintain an anti-apoptotic potential through the activation of critical genes associated with drug resistance and the overexpression of prosurvival proteins as Bcl-2. Hypoxia inducible factor1α (HIF1α) is the master regulator of hypoxic response and its overexpression and enhanced transcriptional activity are linked to tumour initiation and progression by inducing expression of genes mediating angiogenesis and motility, as well as tumor metabolism and survival. Increased level of HIF-1α are associated with resistance to therapy in head and neck, ovarian, esophageal and prostate cancer, thus suggesting that HIF-1α is a key contributor involved in drug resistance acquired by hypoxia. Besides inducing a glycolytic shift of tumour cells, hypoxia also paradoxically increases the intracellular level of reactive oxygen species (ROS) such as superoxide and hydrogen peroxide. Mitochondria appear to be their main source, although hypoxia may affect the intracellular redox state through NADPH oxidase engagement by autocrine growth factor production.

We observed that 1% hypoxia increases the resistance of human Hs29-4T metastatic melanoma to conventional chemotherapy with etoposide, mediating a signalling leading to tumor cells survival. The aim of our work is to identify the role of the redox component of this pro-survival spur in hypoxia signalling. In melanoma cells hypoxia leads to a strong and sustained increase of intracellular ROS, indicating mitochondria as the main source. Mitochondria-derived ROS are both necessary and sufficient to stabilize and activate HIF-1α both in normoxic and hypoxic conditions. In addition, inhibition of HIF-1α with specific siRNA, as well as inhibition of ROS production impairing mitochondrial source (rotenone) or NADPH oxidase (DPI), rescues the hypoxic protection from etoposide-induced apoptosis. Moreover VEGF and VEGF receptor neutralizing antibodies fully abrogate hypoxia-induced survival, thus suggesting a participation of VEGF signalling to sustain survival. Again, removal of mitochondria-derived ROS abrogates VEGF downstream signalling and survival. We therefore propose hypoxia-derived ROS as key modulators of the HIF-1α and VEGF-mediated resistance to chemotherapy of metastatic melanoma.

SCI.4

POSSIBLE ROLE OF OXIDATIVE STRESS IN THE PATHOGENESIS OF CHRONIC VENOUS INSUFFICIENCY. PRELIMINARY RESULTS OF A POPULATION STUDY

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BACKGROUND: It is now appreciated that Oxidative Stress (OS) is involved in the pathogenesis of the lesions in certain chronic arterial diseases (as in atherosclerosis)[1]. In contrast, little information is available on the implication of oxidant species in pathologies affecting the venous system. OBJECTIVES: The aim of our population study was to evaluate whether OS could have role in the onset of chronic venous insufficiency (CVI), one of the most spread and severe venous diseases.

METHODS: For this study we have selected 2 samples of women from a total of 350 patients attending a Vascular Surgery Clinics: the first group included 71 women devoid of any type of pharmacologic intervention; the second consisted of 65 subjects who either intensively or occasionally used medicals. Both samples were subdivided in 3 groups: healthy; patients with varicose veins; and patients with diagnosed CVI. Sera from all subjects were employed for colorimetric assessment of Oxidant Power (PO) given by hydroperoxides concentration measured by D-Roms test and total antioxidant power (TAP), determined through a ferric reduction (FRAP) technique.

RESULTS: In the first sample a slight, but not significant ($p>0.05$), increase in Oxidant and Antioxidant power is observed in ill with respect to healthy subjects. The average values of both parameters are higher in patients with CVI than those with varixes. The use of medications appears to indistinctly increase PO ($p<0.001$) in healthy, varixes and CVI groups while the increment in TAP is only significant in CVI patients.

DISCUSSION: Our preliminary results are not consistent with an implication of OS in pathogenesis of CVI. Indeed, the progression of venous disease, from the mild varicous veins to the severe CVI is not accompanied by an increase of OS. Interestingly, the use of medicals for the cure of this venous disease seems to improve the antioxidant defense.

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SCI.5

SYNTHESIS AND ACTIVITY IN PHOTODYNAMIC THERAPY OF POLY(ETHYLENE GLYCOL) CONJUGATED PHEOPHORBIDE.

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In recent years porphyrin-based photosensitizers have experienced enormous interest due to their potential use in photodynamic antitumoral theraphy (PDT). However, insolubility of many porphyrins derivatives in aqueous solution causes serious problems in biological application. Poly(ethylene glycol) (PEG) conjugation is one of the delivery systems that have been employed to increase solubility, bioavailability and pharmacokinetics of many drugs.

In the present study, we have conjugated pheophorbide (Pba) to a new orthogonally protected bifunctional PEG (M.W. = 5000 Da), followed by the addition of a fluorescent moiety to the other extremity of the polymeric chain to analyze its cellular trafficking. Alternatively, a galactose unit was also added since glycoconjugation can be a potentially effective strategy for targeting photosensitizers toward tumor cells. The biological activity of Pba and PEG-Pba have been initially evaluated in a number of different cancer cells, including HeLa, HepG2, MCF7 and B78. Both porphyrins induced a strong dose-dependent PDT effect with IC_{50} values up to 800 nM. At high doses of porhyrin (>800 nM for Peg-Pba or >100 nM for Pba) the treated cells were found to die by apoptosis or necrosis, as indicated by FACS and caspase 3/7 assays. At lower doses the porphyrins, the FACS analyses showed that the cells were arrested in the G2/M phase. Moreover, the fact that both porphyrins induced in the treated cells the formation of relatively high levels of malondialdehyde (MDA) suggests that these compounds cause peroxydation of the membrane phospholipids.

SCI.6

THE CHIRAL SEQUENCE OF A NATURAL PEPTIDE INHIBITOR OF HIV-1 INTEGRASE ELUCIDATED

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Integramide A, an efficient inhibitor of the coupled reaction of HIV-1 integrase, is a 16-mer linear peptide characterized by 9 C^{α}-methylated α-amino acids (5 Iva, isovaline, and 4 Aib, α-aminoisobutyric acid, residues) that was isolated from fungal extracts of *Dendrodochium* sp. The amino acid sequence was fully elucidated by the Merck group a few years ago.[1] On the other hand, the chiral sequence was only partially determined. In particular, the precise stereochemistry of the Iva^{14}-Iva^{15} dipeptide (known to contain one D- and one L-residue) near the C-terminus was not reported.

To solve this unsettled issue and to assess integramide A primary structure-bioactivity relationship we performed by solution methods the total chemical independent syntheses of both L-D and D-L 16-mer diastereomers and compared their properties with those of the natural inhibitor. For an unambiguous, complete stereochemical assignment of integramide A we relied heavily on HPLC and NMR techniques. Our results clearly indicate that the chirality sequence of the Iva^{14}-Iva^{15} dipeptide of the natural product is L-D. The two integramide A diastereomers were also evaluated as inhibitors of HIV-1 integrase in the coupled reaction of proviral DNA into the host cell DNA.

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SCI.7

ANTIBACTERIAL ACTIVITY AND RESISTANCE TO PROTEOLYTIC DEGRADATION OF TRICHOGIN GA IV AND SELECTED ANALOGUES

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Trichogin GA IV is the most extensively investigated member of the class of lipopeptaibols that are linear peptide antibiotics of fungal origin, characterized by the presence of a variable, but remarkable, number of Aib residues, a fatty acyl group at the N-terminus, and a 1,2-amino alcohol at the C-terminus.

Several analogues of trichogin GA IV with amino acid substitutions or deletions were designed which allowed determination of the minimal inhibition concentration against Gram-positive and Gram-negative bacteria and various pathogenic fungal cells. The natural peptide exhibits a specific activity against *S. aureus* and only a marginal hemolytic effect. Interestingly, trichogin GA IV is active also against several methicillin-resistant *S. aureus* strains. Studies on synthetic analogues demonstrated that substitution of the C-terminal leucinol by Leu-OMe, or substitution of one Aib residues by the EPR label TOAC do not perturb significantly the biological activity of the peptide. On the other hand, removal of 3 or 7 N-terminal residues eliminated any antibacterial activity. Finally, studies of proteolytic degradation on trichogin GA IV and analogues where the 3 Aib residues are replaced by Leu demonstrated that the presence of several non-coded Aib residues endows the natural peptaibol with remarkable resistance to proteolysis. The present results indicate that trichogin GA IV is a promising lead compound for the development of new, selective and protease-resistant, antibacterial drugs.

SCI.8

ARE LYMPHOCYTES DOPAMINERGIC CELLS? QUESTIONING THE PROTEOMICS RESPONSE OF A T-CELL MODEL TO DOPAMINE TREATMENT.

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PBLs possess a complex dopaminergic system. Indeed, they express tyrosine hydroxylase and store catecholamines into vesicles that are released through a synaptic-like mechanism[1]. They possess transporters like DAT and VMAT, therefore they can internalize dopamine[2].

PBLs are easy to obtain from patients with little discomfort and may represent sensitive cellular sensors to be used for the evaluation of gene expression modification in physiological and pathological conditions, providing a unique and easily available biological model for integrated studies of gene expression in humans[3].

Our final purpose will be that of using lymphocytes as a source to look for biomarkers of Parkinson's disease.

As a preliminary study we wanted to better understand which are the proteins whose level change in response to pharmacological doses of dopamine, to detect probable biochemical pathways "sensitive" to dopamine.

To this purpose, we treated a T-cell model (human T cell leukemia Jurkat cells) with 50 μM dopamine and 700 U/ml catalase, in order to correct for aspecific effects due to auto-oxidation of dopamine in the extracellular space. The differential analysis of the proteome was performed using 2D-electrophoresis. Our maps were rich in proteins with basic and acidic pI. Moreover, lymphocytes have many plasma membrane proteins, whose hydrophobicity could prevent the proteins to enter the IEF gel. To overcome this limit and to obtain a more complete analysis, we also separated proteins with ion-exchange chromatography and we further analyzed the different fractions by classical SDS-PAGE.

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SCI.9

INHIBITION OF ALPHA CRYSTALLIN AMYLOID FIBRILS FORMATION BY CARNOSINE

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Posttranslational modifications of lens crystallin, consequent to aging or diseases such as diabetes, lead to lens opacification and cataract formation[1]. Cataract is seen as a conformational disorder where unfolding or destabilization of the crystallin proteins drives their aggregation. Alpha-crystallin is the major structural protein within the eye lens and is therefore predominant in the aggregates.

Under mildly denaturing conditions *in vitro*, bovine alpha-crystallin assembles into fibrillar structures[2]. The crystallins conversion into fibrils under destabilizing conditions suggests that this process could contribute to the development of cataract with aging.

Carnosine (ß-alanyl-histidine) is present in long-lived tissues in high amounts and has been shown to delay aging of cultured cells[3], to disaggregate glycated alpha-crystallin and to have potential biological and therapeutic significance.

In the present study we examined the ability of L and D-carnosine to affect the alpha-crystallin amyloid fibril formation under destabilizing conditions and its property in dissolving preformed fibrils. Morphological changes of whole lens, dissected from Rattus norvegicus, under organ culture were examined after treatment with guanidine hydrochloride and in the presence of carnosine. The observed dose and time dependent lens opacification was prevented in the presence of carnosine. Interestingly carnosine addition to already damaged lenses provided a significant recovery.

Studies on conformational stability of alpha-crystallin have been carried out in the presence of guanidine and increasing temperature, by calorimetric (DSC) microscopic (AFM) and fluorescence analyses. The observed formation of fibrillar structures and aggregates of alpha-crystallin was reversed in the presence of carnosine.

Either considering alpha-crystallin alone or the more complex model of lens organ culture, the presence of carnosine prevented fibrils formation and disassembled already formed fibrils restoring most of lens transparency. Protein misfolding is particularly relevant in the lens opacification, but it's possible to counteract this process using agents with the common feature protein structures stabilization[4].

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SCI.10

ANTIOXIDANT AND ANTIPROLIFERATIVE ACTIVITY OF SEEDS FROM *BETA VULGARIS CICLA*

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Seeds of several plants contain a large number of chemical compounds which could be examined for useful phytochemicals. These compounds probably function in the protection of seeds from microbial degradation until conditions are favourable for germination. Given the easily availability of seeds from food plants in horticulture, these seeds are now currently evaluated for isolation of phytocomplexes and pure phytochemicals to be used for pharmaceutical and nutritional purposes.

This report focuses on the antioxidant and antiproliferative activity of seeds from *Beta vulgaris cicla*.

Values for total phenolics, flavonoids and antioxidant capacity were determined in the raw seed powder and after each step of organic extraction and preparative chromatography on Sepadex LH 20 and RP 18 silica media. A phytocomplex, labelled P2, with remarkable antioxidant capacity and antiproliferating activity was obtained. P2 was tested for growth inhibition of cancer cell lines: MCF-7, RKO and G401. The most responsive cells were RKO which showed a 50% proliferating inhibition with 60 ug/ml phenolic concentration. The main phytochemical present in P2, vitexin-2-O-rhamnoside, a glucoside of the flavone apigenine, was not able alone to exploit the antiproliferating effect showed by P2. The number of apoptotic cells evaluated cytofluorimetrically was 10% only thus suggesting an effect of P2 on some cell cycle regulatory proteins and not on the apoptotic mechanism.

P2 resulted bioavailable by searching with an HPLC method, the aglicone apigenine in the blood and liver of mice which received 5% P2 in food for 5 days. Flavonoid presence in mice plasma was also confirmed by an ELISA assay based on specific antibodies previously developed in mouse by creation of haptens to obtain a proper immune response.

The results show that *Beta vulgaris cicla* seeds are an excellent source for phytochemicals of potential utility in chemoprevention and for preparation of antioxidant nutraceutical products.

SCI.11

STUDIES ON *MYCOBACTERIUM TUBERCULOSIS* GLUTAMYL-tRNA SYNTHETASE

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Mycobacterium tuberculosis glutamyl-tRNA synthetase (Mt-GluRS) is an essential enzyme which provides Glu-$tRNA^{Glu}$ for protein biosynthesis and for that of δ-aminolevulinic acid (ALA), the first common precursor of tetrapyrrole biosynthesis.

Soluble recombinant protein was obtained in large amounts and purified to homogeneity. The catalytic properties of Mt-GluRS are being investigated using the well characterized *E. coli* GluRS (Ec-GluRS) as a reference in order to highlight peculiar properties of the *M. tuberculosis* enzyme. The steady-state kinetic parameters of Mt-GluRS reaction were found to be similar to those exhibited by other GluRS except for a higher value of the K_m for L-Glu (2.7 mM instead of 0.1 mM for Ec-GluRS). The enzyme reaction mechanism and the Glu-AMP intermediate formation was studied by using a colorimetric method for pyrophosphate (PP_i) detection, [^{3}H]-labelled ATP and chromatographic separation of reaction components and by monitoring the [^{32}P]-PP_i-ATP exchange reaction. Mt-GluRS was shown to catalyze the formation of Glu-AMP only in the presence of bound $tRNA^{Glu}$ as reported for GluRS from other sources. Among the several GluRS substrate and product analogs tested, only the reaction product PP_i and the Glu-AMP analog glutamol-AMP (GOM) had an effect on GluRS activity. PP_i was a non competitive inhibitor with respect to ATP, in agreement with the observed [^{32}P]-PP_i-ATP exchange. GOM was a competitive inhibitor with respect to both L-Glu and ATP (K_i 3.9 and 1.5 μM, respectively) suggesting random binding of these substrates to the enzyme•tRNA complex. At variance with other GluRS, Mt-GluRS was found to catalyze the hydrolysis of ATP to ADP+P_i in the absence of tRNA and Glu, although at a rate of 5.1 min^{-1}, corresponding to only 4% of the turnover rate of the physiological reaction.

Analysis of *M. tuberculosis* genome revealed the absence of genes encoding Gln-$tRNA^{Gln}$ synthetase indicating that Mt-GluRS belongs to the class of the non discriminating GluRS, which misacylate $tRNA^{Gln}$ with L-Glu forming Glu-$tRNA^{Gln}$. The misacylation is corrected by the specific amidotransferase generating Gln-$tRNA^{Gln}$. Genes encoding the amidotransferase have indeed been identified in *M. tuberculosis* genome. However, the overexpression of Mt-GluRS in *E. coli*, which expresses a Gln-$tRNA^{Gln}$ synthetase and lacks the amidotransferase, was not toxic, raising the question of the tRNA specificity of Mt-GluRS.

Genome analyses indicated that in *M. tuberculosis* ALA is formed from Glu-$tRNA^{Glu}$ by glutamyl-tRNA reductase (GluTR), in the presence of NADPH, and glutamate 1-semialdehyde aminomutase in the so-called C5 pathway. Thus, we tested the hypothesis of the possible formation of a complex between GluRS and GluTR, which would commit Glu-$tRNA^{Glu}$ to tetrapyrrole biosynthesis as opposed to protein synthesis. By affinity chromatography with immobilized His-tagged forms of GluRS or of GluTR as the bait to fish out the second enzyme, we obtained data consistent with complex formation.

SCI.12

STRUCTURE, STABILITY AND DYNAMICS OF A MESOPHILIC-PSYCROPHILIC PAIR

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Superoxide dismutases are a family of metalloenzymes that catalyse the dismutation of superoxide anion radicals into molecular oxygen and hydrogen peroxide. Here, we present the first crystal structure of a cold-active iron superoxide dismutase, from the antarctic eubacterium *Pseudoalteromonas haloplanktis (Ph*SOD)[1-2]. The structural information is combined with a characterization of stability and dynamics of the enzyme and with a comparative study with the mesophilic counterpart from *E. coli* (*Ec*SOD)[3], which shares the same number of residues and a very high sequence identity with *Ph*SOD (70%). The temperature- and denaturant-induced unfolding of *Ph*SOD and *Ec*SOD have been studied by circular dichroism (CD) and fluorescence measurements. The denaturation temperature at the transition midpoint of *Ph*SOD is comparable to that of *Ec*SOD, and higher than that expected for a psychrophilic enzyme. On the contrary, the values of the denaturant concentration at the transition midpoint are in line with the psychrophilic/mesophilic origin of the two proteins.

*Ph*SOD and *Ec*SOD share a large structural similarity: conserved tertiary structure and arrangement of two monomers, almost identical total number of inter- and intramolecular hydrogen bonds and salt bridges, polar and non polar surface, number of internal cavities. However, the cold-adapted protein shows an increased flexibility of the active site residues with respect to its mesophilic homologue.

These data provide an additional support for the hypothesis that cold-adapted enzymes achieve efficient catalysis at low temperatures by increasing active site flexibility. Our results also illustrate how fine structural modifications can alter enzyme active site flexibility, without compromising the overall protein structure and stability.

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Name index

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Index

E. ANTONINI LECTURE

THE BACTERIAL PAN-GENOME AND THE GLOBAL GENE POOL
R. Rappuoli

SPEAKERS' PRESENTATIONS

R.1 PROTEOMICS IN NEURODEGENERATIVE DISEASES
K. Marcus

R.2 FIELD-FLOW FRACTIONATION FOR THE ANALYSIS OF BIOPHYSICAL CHARACTERISATION OF BIOLOGICAL SYSTEMS
P. Reschiglian

R.3 AN INTEGRATED PLATFORM FOR CLINICAL ANALYSIS OF GENE EXPRESSION
M. Simmaco

R.4 NETWORK BIOLOGY: TOWARDS THE COMPREHENSION OF COMPLEX BIOLOGICAL SYSTEMS.
A. Zanzoni

R.5 ROLE OF MASS SPECTROMETRY IN PROTEIN BIOMARKERS DISCOVERY: THE CASE OF RENAL CLEAR CELL CARCINOMA.
F. Magni

R.6 THE PROTEOME OF SKELETAL MUSCLE IN NEURODEGENERATIVE CONDITIONS
C. Gelfi

R.7 SEQUENCE-DEPENDENT COLLECTIVE PROPERTIES OF DNA AND THEIR ROLE IN BIOLOGICAL SYSTEMS
P. De Santis

R.8 QUADRUPLEX DNA: STRUCTURAL FEATURES AND POTENTIAL APPLICATIONS.
A. Galeone

R.9 PEG-ADDITION TO OLIGONUCLEOTIDES: A STORY WITH A FUTURE?
G.M.Bonora

R.10 NEW INHIBITORS OF METALLOPROTEASES VIA STRUCTURE/LIGAND BASED DESIGN: A CASE STUDY.
A. Topai

R.11 NMR IN STRUCTURAL BIOLOGY AND DRUG DISCOVERY
C. Luchinat

R.12 PEPTIDE ANTAGONISTS OF PROTEIN-PROTEIN INTERACTIONS IDENTIFIED BY SCREENING PROTEIN FRAGMENTS.
M. Ruvo

R.13 STRUCTURAL AND FUNCTIONAL CHARACTERIZATION OF HELICOBACTER PYLORI PROTEINS. IDENTIFICATION OF NEW PHARMACOLOGICAL TARGETS.
G. Zanotti

R.14 STRUCTURE-BASED DISCOVERY OF ANTIVIRALS
R. Hilgenfeld

R.15 ALLOSTERIC MODULATORS OF IL-8/CXCL8 RECEPTORS.
M. Allegretti

R.16 THE FUNCTIONAL EFFECTS OF THE NEW HUMAN HORMONES ABSCISIC ACID ON ANIMAL CELLS ARE MEDIATED BY THE SECOND MESSANGER CYCLIC ADP-RIBOSE
E. Zocchi

R.17 TARGETING MET IN TUMOR AND METASTASIS: A MATTER OF ADDICTION AND SENSITIVITY
S. Giordano

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R.18 **TARGETING CK2 AS A GENERAL STRATEGY IN SIGNAL TRASDUCTION THERAPY**
L. Pinna

R.19 **TARGETING HYPOXIA INDUCIBLE FACTOR 1 (HIF-1) FOR CANCER THERAPY.**
G. Melillo

R.20 **DIOXYGENASE ENZYMES FROM ARENE-DEGRADING BACTERIA: CAN WE DEVELOP THEIR POTENTIAL FOR APPLICATION IN BIOCATALYSIS?**
C. Allen

R.21 **METABOLIC ENGINEERING: A TOOL FOR BIOREMEDIATION STRATEGIES**
A. Di Donato

R.22 **MICROBIAL REDUCTIVE DEHALOGENATION FOR THE SUSTAINABLE REMEDIATION OF PCB-CONTAMINATED SEDIMENTS.**
F. Fava

R.23 **SENSOR FOR CHEMICAL RISK ASSESSMENT**
L. Campanella

R.24 **LIFE ON THE EDGE: THE NATURE AND ORIGINS OF PROTEIN MISFOLDING DISEASES**
C. M. Dobson

R.25 **B_2-MICROGLOBULIN REFOLDING INTERMEDIATES AND FIBRILLOGENESIS**
G. Esposito

R.26 **MISFOLDING AND TISSUE LOCALIZATION OF AMYLOIDOGENIC PROTEINS: COMPLEXITIES AND BREAKTHROUGHS**
V. Bellotti

R.27 **PRION PROTEIN, PRION DISEASES AND TETRACYCLIC COMPOUNDS**
F. Tagliavini

R.28 **PROTEIN FOLDING AND SIGNALING IN THE EARLY SECRETORY APPARATUS.**
R.Sitia

R.29 **"SEEING" AT THE NANOSCALE WITH THE ATOMIC FORCE MICROSCOPE**
A. Relini

R.30 **ORTHOGONAL STRATEGIES IN PROTEOMICS**
P. Pucci

R.31 **MANIPULATION AND IMAGING OF SINGLE BIO-MOLECULES**
F. S. Pavone

R.32 **MOLECULAR FUNCTIONS OF SIRTUINS.**
J. M. Denu

R.33 **miR "STEMNESS" & SKIN**
G. Melino

R.34 **MANY FACES OF THE RNA BINDING PROTEIN KSRP**
R. Gherzi

R.35 **RNA INTERFERENCE-BASED SCREENING OF GENES INVOLVED IN EMBRYONIC STEM CELL SELF-RENEWAL AND DIFFERENTIATION**
T. Russo

R.36 **"miR GENES IN PAEDIATRIC CANCER"**
M. Zollo

R.37 **THE ROLE OF OXIDATIVE STRESS IN INSULIN SECRETION AND AGING**
Fatima Mechta-Grigoriou

R.38 **OXIDATION-COUPLED TRANSCRIPTION : DEMETHYLATION OF HISTONE H3 LYSINE 9 INDUCES DNA OXIDATION AND DRIVES TRANSCRIPTION INITIATION BY ESTROGENS**
E. V. Avvedimento

R.39 **IDENTIFICATION AND FUNCTIONAL CHARACTERIZATION OF A NOVEL ANTIAPOPTOTIC GENE INVOLVED IN ADAPTATION TO OXIDATIVE STRESS AND CHEMORESISTANCE.**
F. Esposito

R.40 **REDOX REGULATION OF CELLULAR STRESS RESPONSE IN AGING AND NEURODEGENERATIVE DISORDERS: ROLE OF VITAGENES**
V. Calabrese

R.41 **CELL COMMUNICATION IN THE NERVOUS SYSTEM: THE MEDIATOR ROLE OF SPHINGOSINE 1-PHOSPHATE**
L. Riboni

R.42 **DIACYLGLYCEROL KINASES AS SWITCH REGULATORS FROM DIACYLGLYCEROL TO PHOSPHATIDIC ACID-MEDIATED SIGNALING: IMPLICATION IN CELL SIGNALING**
A. Graziani

R.43 **SPHINGOLIPIDS AS MODULATORS OF MEMBRANE SIGNALLING COMPLEXES.**
A. Prinetti

R.44 **CELLULAR TARGETS AND ACTIVITIES OF THE GLYCEROPHOSPHOINOSITOLS.**
D. Corda

R.45 **REACTION MECHANISM OF BOVINE HEART CYTOCHROME C OXIDASE**
S. Yoshikawa

R.46 **THE MITOCHONDRIAL PERMEABILITY TRANSITION IN PATHOPHYSIOLOGY**
P. Bernardi

R.47 **NITRIC OXIDE AND CONTROL OF MITOCHONDRIAL RESPIRATORY CHAIN: ELEXTRON FLUX**
P. Sarti

R.48 **MITOCHONDRIAL SUBSTRATE LEVEL PHOSPHORYLATION IMPROVES VIABILITY OF HUMAN CELLS WITH SEVERE OXIDATIVE PHOSPHORYLATION IMPAIRMENT**
A. Baracca

R.49 **MITOCHONDRIAL TRANSPORTERS IN HEALTH AND DISEASE**
F. Palmieri

R.50 **REGULATION BY cAMP DEPENDENT PHOSPHORYLATION OF MITOCHONDRIAL PROTEIN IMPORT**
D. De Rasmo

R.51 **THE MITOCHONDRIAL PROTEASE AFG3L2 IS ESSENTIAL FOR DEVELOPMENT AND MAINTENANCE OF MOTOR AXON AND CEREBELLUM**
G. Casari

ABSTRACTS

1.1 **FULL-LENGTH TRANSGLUTAMINASE 2 AND ITS SHORT ISOFORM ARE OVER EXPRESSED IN HUMAN ASTROCYTOMAS**
V. Macaione, M. Aguennouz, C. Tomasello,I. Barbagallo, A. Conti, M.G. De Pasquale, N. Germanò, F. Tomasello, R.M. Di Giorgio, G. De Luca, A. Campisi, A. Vanella.

1.2 **THE POLYAMINE ANALOGUE N^1,N^{11}-DIETHYLNORSPERMINE CAN INDUCE CHONDROCYTE APOPTOSIS INDEPENDENTLY OF POLYAMINE METABOLISM AND LEVELS**
I. Stanic', S. Cetrullo, A. Facchini, C. Pignatti, C. Stefanelli, R.M. Borzì, C. Guarnirei, C.M. Caldarera, F. Flamigni

1.3 **INVOLVEMENT OF TRANSGLUTAMINASE IN CELL STRESS RESPONSE EVOKED BY HOMOCYSTEINE IN SH-SY5Y NEUROBLASTOMA CELLS**
S.Condello, D.Caccamo, M. Currò, N. Ferlazzo, R.Ientile

1.4 **CARDIOPROTECTION AND ENERGY METABOLISM MODULATION BY 3-IODOTHYRONAMINE, A NEW ENDOGENOUS CHEMICAL MESSENGER**
S. Ghelardoni S. Frascarelli, G. Chiellini, S. Ronca-Testoni, D.K. Grandy, T.S. Scanlan, R. Zucchi

1.5 **EFFECT OF SPERMIDINE ON DNase I ACTIVITY**
C. Seppi, C. Balduini

1.6 **DNA AGGREGATION INDUCED BY SPERMIDINE: A TECHNICAL NOTE**
C. Seppi, C. Balduini

1.7 A REDOX SWITCH AND REGULATION OF ADENINE NUCLEOTIDE TRANSLOCASE PHOSPHORYLATION ARE IMPLICATED IN THE POLYAMINE EFFECTS ON MITOCHONDRIAL PERMEABILITY TRANSITION.
A. Toninello, S. Grancara, E. Tibaldi, V. Battaglia, A. M. Brunati

2.1 ENDOCANNABINOIDS PROLONG PLATELET LIFE SPAN
L. Avigliano, M. V. Catani, V. Gasperi, A. Finazzi Agrò, M. Maccarrone

2.2 P2X7 OVEREXPRESSION IS RESPONSIBLE FOR THE INCREASED INTRACELLULAR CALCIUM AND FUNCTIONAL DERANGEMENT IN SCHWANN CELLS FROM RATS WITH CHARCOT-MARIE-TOOTH TYPE 1A NEUROPATHY
S. Bruzzone, L. Sturla, F. Fiorese, C. Usai, G. Basile, I. Moreschi, F. Benvenuto, E. Zocchi, A. De Flora, A. Schenone, L. Nobbio

2.3 ABSCISIC ACID IS AN ENDOGENOUS STIMULATOR OF INSULIN RELEASE FROM HUMAN PANCREATIC ISLETS WITH CYCLIC ADP-RIBOSE AS SECOND MESSENGER
S. Bruzzone, N. Bodrato, C. Usai, L. Guida, I. Moreschi, R. Nano, F. Fruscione, M. Magnone, S. Scarfi', A. De Flora, E. Zocchi

2.4 DIACYLGLYCEROL KINASE ALPHA REGULATES CELL MIGRATION THROUGH PKCz, RhoGDI AND RAC.
F. Chianale, E. Rainero, C. Cianflone, V. Bettio, P. Porporato, M. Gaggianesi, G. Tur-Arlandis, S. Traini, G. Baldanzi, N. Filigheddu, A. Graziani.

2.5 REACTIVE OXYGEN SPECIES HIF-1α INDUCED SURVIVAL IN METASTATIC MELANOMA
M.Calvani , M. Parri, E. Giannoni, G. Comito, P. Chiarugi

2.6 INCREASE OF INVASIVENESS IN HUMAN MELANOMA CELLS DURING HYPOXIA: ROLE OF REDOX SIGNALLING AND MET UPREGULATION
G. Comito, M. Calvani, C. Migliore, E. Giannoni, M. Parri , S. Giordano e P. Chiarugi

2.7 ABSCISIC ACID PRODUCED BY HUMAN MONOCYTE AND VASCULAR SMOOTH MUSCLE CELL RESPONSES INVOLVED IN THE DEVELOPMENT OF THE ATHEROSCLEROTIC LESION
A. De Flora, M. Magnone, S. Bruzzone, L. Guida, G. Damonte, E. Millo, S. Scarfì, C. Usai, D. Palombo, E. Zocchi

2.8 STROMAL MYOFIBROBLAST AND PROSTATE CANCER CELLS: AN ACTIVE PRO-INVASIVE BIDIRECTIONAL COMMUNICATION
E. Giannoni, F. Bianchini, L. Calorini, G. Raugei, G. Ramponi and P. Chiarugi

2.9 THE ROLE OF DNA-DAMAGE BINDING PROTEIN (DBB1), A COMPONENT OF E3 UBIQUITIN LIGASE COMPLEX IN $p27^{Kip1}$ TURNOVER
B. Iovine, M.L. Iannella, F. Cirillo, G. Madonna, M.A. Bevilacqua

2.10 siRNA-MEDIATED DOWNREGULATION OF PKD1 AND PKHD1 GENES: DIFFERENT EFFECTS ON CALCIUM HOMEOSTASIS AND CELL PROLIFERATION
A.Mangolini, G.Aguiari and L. del Senno.

2.11 EPHA2 (RE)-EXPRESSION PROMPTS INVASION OF MELANOMA CELLS SHIFTING FROM MESENCHYMAL TO AMOEBOID-LIKE MOTILITY STYLE
M. Parri, M. L. Taddei, F. Bianchini, L. Calorini and P. Chiarugi.

2.12 GLOBULAR ADIPONECTIN INDUCES GROWTH AND SURVIVAL OF MESANGIOBLASTS
M. Parri, T. Fiaschi, D. Cirelli, G. Ramponi, G. Cossu and P. Chiarugi

2.13 HAPTOGLOBIN INFLUENCES APOA-I ACTIVITIES IN REVERSE CHOLESTEROL TRANSPORT
C. R. Pugliese, L. Cigliano, E. Santoro, A. d'Alessio, E. Picardi, B. Maresca, A. Salvatore, A. Carlucci, P. Abrescia.

2.14 Sic1 PHOSPHORYLATION BY CK2 IS MODULATED BY CARBON SOURCE IN *Saccharomyces cerevisiae*
F. Tripodi, C. Cirulli, V. Reghellin, O. Marin, M.P. Schiappelli, M. Vanoni, L. Alberghina, P. Coccetti

2.15 EFFECT OF ELLAGIC ACID ON PROSTATIC CANCER CELL LINES
L. Vanella, V. Sorrenti, C. Di Giacomo, R. Acquaviva, V. Cardile, L. Lombardo, A. Vanella

2.16 INTERACTION OF TUMOR CELLS WITH BRAIN VASCULAR ENDOTHELIAL CELLS: A SEARCH FOR CYTOSOLIC PLA_2-PKCα-ERK1/2 SIGNALING PATHWAY
C.D. Anfuso, G. Giurdanella, C. Motta, S. Muriana, G. Lupo, V. Arena, N. Ragusa, M. Alberghino

2.17 CURCUMIN PROTECTS CARDIAC CELLS AGAINST ISCHEMIA-REPERFUSION INJURY: MECHANISM OF ACTION
M. Becatti, C. Fiorillo, C. Cecchi, N. Nassi*, M. Zampagni, P. Nassi

2.18 IDENTIFICATION OF ALTERNATIVELY SPLICED FORMS OF IL-6 GENE IN C2C12 CELLS.
G. Annibalini, M. Guescini, D. Agostini, P. Sestili, C. Martinelli, M. Paolillo, M. Mantuano, P. Tibollo, V. Stocchi

2.19 ROLE OF THE MECHANOTRANSDUCTION IN SKELETAL MUSCLE CELLS STIMULATED BY SPHINGOSINE 1-PHOSPHATE
F. Bini, R. Squecco, C. Sassoli, L.Formigli, F.Francini, E. Meacci

2.20 PHOSPHATIDYLSERINE SYNTHESIS DURING CHROMIUM (VI)-INDUCED APOPTOSIS IN HUMAN LYMPHOBLASTIC CELLS
S. Buratta, A. Gambelunghe, C. Marchetti, G. Migliorati, G. Muzi and R. Mozzi

2.21 TRAP1, A NOVEL ANTIAPOPTOTIC GENE RESPONSIBLE FOR MULTIDRUG RESISTANCE.
S. Calise, E. Costantino, M. Landriscina and F. Esposito

2.22 BIOENERGETIC STUDY OF K-RAS^{-}TRANSFORMED FIBROBLASTS.
G. Casalena, G. Sgarbi, A. Baracca, G. Solaini, F. Chiaradonna, L. Alberghina, G. Lenaz

2.23 THE ROLE OF ERAD IN PLANT CELLS: A PRELIMINARY BIOCHEMICAL CHARACTERIZATION OF PLANT PROTEASOMES
P. Cascio, F. Cerruti, A. Ceriotti

2.24 SIGNAL TRANSDUCTION PATHWAY IN PrP^{C}-DEPENDENT NEURITOGENESIS
B. Pantera, A. Caselli, C. Bini, P. Cirri, P. Paoli, G. Camici, G. Manao

2.25 RELATIONSHIP BETWEEN INTRACELLULAR OXIDATIVE STATUS AND INTERLEUKIN SYNTHESIS IN 18CO CELLS: INVOLVEMENT OF P38 AND ERK1/2 MAPKINASES
S. Catarzi, F. Favilli, T.Marcucci, F.Tonelli, M.T. Vincenzini and T. Iantomasi

2.26 REDOX REGULATION OF PDGF RECEPTOR TRANSACTIVATION INDUCED BY EXTRACELLULAR SPHINGOSINE 1-PHOSPHATE: ROLE OF G_i PROTEINS AND C-SRC-KINASE ACTIVITY.
S.Catarzi, F.Favilli, T.Iantomasi and M.T.Vincenzini

2.27 CYTOCHROME OXIDASE, INHIBITING EFFECTORS AND THE CONTROL COEFFICIENT
Maria Elena Dalmonte, Elena Forte, Alessandro Giuffrè, Daniela Mastronicola, M. Luisa Genova, Giorgio Lenaz, Paolo Sarti

2.28 EXPRESSION AND ACTIVITY OF GPR17, A NEW NUCLEOTIDE-CYSTEINYL-LEUKOTRIENE RECEPTOR, IN NEURONAL PC12 CELL MODEL.
S. Daniele, O. Ciampi, I. Tonazzini, M.L. Trincavelli, S. Cuboni , P. Rosa, M.P. Abbracchio, C. Martini

2.29 LOW OXIGEN TENSION AS A QUIESCENT FACTOR FOR NON-TRASFORMED CELLS.
A. De Donatis, R. Caporale, G. Camici, G. Manao e P. Cirri

2.30 NEW SMALL MOLECULE ANTAGONISTS TO PROTEASE-ACTIVATED RECEPTOR-1 (PAR-1): INHIBITION OF RECEPTOR INDUCED CALCIUM RESPONSE IN A HUMAN MICROVASCULAR ENDOTHELIAL CELL LINE (HMEC-1).
M. T. Dell'Anno, S. Asteriti, A. Gilchrist, M. R. Mazzoni

2.31 ANTITUMOR ACTIVITY OF NEW SUBSTITUTED 3-(5-IMIDAZO[2,1-b]THIAZOLYLMETHYLENE)-2-INDOLINONES
F. Di Giorgio, N. Calonghi, C. Cappadone, J. Hysomema, C. Mangano, C. Parolin, C. Stefanelli, M. Zini, M Voltattorni, L. Masotti.

2.32 R)-9-HSA HAS A MORE REMARKABLE ANTIPROLIFERATIVE EFFECT COMPARE TO (S)-9-HSA.
F. Di Giorgio, C. Parolin, N. Calonghi, C. Mangano, C. Cappadone, J. Hysomema, C. Boga and L. Masotti

2.33 INTRACELLULAR ZINC INCREASE INHIBITS p53$^{-/-}$ PANCREATIC ADENOCARCINOMA CELL GROWTH BY ROS/AIF-MEDIATED APOPTOSIS
M. Donadelli, E. Dalla Pozza, M.T. Scupoli, C. Costanzo, A. Scarpa and M. Palmieri

2.34 RhoA SILENCING REVERSES THE RESISTANCE TO DOXORUBICIN IN HUMAN COLON CANCER CELLS
S. Doublier, C. Riganti, C. Voena, C. Costamagna, E. Aldieri, G. Pescarmona, D. Ghigo, A. Bosia

2.35 CORRELATION BETWEEN THE PROTEIN COMPOSITION OF STAT3-DEPENDENTENHANCEOSOME AND ITS ACTIVATION PATHWAYS IN CANCER CELLS
M. Eufemi, R. Cocchiola, C. Grillo, A. Ferraro, F. Altieri, S. Chichiarelli, C. Turano.

2.36 CORRELATION BETWEEN NEUROINFLAMMATION AND CHOLESTEROL METABOLISM IN GLIAL CELLS
A. Galmozzi, B. Viviani, F. Gilardi, M. Boraso, D. Caruso, M. Crestani, M. Marinovich, E. De Fabiani

2.37 STUDYING THE ERp57 FUNCTION IN LIVING CELLS
E. Gaucci, V. Arcangeli, S. Chichiarelli, A. Ferraro, M. Eufemi, C. Turano

2.38 THE SYNAPTIC PROTEIN NEUREXIN AND THE VASCULAR TYROSINE KINASE RECEPTOR TIE-2 PHYSICALLY ASSOCIATE IN BLOOD VESSELS
S. Graziano, G. Cordara, A. Bottos, A. Rissone, F. Bussolino, M.Arese

2.39 MOLECULAR PROFILING OF DIFFERENTIATED THYROID NEOPLASM: DIAGNOSTIC ANDCLINICAL IMPLICATIONS.
C. Grillo, M. Marinelli, F. Curcio, M. Eufemi, C. Turano, F. Altieri,

2.40 INHIBITION OF NF-KB ACTIVATION PREVENTS UPREGULATION OF SKP2 AND CKS1B BUT NOT OF THEIR mRNA FOLLOWING STIMULATION OF T-LYMPHOCYTES WITH ANTI TCR/CD3 AND ANTI CD28 IMMOBILIZED ANTIBODIES.
E. Lupino, M. Piccinini, B. Buccinnà, A. Lomartire, G. DeMarco, C.Ramondetti, M.T. Rinaudo.

2.41 BIOLOGICAL EFFECTS OF A NEW CLASS I HDAC SELECTIVE INHIBITOR IN HT29 COLON CANCER CELLS.
C. Mangano, N. Calonghi, M. Naldi, F. Di Giorgio, J. Hysomema, C. Parolin, C. Cappadone, A. Mai, L. Masotti

2.42 CYTOTOXICITY EVALUATION OF A POLYAMINE-QUINONE LIBRARY CONJUGATES IN HUMAN COLON CARCINOMA CELLS
C. Mangano' N. Calonghi, C. Parolin, C. Cappadone, F. Di Giorgio, J. Hysomema, M.L. Bolognesi, C. Melchiorre and L. Masotti

2.43 REGULATION OF HEPATIC LIPID AND CARBOHYDRATE METABOLISM BY THE LIVER X RECEPTOR
N. Mitro, P.A. Mak, L. Vargas, C. Godio, V. Molteni, A. Kreusch, E. De Fabiani, D. Caruso and E. Saez

2.44 CATHEPSIN S AND D EXPRESSION AS A BIOCHEMICAL CHECKPOINT FOR THE GENERATION OF TOLEROGENIC DENDRITIC CELLS FROM HSC-CD34+
S. Martino, R. Tiribuzi, E. Ciraci, G. Makrypidi, I. di Girolamo, F. D'Angelo, G.F. Bottazzo, A. Datti, A. C. Berardi , A. Orlacchio.

2.45 PROTEOMIC PROFILING OF VESICLES RELEASED BY 8701-BC CELLS.
G. Palazzolo, M. Frrokaj, G. Gallo, M. L. Vittorelli

2.46 EFFECT OF AN INDOLE-DERIVATIVE IN HUMAN OVARIAN CARCINOMA CELLS.
C. Parolin, N. Calonghi, C. Cappadone, F. Di Giorgio J. Hysomema, C. Mangano, V. Andrisano, J. Fiori, L. Masotti.

2.47 HISTONE POST-TRANSLATIONAL MODIFICATIONS BY HPLC-ESI-MS AFTER HT29 CELL TREATMENT WITH HDACS INHIBITORS.
C. Parolin, N. Calonghi, M. Naldi, C. Mangano, F. Di Giorgio, J. Hysomema, C. Cappadone, V. Andrisano, A. Mai, L. Masotti

2.48 ANTI-APOPTOTIC EFFECT OF ROS PRODUCED BY NAD(P)H OXIDASE IN HUMAN LEUKAEMIA CELLS
C. Prata, T. Maraldi, D. Fiorentini, L. Zambonin, E. Leoncini; L. Landi, G. Hakim

2.49 GENOMIC APPROACHES TO THE DISCOVERY OF MOLECULAR TARGETS OF LOW MOLECULAR WEIGHT PHOSPHOTYROSIN PROTEIN PHOSPHATASE (LMW-PTP) IN TUMORIGENESIS.
G. Raugei, D. Cirelli, C. De Filippo, D. Cavalieri, R. Marzocchini

2.50 ENZYMATIC METHYLATION OF DEAMIDATED PROTEINS PREVENTS THE APOPTOSIS INDUCED BY OXIDATIVE STRESS OF ENDOTHELIAL CELLS
I. Sambri, R. Capasso, A. Cimmino, L. Masella, V. Zappia, P. Galletti, D. Ingrosso.

2.51 QUALITATIVE AND QUANTITATIVE ANALYSIS OF CARBOXYLIC ACIDS OF CHEMICAL-CLINICAL INTEREST THROUGH THE USE OF COUPLED TECHNIQUES AS HPLC-UV, HPLC-FLD E HPLC-ESI-MS/MS.
C. Scapolla, ,A. Armirotti, A.Salis, E.Millo, U.Benatti, A.Galatini, V.Rocca and G. Damonte

2.52 OSTEOBLAST RESPONSE TO MECHANICAL STRESSES IN VITRO IN THE PRESENCE OF ESTRADIOL
B Cerboni, C Alessandrini, T Doldo, V Micheli, G Pierucci, G Nardi, S Sestini

2.53 microRNA EXPRESSION DURING THE INDUCTION OF CELLULAR SENESCENCE
R. Faraonio, T. C. Nappi, M. Comegna, C. Sedia, M. Santoro, and F. Cimino

3.1 INCREASED ALANINE CONCENTRATION AS METABOLIC BIOMARKER OF EXPOSURE TO ORGANOPHOSPHATES IN INVERTEBRATES.
P. Parenti, M. Forcella, R. Giacchini, S. Bonzini

3.2 THE EFFECTS OF DIFFERENT ANAESTHETICS ON PLASMA SEROTONIN IN *DICENTRARCHUS LABRAX*
A.M. Ferlazzo, G. Bruschetta, E. Burrascano, S. Campo, P. Di Pietro, E. Fazio, F. Gallo, F. Marino, P. Medica

3.3 ANALYSIS OF THE CATHELICIDIN PROTEIN FAMILY IN SALMONIDS
M. Scocchi, A. Pallavicini, G. Cannone, K. Bociek, R. Gennaro

3.4 STRUCTURAL CHARACTERIZATION OF SILICATEIN FROM THE MARINE SPONGE PETROSIA FICIFORMIS
M. Giovine, A. Armirotti, M. Pozzolini, F. Mussino, C. Cerrano, U. Benatti, G. Damonte

3.5 FUNCTIONAL METAPROTEOME ANALYSIS OF MICROBIAL POPULATIONS IN ENRICHMENT CULTURES ON POLYCYCLIC AROMATIC HYDROCARBONS
A. Magini, E. Federici, L. Urbanelli, M. A. Giubilei, B. Tancini, F. Trivelli, G. Cenci, C. Emiliani

3.6 MURINE PULMONARY INFLAMMATORY RESPONSE FOLLOWING INSTILLATION OF SIZE-FRACTIONATED TYRE PARTICLES (TP)
F. Farina, P. Mantecca, G. Sancini, E. Moschini, M. Gualtieri, G. Miserocchi, M. Camatini and P. Palestini

4.1 NUTRITIONAL MODULATION OF IL-23 RECEPTOR AS POTENTIAL REGULATOR OF BOWEL INFLAMMATION.
F. Danesi, M. Philpott, C. Huebner, A. Bordoni, L. Ferguson

4.2 PRO-APOPTOTIC EFFECTS OF *ORIGANUM VULGARE* IN HUMAN COLON CANCER CACO2 CELLS
I. Savini, R. Arnone, M.V. Catani, L. Avigliano

4.3 POLYUNSATURATED FATTY ACIDS IN THE CONTROL OF CHOLESTEROL AND TRYGLICERIDE SYNTHESIS: ROLE OF SREBP
M. Di Nunzio, D. VanDeursen, A. Verhoeven, A. Bordoni

4.4 SUPPLEMENTATION WITH ISOFLAVONES: METABOLIC EFFECTS IN YOUNG HEALTHY WOMEN
L. Massaccesi, D. Erba, P. Foti, G. Testolin, G. Goi

4.5 KINETICS OF THE LIPOPEROXYL RADICAL-SCAVENGING REACTION OF BETANIN AND BETANIDIN IN SOLUTION.
M. Allegra, K. Pawlicka, C. Gentile, L. Tesoriere, M.A Livrea.

4.6 ASCORBIC ACID ANTIOXIDANT ACTIVITY IN PRESENCE OF STABILIZATION AGENTS.
E. Bellocco, D. Barreca, G. Laganà, S. Ficarra, E. Tellone, S. Magazù, F. Migliardo, U. Leuzzi, A. Galtieri

4.7 FIRST EVIDENCE OF POTENTIAL ANTIOXIDANT ACTIVITY OF *ALLIUM SATIVUM* PROTEIN EXTRACT.
E. Bellocco, D. Barreca, G. Laganà, S. Ficarra, E. Tellone, S. Magazù, F. Migliardo, U. Leuzzi, A. Galtieri

4.8 ANTIOXIDANT ACTIVITY IS INCREASED IN *XENOPUS* EMBRYOS DEVELOPED IN SIMULATED MICROGRAVITY
M. Negroni, G. Montorfano, P. Corsetto, P. Berselli, S. Zava, B. Berra and A. M. Rizzo

4.9 LONG CHAIN PUFAS OMEGA-3 INCORPORATION IN CELL MEMBRANE MICRODOMAINS OF BREAST CANCER CELLS
P. Corsetto, G. Montorfano, M. Negroni, A. M. Rizzo and B. Berra

4.10 EFFECT OF POTASSIUM METABISULFITE IN HUMAN FIBROBLASTS
C. Bietolini, G. Cardinali, L. Corte, L. Roscini, A. Magini, V. Ciccarone, B. Tancini, F. Trivelli, C. Emiliani

4.11 AUTOCATALYTIC AND MITOCHONDRIA-ASSISTED FLAVINYLATION OF RECOMBINANT RAT DIMETHYLGLYCINE DEHYDROGENASE.
C. Brizio, S. Iametti, F. Bonomi, P. Ferranti, E. Gianazza, M. Barile

4.12 BODY FAT DISTRIBUTION MAY INFLUENCE BOTH LIPIDEMIC AND OXIDATIVE PROFILES IN WOMEN. RESULTS OF CROSS-SECTIONAL STUDY
C. Cervellati, M. Pedriali, G. Pascale, C. Castaldini, G. La Marca, P. Christodoulou, A. Guariento, G. Bonaccorsi, C.M. Bergamini, F. S. Pansini, F. Casali

4.13 POSSIBLE ROLE OF OXIDATIVE STRESS IN THE PATHOGENESIS OF CHRONIC VENOUS INSUFFICIENCY. PRELIMINARY RESULTS OF A POPULATION STUDY
C. Cervellati, E. Franzoni, E. Masotti, E. Cremonini, A. Guariento, C.M. Bergamini

4.14 STRUCTURE-FUNCTION RELATIONSHIP EVALUATION OF SYNTHETIC VITAMIN E ANALOGUES IN C6 MURINE GLIOMA CELLS AND PERIPHERAL BLOOD LEUKOCYTES.
M. Betti, F. Mazzini, A. Floridi, B. Canonico, F. Luchetti, M. Piroddi, F. Pilolli, M.C. Aisa and F .Galli

4.15 MATERNAL SUPPLEMENTATION WITH HIGH DOSES OF α-TOCOPHEROL INFLUENCES HIPPOCAMPAL PKC ACTIVITY OF RAT OFFSPRING.
M. Betti, A. Floridi, A. Minelli, R. Cuppini, F. Pilolli, M. Piroddi, F. Galli

4.16 DISTRIBUTION OF HOMOCYSTEINE AND CYSTEINE IN PLASMA LIPOPROTEIN FRACTIONS
M.C. Gueli

4.17 SULFORAPHANE MODULATES NRF2/ARE PATHWAY IN CARDIAC CELLS
E. Leoncini, C. Angeloni, M. Malaguti, S. Angelini, P. Hrelia, S. Hrelia.

4.18 IS DNA DOUBLE STRAND BREAKS RECOGNITION RELATED TO LONGEVITY ?
A. Lorenzini, A. Oliver, M. Tresini, V.J. Cristofalo, M. Malaguti, P. Biagi, S. Hrelia and T.D. Stamato

4.19 SULFORAPHANE TREATMENT PREVENTS ACUTE EXHAUSTIVE EXERCISE INDUCED MUSCLE DAMAGE IN RATS.
M. Malaguti, C. Angeloni, M. Baldini, N. Garatachea, J. Gonzalez Gallego, P.L. Biagi, and S. Hrelia

4.20 MODULATION OF THE MMP-9 EXPRESSION BY OLIVE OIL PHENOLS
O. Maschi, M. Dell'Agli, R. Fagnani, E. DeFabiani, G.V. Galli, F. Gilardi, E. Bosisio, D.Caruso

4.21 CHAMOMILE INFUSIONS INHIBIT PROTEASES INVOLVED IN GASTRIC INFLAMMATION.
O.Maschi, M. Dell'Agli, G.V. Galli, E. Bosisio and D. Caruso

4.22 HOMOCYSTEINE-INDUCED ENDOTHELIAL DYSFUNCTION: PROTECTIVE EFFECTS OF OLIVE OIL POLYPHENOLS.
D. Napoli, M. Porcelli, G. Cacciapuoti, I. Peluso, V. Zappia and C. Manna.

4.23 ANTIOXIDANT ACTIVITY IN SOLUTION AND BIOLOGICAL MEMBRANES OF SEVEN CULTIVARS OF *SICILIAN PEACH* (*PRUNUS PERSICA*, L. MILL.)
M. Fazzari, D. Butera, C. Scazzone, G. Volpe, A.M. Pintaudi, L. Di Marco, A. Bono, L. Tesoriere, M. A. Livrea.

4.24 MITOCHONDRIAL LOCALISATION OF THE HUMAN FAD SYNTHETASE ISOFORM 1
E.M. Torchetti, C. Brizio, M. Galluccio, M.R. Napola, M. Roberti, M. Barile

5.1 PRECISION AND ACCURACY IN NON-OPTIMAL REAL-TIME PCR: ADVANTAGES OF A NEW DATA ANALYSIS METHOD
M. Guescini, D. Sisti, M.B.L. Rocchi, L. Stocchi, V. Stocchi

5.2 XYLab: AN INTERACTIVE PLOTTING TOOL FOR MIXED MULTIVARIATE DATA OBSERVATION AND INTERPRETATION
M. Ramazzotti, E. Monsellier, D. Degl'Innocenti

6.1 STRESS BY AROMATIC COMPOUNDS IN SULFOLOBUS SOLFATARICUS: DETOXIFICATION, REGULATION AND BIOMONITORING
G. Fiorentino, R. Ronca, A. Vitale, S. Bartolucci

6.2 TOLUIDINE BLUE-MEDIATED PHOTODYNAMIC EFFECTS ON STAPHYLOCOCCAL BIOFILMS
A. Di Poto, M. S. Sbarra, L. Visai, P. Speziale

6.3 DESIGN, HETEROLOGOUS EXPRESSION AND CHARACTERIZATION OF A CHIMERIC MOLECULE COMPOSED OF TWO PLANT DEFENSE PROTEINS
V. Capuzzi, A.G. Ficca, E. Poerio, N. Bruni and A. Di Maro

6.4 CONJUGATES BETWEEN FOOD PROTEINS AND MAGNETIC NANOPARTICLES: PRODUCTION AND PROPERTIES
M. Marengo, F. Bonom, M. Miriani, S. Cosentino, A. Ferraretto, R. Hempelmann

6.5 GENETIC VARIABILITY ANALYSIS OF APULIA SHEEP BREEDS BY USING MICROSATELLITES.
L. Siculella, F. Damiano, P. Priore and G.V. Gnoni

6.6 FUNGAL LIGNINOLYTIC ENZYMES IN TEXTILE INDUSTRIAL WASTE DEPURATION
F. Balestri, S. Di Gregorio, L. Mariotti, V.Matteini, M.G.Tozzi and R. Lorenzi.

6.7 BINDING EQUILIBRIUM ASSESSMENT BETWEEN MUCIN AND ARABINOGALACTAN, A NO-VISCOUS POLYMER USEFUL IN THE TREATMENT OF DRY EYE
F. Gini, R. Moschini, G. Falcone, E. Boldrini, U. Mura, A. Del Corso

6.8 STRATEGIES COMBINING CELLS AND SCAFFOLDS FOR BONE TISSUE ENGINEERING
E. Saino, V. Maliardi, L. Fassina, M. S. Sbarra, M. G. Cusella De Angelis, G. Magenes, F. Benazzo, L. Visai

8.1 ANTIOXIDANT ACTIVITY OF *OLEA EUROPAEA* DERIVATIVES
L. Bovalini; M. Andreassi, M. Centini, R. Maffei Facino, C. Anselmi.

8.2 NEW APPROACHES TO THE EXPANDED NEWBORN SCREENING PROGRAMS BY TANDEM MASS SPECTROMETRY: REDUCTION OF FALSE-POSITIVES FOR C5, C5OH, C6DC.
F. Di Gaudio, M. D'Ancona, G. La Marca.

8.3 EFFECTS OF HISTONE DEACETYLASE INHIBITORS ON ENERGY METABOLISM
A. Galmozzi, E. Gers, C. Godio, N. Mitro, F. Gilardi, E. Scotti, D. Caruso, M. Crestani, E. De Fabiani.

8.4 PHARMACOLOGICAL POTENTIAL OF INHIBITORS OF 6-PHOSPHOGLUCONATE DEHYDROGENASE FROM *T. brucei*: DIFFERENTIATION OF SUBSTRATE ANALOGUES FROM REACTION-INTERMEDIATE ANALOGUES BY ISOTHERMAL TITRATION CALORIMETRY
S. Hanau, K. Montin, M. Magnani, F. Dall'Occhio.

8.5 USE OF HUMAN RECOMBINANT PROLIDASE TO INVESTIGATE THE ENZYME ACTIVE SITE.
A. Lupi, S. Alleva, V. Minicozzi, S. Morante, F. Stellato, E. Campari, R. Gioia, A. Profumo, A. Rossi, G. Cetta, R. Tenni, A. Forlino.

8.6 THE COMBINED MUTATION OF INTERFACIAL AND ACTIVE-SITE RESIDUES *OF TREPONEMA DENTICOLA* CYSTALYSIN LEADS TO A FOLDED PLP-BOUND MONOMER
R. Montioli, B. Cellini, A. Paiardini, M. Bertoldi and C. Borri Voltattorni.

8.7 CYSTATHIONINE GAMMA-LYASE IN BOVINE LENS
R. Moschini, E. Calvani, A. Del Corso, M. Cappiello, U. Mura.

8.8 STREPTOZOTOCIN-INDUCED DIABETES REDUCES ACTIVITY AND EXPRESSION OF MITOCHONDRIAL CITRATE CARRIER IN RAT LIVER.
P. Priore, A. Giudetti, G. Gnoni.

8.9 SILDENAFIL INHIBITS THE ROS PRODUCTION BY XANTHINE OXIDASE
G. Taibi, G. Carruba, L. Cocciadiferro, A. Cucchiara, and C.M. A. Nicotra.

9.1 PROTEOGLYCAN UNDERSULFATION OF THE GROWTH PLATE IN A SULFATE TRANSPORTER KNOCK-IN MOUSE
M. Facchini, B. Gualeni, M. Viola, A. Passi, A. Forlino, F. Pecora, G. Cetta, A. Rossi

9.2 EXPRESSION AND SUBCELLULAR LOCALIZATION OF SIALIDASE NEU3 IN MDCK CELLS.
G. Zanchetti, N. Papini, M. Franguelli, M. Manzoni, C. Tringali, G. Tettamanti, B. Venerando, L. Caimi, A. Preti, E. Monti, R. Bresciani

9.3 PURIFIED HUMAN GLYCOSAMINOGLYCANS REDUCED NF-kB ACTIVATION, PRO-INFLAMMATORY CYTOKINE PRODUCTION AND APOPTOSIS INDUCED BY LPS IN MOUSE CHONDROCYTES
G. M. Campo, A. Avenoso, S. Campo, A. D'Ascola, P. Traina, D. Samà, A. Calatroni.

9.4 THE ANTIOXIDANT ACTIVITY OF CHONDROITIN-4-SULPHATE, IN CARBON TETRACHLORIDE-INDUCED ACUTE TOXIC HEPATITIS IN MICE, INVOLVES NF-kB AND CASPASE ACTIVATION
G.M. Campo, A. Avenoso, S. Campo, G. Nastasi, P. Traina, A. D'Ascola, A. Calatroni.

9.5 NEU4L SIALIDASE ACCELERATES THE PROLIFERATION RATE IN THE HUMAN NEUROBLASTOMA CELLS, SK-N-BE, THROUGH CELL CYCLE DEREGULATION
F.Cirillo, C.Tringali, B.Lupo, L.Anastasia, N.Papini, G.Lamorte, E.Monti, G.Tettamanti, B.Venerando

9.6 A GLANCE AT THE PHOSPHOPROTEOME OF AZT-TREATED K562 CELLS.
G. D'Andrea, F. Brisdelli, M.E. Schininà, L. Di Francesco, V. Bernabei, A. Bozzi

9.7 SODIUM CARBONATE TREATMENT CAUSES THE RELEASE OF ACIDIC SIALIDASE PRESENT ON HUMAN ERYTHROCYTE MEMBRANE
F. D'Avila, C. Tringali, N. Papini, L. Anastasia, G. Tettamanti, B. Venerando

9.8 SILENCING OF THE MEMBRANE SIALIDASE NEU3 TRIGGERS MEGAKARYOCYTIC DIFFERENTIATION IN CHRONIC MYELOID LEUKEMIC CELLS, K562, THROUGH THE INCREASE OF GANGLIOSIDE GM3 CONTENT
B.Lupo, C.Tringali, F.Cirillo, N.Papini, L. Anastasia, E.Monti, G.Tettamanti, B.Venerando

9.9 NEU3 SIALIDASE STRICTLY MODULATES GM3 LEVELS IN SKELETAL MYOBLASTS C2C12 THUS FAVORING THEIR DIFFERENTIATION AND PROTECTING THEM FROM APOPTOSIS
G. Palazzolo, L. Anastasia, N. Papini, F. Colazzo, C. Tringali, L. Dileo, M. Piccoli, E. Conforti, C. Sitzia, E. Monti, M. Sampaolesi, G. Tettamanti, and B. Venerando

9.10 CHARACTERIZATION OF HUMAN LYSOSOMAL α-D-MANNOSIDASE IN LEUKAEMIC AND NON LEUKAEMIC CELLS
L. Urbanelli, F. Trivelli, A. Magini, B. Tancini, A. Tabilio, C. Emiliani

9.11 RAS MUTANTS SPECIFICALLY REGULATES LYSOSOMAL GLYCOHYDROLASES ACTIVITY IN DIFFERENT CELL MODELS
L. Urbanelli, T. Trivelli, V. Ciccarone, A. Magini, B. Tancini, G. Pelicci, C. Emiliani

9.12 THE CYTOKINE REDUCTION BY GLYCOSAMINOGLYCANS IN LPS-STIMULATED MOUSE CHONDROCYTES MAY INVOLVE TOLL-LIKE RECEPTOR-4 MODULATION
A. Calatroni, A. Avenoso, S. Campo, A. D'Ascola, P. Traina, G. M. Campo.

9.13 STIMULATION/BLOCK OF CD44 RECEPTOR DURING INFLAMMATION BY HYALURONAN AT DIFFERENT MOLECULAR SIZE
A. Calatroni, A. D'Ascola, A. Avenoso, S. Campo, P. Traina, G.M. Campo.

10.1 PROTEIN CHANGES INDUCED BY GROUP B STREPTO-COCCUS INFECTION OF PERITONEAL MACROPHAGES
D. Chiasserini, F. Susta, P. Orvietani, K. Fettucciari, F. Quotadamo, P. Cornacchione, P. Marconi, L. Binaglia

10.2 A FAST MUTAGENESIS APPROACH TO BYPASS INTERNAL AMBER STOP CODONS IN scFvs AND PURIFY RECOMBINANT ANTIBODIES
M. Ramazzotti, F. Niccheri, M.Berardi, M. Belli, D. Degl'Innocenti

10.3 EFFECT OF pH ON MMP-9 RELEASE BY LPS-STIMULATED NEUTROPHILS
A.Trentini, M.C.Manfrinato, M.Castellazzi, E.Fainardi, G.Dallocchio, C.A.Volta and T.Bellini.

11.1 DEFICIENCY OF POLYCYSTIN-1 LEADS TO UPREGULATION OF ADENOSIN A_3 RECEPTOR EXPRESSION IN HUMAN KIDNEY CELL LINES: POSSIBLE MODULATION OF CELL PROLIFERATION BY SPECIFIC RECEPTOR AGONIST.
G. Aguiari, K. Varani, M. Bogo, A Mangolini, C.Durante, P. A. Borea, L. del Senno.

11.2 ANTIOXIDANTS PREVENTS MITOCHONDRIAL DAMAGE AND CELL DEATH IN KERATINOCYTES FROM PERILESIONAL SKIN IN VITILIGO
M. Becatti, C. Fiorillo, F. Prignano, L. Pescitelli, P. Nassi, T. Lotti, N. Taddei.

11.3 CYTOSTATIC EFFECT INDUCED BY 2-CHLOROADENOSINE SENSITISES PC3 CELLS TO DOCETAXEL.
I. Bellezza, C. Conte, A. Tucci, A. Minelli.

11.4 PROTEASOMES ARE NOT A TARGET OF DOXORUBICIN IN FELINE-INJECTION SITE SARCOMA
P. Cascio, M. Martano, E. Morello, R. Bruno, P. Buracco, F. Cerreti.

11.5 NEW LOW CALCEMIC VITAMIN D ANALOGS: 2-METHYLENE-22E-19,26-DINOR-1A,25-DIHYDROXY VITAMIND3 COMPOUNDS, SYNTHESIS AND BIOLOGICAL PROPERTIES.
G.Chiellini, P.K. Grzywacz, L.E. Plum, M. Clagett-Dame, R.Zucchi, H.F. DeLuca.

11.6 SURFACE-ENHANCED RAMAN SPECTROSCOPY (SERS) INVESTIGATION ON ANTI-INFLAMMATORY DRUGS INTERACTING WITH METAL IONSM.
Di Foggia, S. Bonora, A. Maris.

11.7 PROTEOMICS OF PARATHYROID GLANDS: A PRELIMINARY STUDY
F. Ciregia, L. Giusti, F. Cetani, G. Giannaccini, C. Banti, C. Marcocci, A. Pinchera, A. Lucacchini.

11.8 HUMAN COLORECTAL CANCER: GENE EXPRESSION OF PURINE METABOLISM.
R. Leoncini, S. Giglioni, D. Vannoni, E. Aceto, A. Chessa, S. Civitelli, A. Bernini, G. Tanzini, E. Marinello.

11.9 APPLICATION OF PROTEOMICS IN THE STUDY OF ANTIAGGREGANT THERAPY EFFECT IN PATIENTS UNDERWENT PERCUTANEOUS CORONARY INTERVENTION (PCI)
L. Giusti, E. Volpi, F. Ciregia, G. Giannaccini, A. Clerico, A. Lucacchini.

11.10 DEHYDROEPIANDROSTERONE INHIBITS PLATELET AGGREGATION WITH A cGMP-DEPENDENT MECHANISM
A. Rastoldo, C. Sarasso, S. Baldassarri, M. Zanfa, A. Bagarotti, C. Di Vito, A. Bertoni, F. Sinigaglia.

11.11 NICOTINAMIDE N-METHYLTRANSFERASE: A new PROGNOSTIC MARKER FOR ORAL SQUAMOUS cell CARCINOMA.
D.Sartini, A.Santarelli, R.Rocchetti, M.Tomasetti, V.Rossi, V.Pozzi, L.Lo Muzio and M.Emanuelli.

11.12 STREPTOZOTOCIN-INDUCED DIABETES AFFECTS TWO REGULATIVE STEPS OF THE CITRATE CARRIER GENE EXPRESSION IN RAT LIVER
L. Siculella, E. Mercuri, F. Damiano, S. Alemanno and G.V. Gnoni.

11.13 A NOVEL ND5 GENE NUCLEOTIDE VARIANTS ASSOCIATED WHIT OPTICAL SUBATROPHY AND RENAL INVOLVEMENT
Dell'Aglio R., Artuso L., Carrozzo R., Negro R., Iuso A., Papa, S., Torroni A., Guerriero S., Petruzzella V.

11.14 LHON "*PLUS*" PHENOTYPE IN A FAMILY WITH A 3460/ND1 MITOCHONDRIAL DNA MUTATION
Artuso L., Dell'Aglio R., Negro R., Papa S., Carelli V., Guerriero S., Petruzzella V.

12.1 PROBING STRUCTURAL AND DYNAMIC FEATURES IN THE MITOCHONDRIAL OXOGLUTARATE CARRIER BY SITE DIRECTED SPIN-LABELING.
G. Lauria, P. Sanchez, B. Morozzo della Rocca, C.L. Pierri, F. Polizio, I. Stipani, A. Desideri

12.2 EFFECT OF IBUPROFEN ON THE THERMAL BEHAVIOUR OF DMPC LIPOSOMES AS A FUNCTION OF pH
M. Di Foggia, S. Bonora, and A. Torreggiani

12.3 OPA1 LINKS MITOCHONDRIAL GENOME MAINTENANCE TO NETWORK DYNAMICS
S. Vidoni, G. Elachouri, C. Zanna, G. Lenaers and M. Rugolo

12.4 BCL-2 ANTIOXIDANT FUNCTION IN CELLS WITH DEFECTIVE RESPIRATORY COMPLEX I
M. Rugolo, A. Ghelli, L. Iommarini, E. Mariani,M. Hoque, G. Gasparre,A. M. Porcelli

12.5 LIPID RAFT ISOLATION AND POTENTIAL CORRELATION WITH GLUCOSE TRANSPORT AND NOX ACTIVITY IN ACUTE LEUKAEMIA CELLS
C. Prata, L. Zambonin, C. Caliceti, F. Vieceli, D. Fiorentini, G. Hakim, L. Landi

12.6 THE EFFECTS OF SERUM AND CAMP PATHWAY MODULATION ON MITOCHONDRIAL RESPIRATORY CHAIN: TRANSCRIPTIONAL AND POST-TRANSLATIONAL PROCESSES.
Scaringi R, Nicastro A., Iuso A., Scivetti M., Papa F.,Scacco S.

12.7 ANALYSIS OF *D-AKAP1* GENE EXPRESSION IN HUMAN CELLS
M. Carone, R. Vitale, D. Panelli A. M. Sardanelli, and S. Papa

12.8 GENETIC AND PATHOGENETIC MECHANISMS OF TWO TYPES OF HEREDITARY DYSFUNCTION OF COMPLEX I
Iuso A., Scacco S, Trentadue R, Panelli D, Papa S.

12.9 N-ARACHIDONYL-GLYCINE INTERACTION WITH THE RESPIRATORY CHAIN OF RAT LIVER MITOCHONDRIA
Saltarella M., Zaccagnino P., D'Oria S. , Lorusso M.

12.10 β-ADRENERGIC SIGNALLING PATHWAY IN THE MODULATION OF COMPLEX I AND ROS BALANCE
Signorile A., Santeramo A., Scivetti M., Papa F., Papa S.

12.11 TRANS- RETINOIC ACID EFFECT ON RESPIRATORY CHAIN COMPLEX I: PROTEOLYTIC STABILIZATION OF GRIM-19 SUBUNIT IN HUMAN KERATINOCYTES
A. Nicastro, R. Scaringi, A. Iuso, F. Papa, S. Scacco

12.12 RESHAPING OF LIPID MICRODOMAINS IN BOAR SPERM DURING CAPACITATION
L. Botto, N. Bernabò, B. Barboni and P. Palestini

12.13 FUNCTIONAL ANALYSIS OF UCP3 NATURAL MUTANTS IDENTIFIED IN SEVERELY OBESE CHILDREN LIVING IN SOUTHERN ITALY
C.V. Musa, A. Mancini, A. Alfieri, G. Labruna, G. Valerio, A. Franzese, F. Pasanisi, L. Sacchetti and P. Buono

12.14 METABOLIC CONTROL ANALYSIS REVEALS A SUPERASSEMBLED RESPIRATORY CHAIN IN POTATO TUBER MITOCHONDRIA.
M. L. Genova, M. E. Dalmonte, M. Faccioli, A. I. Falasca and G. Lenaz

12.15 CHANGES IN MEMBRANE PHOSPHOLIPIDS COMPOSITION AFFECT ABETA PEPTIDE TOXICITY ON NEURONAL CELL .
E. Cazzaniga, A. Bulbarelli, E. Lonati, M. Masserini

12.16 STRUCTURAL STUDIES ON THE HUMAN ABCC6 TRANSPORTER
MF Armentano, A.Ostuni, R. Miglionico, M.A. Castiglione Morelli and F.Bisaccia

12.17 NEW ASPECTS OF INHIBITORY EFFECT OF NITRIC OXIDE ON THE RESPIRATORY ACTIVITY OF LIVER MITOCHONDRIA
D. Laraspata, V. Palmitessa, V. Gorgoglione, G. La Piana, D. Marzulli, N. E. Lofrumento

12.18 STRUCTURAL STUDIES ON THE HUMAN MRP6
MF. Armentano, F.Bisaccia

12.19 EVIDENCE FOR AEROBIC METABOLISM IN RETINAL ROD OUTER SEGMENT DISKS.
I. Panfoli, D. Calzia , S. Ravera, P. Bianchini, A. Diaspro, G. Candiano, A. Bachi, C. Tacchetti, A. Morelli, I.M. Pepe

13.1 EFFECTS OF PEROXYNITRITE ON LCAT ACTIVITY IN CEREBROSPINAL FLUID
A. Salvatore, A. Carlucci, L. Cigliano, B. Maresca, C.R. Pugliese, G. Puoti, G. Lus, R. Cotrufo, P. Abrescia.

13.2 EFFECT OF NEUROSTEROIDS ON NESTIN, NEUROFILAMENT, B-TUBULIN AND MAP-KINASE ESPRESSION ON RAT BONE MARROW MESENCHYMAL STEM CELL CULTURES
V. Bramanti, D. Bronzi, F. Laureanti, D. Tomassoni, F. Amenta, G. Li Volsi, R. Avola

13.3 CHARACTERIZATION OF BIOTIN-ANANDAMIDE, A NOVEL TOOL FOR THE VISUALIZATION OF ANANDAMIDE ACCUMULATION.
C. De Simone, F. Fezza, S. Oddi, M. Di Tommaso, C. Rapino, N. Pasquariello, E. Dainese, A. Finazzi-Agrò, M. Maccarrone

13.4 EFFECT OF CARNOSINE AND TREHALOSE ON PARP-1 AND 2 EXPRESSION UNDER PROINFLAMMATORY STRESS CONDITIONS
V. Spina Purrello, S. Giliberto, G. Grasso , R. Greca, V. G. Nicoletti, E. Rizzarelli and A. M. Giuffrida Stella.

13.5 AGGREGATION AND ASSEMBLY OF Aβ(25-35) IN WATER
L. Millucci, R. Raggiaschi, D. Franceschini, G. Gaviraghi, P. Martelli, G.C. Terstappen, A. Santucci

13.6 TSPO EXPRESSION ON MURINE MODEL OF OBESITY
L. Schmid, A. Pirone, L. Betti, L. Fabbrini, M. Lanza, L. Palego, G. Mascia, C. Lenzi, O.Fabiani, G. Giannaccini, A. Lucacchini

13.7 PIN1: A NEW OUTLOOK IN THE STUDY OF ALZHEIMER'S DISEASE
E. Lonati, E. Cazzaniga, M. Masserini and A. Bulbarelli.

13.8 DISTRIBUTION OF THE NUCLEAR PROTEIN TDP-43 IN THE CYTOPLASM AS FULL LENGTH MOLECULE AND UBIQUITIN-TAGGED-DERIVATIVE IS A FEATURE OF BRAIN CORTEX AND PINAL CORD IN SPORADIC AMYOTROPHIC LATERAL SCLEROSIS.
G. De Marco, M. Piccinini, B. Buccinnà, E. Lupino, C. Ramondetti, A. Lomartire, S. Grifoni, M.T. Giordana, M.T. Rinaudo

13.9 PROTEOMIC ANALISYS OF GLYCOPROTEIC PATTERN IN MILD COGNITIVE IMPAIRMENT AND ALZHEIMER DISEASE HIPPOCAMPUS
F. Di Domenico, C. Cini,C. Blarzino, C.Foppoli, F. De Marco, D.A. Butterfield, R. Coccia

13.10 PROTEIN OXIDATION AND CELLULAR STRESS RESPONSE IN AGING BRAIN: A REDOX PROTEOMICS APPROACH
M. Perluigi, F. Di Domenico, M. Cavallaro, F. Bellia, M.T. Cambria, A.M. Giuffrida Stella, E. Rizzarelli, V. Calabrese and C. Cini

13.11 RESTRICTED EXPRESSION OF RELAXIN 3 GENE IN THE BRAIN OF DEVELOPING ZEBRAFISH EMBRYO
A. Donizetti, M. Grossi, P. Pariante, S. Minucci , F. Aniello.

13.12 GLIOMA AND MITOCHONDRIAL PERMEABILITY TRANSITION: NEW CHEMOTHERAPY TARGET.
E.Da Pozzo, B.Chelli, B.Costa, A.Lena, M.R.Rechichi, A.Salvetti, P.Gabelloni, V.Gremigni, C.Martini, L.Rossi

13.13 IMPAIRMENT OF METHYLATION CYCLE IN TREATED PATIENTS WITH PARKINSON'S DISEASE
M. C. Gueli

13.14 ANT1 EXPRESSION AND RAGE-NF-KB PATHWAY ACTIVATION IN SPORADIC INCLUSION BODY MYOSITIS
V. Macaione, M. Aguennouz,De Pasquale MG, R. Crupi, F. Festa, G. De Luca, R.M. Di Giorgio, G. Vita, A. Toscano, C. Rodolico

13.15 SILENCING OF HEXA AND HEXB GENES REVEALES A ROLE OF β-HEXOSAMINIDASE IN NEUROINFLAMMATION
S. Martino, R. Tiribuzi, I. di Girolamo, G. Makrypidi, A. Tortori, A.C. Berardi, G.F. Bottazzo, and A. Orlacchio

13.16 OXYGEN CONSUMPTION AND ATP SYNTHESIS IN ISOLATED MYELIN: A NEW TROPHIC ROLE OF MYELIN SHEATH.
S. Ravera, I. Panfoli, D. Calzia, P. Bianchini, C. Tacchetti, A. Diaspro, G. Mancardi, A.Morelli

13.17 pLG72 BINDING TO D-AMINO ACID OXIDASE MODULATES D-SERINE CONCENTRATION IN HUMAN BRAIN.
S. Sacchi, G. Bernasconi, L. Caldinelli, M. Martineau, J.P. Mothet, L. Pollegioni and G. Molla

13.18 ALTERATION OF TRANSMETHYLATION REACTIONS IN AMYOTROPHIC LATERAL SCLEROSIS
A. Salvatore, M.L. De Bonis, S. D'Angelo, M.R. Monsurrò, G. Tedeschi, P. Galletti

13.19 EVALUATION OF NEUROACTIVE STEROID LEVELS, BIOCHEMICAL AND FUNCTIONAL PARAMETERS IN AN EXPERIMENTAL MODEL OF NERVE CRUSH INJURY
S. Scurati, I. Roglio, S. Giatti, M. Pesaresi, F. Giavarini, R.C. Melcangi and D. Caruso

13.20 TRANSLOCATOR PROTEIN (TSPO) AND IDENTIFICATION OF NEW MOLECULAR DIAGNOSIS TOOLS.
E.DaPozzo, B.Costa, B.Chelli, S.Taliani, M.Bellandi, F.Da Settimo, S.Daniele, V.Gremigni, L.Rossi, C.Martini.

13.21 LIPID RAFT CHOLESTEROL PROTECTS AGAINST AMYLOID CYTOTOXICITY.
M. Zampagni, G. Liguri, C. Bernacchioni, A. Pensalfini, M. Stefani, A. Relini, D. Nichino, A. Gliozzi, C. Cecchi

13.22 EXTRACELLULAR UPTAKE OF AMYLOID BETA AGGREGATES BY A CLATHRIN-DEPENDENT, RAFT-MEDIATED MECHANISM.
M. Zampagni, C. Cecchi, A. Pensalfini, M. Becatti, E. Evangelisti, F. Romano, G. Liguri

13.23 POTENTIAL CYTOPROTECTIVE EFFECTS OF A NEW POLY(ADP-RIBOSE)POLYMERASE INHIBITOR
L. Mosca, I. De Zottis, C. Masi, C. Potestà, I. Tempera, A. Masci, A. Mai, M. d'Erme

14.1 PYRIMIDINE NUCLEOSIDE TRIPHOSPHATES ARE MAINTAINED AT HIGH LEVELS DURING MASSIVE ATP BREAKDOWN INDUCED BY ANOXIC-LIKE CONDITIONS IN CENTRAL NERVOUS SYSTEM CELLS.
F. Balestri, C. Barsotti, M. Camici, M.G. Tozzi, P.L. Ipata.

14.2 TEMPERATURE DEPENDENCE AND KINETIC STUDIES ON THE HUMAN CYTIDINE DEAMINASE MUTANT ENZYME Y33G.
F.M. Carpi, S. Vincenzetti, D. Micozzi, S. Pucciarelli, P. Mariani, V. Polzonetti, P. Natalini, A. Vita.

14.3 EARLY PRESENCE OF A MYCOVIRUS IN CHERRY RUSTY SPOT DISEASE BEFORE EVIDENCE OF LEAF ALTERATION.
R. Carrieri, I. D'Elia, A. Ragazzino, G. Geraci, R. del Gaudio

14.4 CYCLIC NUCLEOTIDES LEVELS AND cAMP/cGMP PHOSPHODIESTERASES ACTIVITY EVALUATION IN STEM CELLS OF THE HUMAN PERIODONTAL LIGAMENT (PDL-MSCs)
L. D'Alessandro, M. Paludi, D. Vitale, S. Di Marco, M. Iannetti, O. Trubiani and G. Spoto

14.5 DIFFERENT EXPRESSION OF NICOTINAMIDE MONONUCLEOTIDE ADENYLYLTRANSFERASE ISOZYMES IN NORMAL AND PATHOLOGICAL TISSUES AND CELL LINES.
M. Di Stefano, S. Scotti, L. Galassi, F. Mazzola, A. Poloni and G. Magni

14.6 EVALUATION OF BIOMARKERS IN PATIENTS WITH HAND OSTEOARTHRITIS
L.D. Kozaci, O.F. Sendur, P. Ruggeri, N. Franceschini

14.7 ALA147THR SUBSTITUTION IN TSPO IS ASSOCIATED TO ADULT SEPARATION ANXIETY IN PATIENTS WITH DEPRESSION
P. Gabelloni, B. Costa, S. Pini, M. Abelli, G.B. Cassano, C. Martini.

14.8 A GENE EXPRESSION STUDY FOR B-CELL CHRONIC LYMPHOCYTIC LEUKEMIA
E. Marinello, V.Tommassini, F. Carlucci, B. Pisano, A. Tabucchi

14.9 LEVELS OF HOMOCYSTEINE AND TRANSAMINASE
E. Marinello, L. Terzuoli, R. Zanaboni, B. Porcelli, M.G. Coppola, A. Cito, R. Pagani

14.10 IDENTIFICATION OF INOSINE NUCLEOTIDES IN CELLULAR RNA BY AN HPLC-LINKED METHOD: POSSIBLE UTILIZATION IN THE STUDY OF mRNA EDITING
A.. Guerrini, G. Jacomelli, M.R. Corradi, M. Bertelli, G. Pompucci, V. Micheli

14.11 MECHANISTIC STUDIES ON STRUCTURE AND FUNCTION OF CYTOSOLIC 5'-NUCLEOTIDASE (cN-II): EFFECTOR SITES AND SUBUNITS INTERACTION.
R. Pesi, M.G. Careddu, S. Allegrini, E. Lazzari, G. Orsini, F. Zucchi , MG. Tozzi

14.12 MECHANISTIC STUDIES ON STRUCTURE AND FUNCTION OF CYTOSOLIC 5'-NUCLEOTIDASE: POINT MUTANT Y55G.
R. Pesi, M.G. Careddu, S. Allegrini, D. Filoni, E. Piccinini, M.G. Tozzi

14.13 CHARACTERIZATION OF THE TRANSCRIPTIONAL REGULATOR OF THE NRTR FAMILY CONTROLLING NAD SALVAGE IN g-PROTEOBACTERIA
C. Mancini, L. Galeazzi, J. De Ingeniis, A. Amici, N. Raffaelli

14.14 PARTIAL INHIBITION OF *ESCHERICHIA COLI* DIHYDROFOLATE REDUCTASE BY SPERMINE
G. S. De Fronzo, M. R. Carradori, P. Natalini, S. Vincenzetti, V. Polzonetti, L. Signorello, A. Vita, and S. Pucciarelli.

15.1 ARCHAEAL PROTOGLOBIN STRUCTURE SUGGESTS NOVEL LIGAND DIFFUSION PATHS AND HEME-REACTIVITY MODULATION.
M. Nardini, A. Pesce, L. Thijs, J.A. Saito, S. Dewilde, M. Alam, P. Ascenzi, M. Coletta, L. Moens, M. Bolognesi

15.2 MOBILITY OF MEMBRANE PROTEINS EXPLORED BY SINGLE PARTICLE TRACKING (SPT): THE LATERAL DIFFUSION OF THE GLYCINE RECEPTOR DEPENDS ON THE OLIGOMERIZATION STATE OF THE SCAFFOLDING PROTEIN GEPHYRIN.
M. Calamai, C. Specht, A. Triller

15.3 ESPRESSION OF THE RHODANESE-LIKE PROTEIN RhdA IN A. VINELANDII UNDER OXIDATIVE STRESS CONDITIONS.
W. Remelli, A. Cereda , S. Pagani and F. Forlani

15.4 ROLE OF RESIDUES Asp161, Arg164 AND Glu211 IN *MYCOBACTERIUM TUBERCULOSIS* NADPH-FERREDOXIN REDUCTASE
F. Ciriello, P. Vella, A. Pennati, V. Pandini, G. Zanetti, A. Aliverti

15.5 A PLANT-TYPE ENZYME AS A PUTATIVE TARGET FOR NOVEL ANTIMALARIAL DRUGS: PROPERTIES OF THE *PLASMODIUM FALCIPARUM* FERREDOXIN-NADP$^+$ REDUCTASE.
D. Crobu, M. Milani, P. Vella, V. Pandini, M. Bolognesi, G. Zanetti, A. Aliverti.

15.6 SMALL ANGLE X-RAY SCATTERING STUDIES REVEAL IMPORTANT CLUES FOR MEMBRANE BINDING AND ACTIVITY OF FATTY ACID AMIDE HYDROLASE (FAAH)
E. Dainese, A. Sabatucci, C. Angelucci, C. Di Pancrazio, N. Pasquariello, Cravatt B. F. and M. Maccarrone

15.7 MODULATION OF THE ENZYMATIC ACTIVITY AND MEMBRANE BINDING PROPERTIES OF SOYBEAN LIPOXYGENASE-1 THROUGH LIMITED PROTEOLYSIS AND METAL SUBSTITUTION
E. Dainese, C. Angelucci, A. Sabatucci, V. De Filippis, G. Mei, I. Cozzani and M. Maccarrone

15.8 OXIDATIVE RESPONSES IN DIFFERENT HUMAN GASTRIC NEOPLASMS
M. Monari, J. Foschi, P. Cortesi, C. Calabrese, G. Di Febo, G.P. Serrazanetti

15.9 IDENTIFYING DISORDERED REGIONS IN PROTEINS BY LIMITED PROTEOLYSIS
A. Fontana, P. Polverino de Laureto, B. Spolaore, E. Frare and M. Zambonin

15.10 L-ASPARTATE OXIDASE AND QUINOLINATE SYNTHASE FROM *B. SUBTILIS*
S. Nonnis, I. Marinoni, A. Alberini, A., Negri, C. Monteferrante, P. Heathcote, E.,Hartig, L. H. Böttger, A. X. Trautwein, S. Ronchi, G. Tedeschi

15.11 PROTEOMIC ANALYSIS OF XENOPUS EMBRYOS EXPOSED TO SIMULATED MICROGRAVITY
S. Nonnis, L. Pagliato, A. Negri, M. Negroni , S. Ronchi, A.M. Rizzo, G . Tedeschi

15.12 INTERACTION OF PROTEASE INHIBITORS FROM MUCUNA PRURIENS SEEDS EXTRACT (MPE) WITH ECHIS CARINATUS VENOM PROTEASES.
N. Hope-Onyekwere, R. Guerranti, C. Muzzi, A. Cortelazzo, E. Marinello, R. Pagani.

15.13 PROTEOMIC ANALYSIS AS NEW RESEARCH TO IDENTIFY VULNERABLE PLAQUE
R. Pagani, I. Ciari, C. Felici, L. Terzuoli, G. de Donato, M. Giubbolini, C. Setacci, C. Banfi, M. Brioschi, E. Tremoli, B. Porcelli

15.14 STUDIES ON *MYCOBACTERIUM TUBERCULOSIS* GLUTAMYL-tRNA SYNTHETASE
S. Paravisi, M. A. Vanoni

15.15 THE INTERACTION OF APOMYOGLOBIN WITH OLEIC ACID LEADS TO A PROTEIN COMPLEX THAT DISPLAYS CELLULAR TOXICITY
O. Pinato, B. Spolaore, M. Canton, P. Polverino de Laureto and A. Fontana

15.16 DPS PROTEINS AND OXIDATIVE PROCESSES IN THE THERMOPHILIC CYANOBACTERIUM T. ELONGATUS
F. Alaleona, P. Ceci, S. Franceschini, A. Ilari, E. Chiancone

15.17 INVESTIGATING THE DRUG-STABILIZING ABILITY OF BETA-LACTOGLOBULIN
A. Barbiroli, P. Rasmussen, T. Beringhelli, S. Scanu, P. Ferranti, F. Bonomi, S. Iametti

15.18 INFLUENCES OF TERNARY AND QUATERNARY COMPLEXES ON ORNITHINE TRANSCARBAMOYLASE STABILIZATION PROCESS
E. Bellocco, D. Barreca, G. Laganà, S. Ficarra, E. Tellone, S. Magazù, F. Migliardo, U. Leuzzi, A. Galtieri

15.19 THE THREE-FOLD "FERRITIN-LIKE" PORES CONTROL BOTH THE IRON UPTAKE AND RELEASE PROCESSES IN LISTERIA INNOCUA DPS. A STUDY WITH SITE-SPECIFIC MUTANTS.
G. Bellapadrona, S. Stefanini, E. Theil, and E. Chiancone.

15.20 ESI-MS/MS DETERMINATION OF LYSOZYME CONFORMATION CHANGES IN PRESENCE OF TREHALOSE.
E. Bellocco, D. Barreca, G. Laganà, S. Ficarra, E. Tellone, S. Magazù, F. Migliardo, U. Leuzzi, A. Galtieri

15.21 A MODEL FOR THE AGGREGATION OF THE ACYLPHOSPHATASE *FROM SULFOLOBUS SOLFATARICUS* IN ITS NATIVE-LIKE STATE
F. Bemporad, T. Vannocci, L. Varela, A. Azuaga, F. Chiti

15.22 FLUORESCENCE STUDIES ON HUMAN SERUM BUTYRYLCHOLINESTERASE: EFFECT OF TEMPERATURE AND PALMITOYLETHANOLAMIDE, AN ENDOGENOUS ANTI-INFLAMMATORY LIPID.
E.Bertoli, L.Ragni, I.Pirisinu, M.Camponi, G.Rosi, R.Romani, A.Ambrosini, G.Zolese

15.23 OLIGO-BRANCHED PEPTIDES AS MULTIVALENT AND MODULAR TUMOR TARGETING AGENTS
C. Falciani, B. Lelli, S. Pileri, J. Brunetti, A. Pini, A. Cappelli, N. Ravenni, C. Pagliuca, L. Bencini, S. Menichetti, R. Moretti, L. Bracci

15.24 STRUCTURALLY DISTINCT PROTOFIBRILS OF HYPF-N SHOW DIFFERENT CYTOTOXIC EFFECTS
S. Campioni, B. Mannini, C. Cecchi, C. Parrini, A. Relini, M. Stefani, F. Chiti

15.25 REGULATION OF AMYLOID PRECURSOR PROTEIN PROTEOLYSIS BY CALMODULIN BINDING
I. Canobbio, L. Cipolla, C. Balduini, M. Torti

15.26 ENZYMATIC PROPERTIES OF RECOMBINANT PEPSINS FROM THE ANTARCTIC ROCK COD *TREMATOMUS BERNACCHII*.
V. De Luca, G. Maria, V. Carginale, A. Capasso, G. De Mauro, S. Brier, J.R. Engen, C. Capasso

15.27 ON THE MECHANISM OF SELF-REGULATION OF GLUTAMATE SYNTHASE: THE ROLE OF K937 AND M479
G. Caprini, M. A. Vanoni

15.28 HUMAN CYTIDINE DEAMINASE MUTANT ENZYME Y33G: STUDIES OF PROTEIN STABILIZATION MEDIATED BY CHAPERONES.
D. Micozzi, S. Vincenzetti, F.M. Carpi, S. Pucciarelli, P. Mariani, V. Polzonetti, P. Natalini, A. Vita

15.29 EXPRESSION IN *PICHIA PASTORIS* AND CHARACTERIZATION OF CERATO-POPULIN, A FUNGAL PROTEIN BELONGING TO THE CERATO-PLATANIN FAMILY
L. Carresi, E. Pagni, F. Martellini, C. Comparini, B. Pantera, A. Scala, G. Cappugi, L. Pazzagli

15.30 MOLECULAR APPROACHES TO STUDY THE MITOCHONDRIAL BEHAVIOUR DURING A MYOGENIC PROGRAM
L. Casadei, E, Barbieri, M. Guescini, G. Piccoli, M. Battistelli, P. Ceccaroli, L. Vallorani, A. D'Emilio, E. Falcieri, V. Stocchi

15.31 PROTEOMIC APPROACH IN C2C12 MYOBLAST DIFFERENTIATION
L. Casadei, L.Vallorani, A.M Gioacchini, M. Guescini, S. Burattini, A. D'Emilio, L. Biagiotti, E. Falcieri, V. Stocchi

15.32 PROTEOMIC ANALYSIS OF THE TOXICITY OF OCHRATOXIN A IN MOUSE KIDNEY
V. Cipriani, N. Ercole, G. Codogno, F. Marsano, M. Cavaletto

15.33 INSIGHTS INTO THE CATALYTIC MECHANISM OF THE BCP FAMILY: FUNCTIONAL AND STRUCTURAL ANALYSIS OF BCP1 FROM SULFOLOBUS SULFATARICUS
K. D'Ambrosio, D. Limauro, E. Pedone, I. Galdi, C. Pedone, S. Bartolucci, G. De Simone

15.34 IDENTIFICATION OF MOLECULAR PARTNERS OF MLL-AF4 ONCOPROTEINS.
F. De Falco, M.R. Imperato, M. D'Antonio, G. Esposito, F. Salvatore

15.35 THE Ses i 2 MAJOR ALLERGEN FROM SESAMUM INDICUM: PURIFICATION, STRUCTURE, STABILITY AND ANTI-THROMBIN ACTIVITY
D. Galla, N. Pozzi, V. De Filippis

15.36 MOLECULAR INSIGHTS INTO THE INTERACTION BETWEEN ALPHA-SYNUCLEIN AND PUFAS
G. De Franceschi, A. Fontana and P. Polverino de Laureto

15.37 SEMI-SYNTHESIS OF CONSENSUS TETRATRICOPEPTIDE REPEAT PROTEINS FOR FOLDING STUDIES
L. De Rosa, A. Romanelli, L. Regan, A. L. Cortajarena, G. Haran, L. D. D'Andrea

15.38 PROTEIN ENGINEERING OF REPRESSOR OF PRIMER (ROP): CONSTRUCTION OF MOLECULAR SCAFFOLDS FOR THE INTRODUCTION OF NEW FUNCTIONS.
G. Di Nardo, A. Di Venere, A. Ortolani, G. Mei, S. Sadeghi, G. Gilardi

15.39 EXPRESSION OF EXTRACELLULAR DOMAINS OF VEGF RECEPTORS AND THEIR USE IN NMR DRUG DISCOVERY STUDIES.
R. Di Stasi, D. Capasso, D. Diana, R. Fattorusso, C. Pedone, L. D. D'Andrea

15.40 THE THREE-DIMENSIONAL STRUCTURE OF THE GLOBIN DOMAIN OF GLOBIN-COUPLED SENSOR FROM GEOBACTER SULFURREDUCENS
M. Nardini, A. Pesce, L. Thijs, S. Dewilde, A. Penco, P. Ascenzi, L. Moens, M. Bolognesi

15.41 THE Na+-BOUND AND Na+-FREE CONFORMATION OF HUMAN Alpha-THROMBIN PROBED BY SOLUTION STUDIES
V. De Filippis,N. Pozzi, and R. Frasson

15.42 ALLOSTERIC AND BINDING PROPERTIES OF A TRUNCATED RECOMBINANT HUMAN SERUM ALBUMIN – AN OPTICAL AND NMR SPECTROSCOPIC INVESTIGATION
G. Fanali , P. Ascenzi, M. Fasano

15.43 NON STEROIDAL ANTI-INFLAMMATORY DRUGS MODULATE MATRIX METALLOPROTEINASES EXPRESSION IN HUMAN RHEUMATOID SYNOVIAL CELLS
P. Ruggeri, S. Vessillier, N. Franceschini

15.44 HUMAN alpha□-THROMBIN EXPRESSED IN *E. COLI*: PRODUCTION AND CHEMICAL, CONFORMATIONAL, AND FUNCTIONAL CHARACTERISATION
R. Frasson, N. Pozzi, D.J. Johnson, J.A. Huntington and V. De Filippis

15.45 REDOX PROTEOMICS OF 3'-NITRO-TYROSINE-CONTAINING PROTEINS IN UREMIC PLASMA
Piroddi M, Pilolli F, Palmese A, Amoresano A, Pucci P and Galli F

15.46 RNase A OLIGOMERIZATION THROUGH 3D DOMAIN SWAPPING IS AFFECTED BY A RESIDUE LOCATED FARAWAY FROM THE SWAPPING DOMAINS
G. Gotte and F. Vottariello

15.47 ACTIVITY OF PROTEASES FROM ECHIS CARINATUS VENOM ON HUMAN PLASMA PROTEINS
N. Hope-Onyekwere., R. Guerranti., A. Cortelazzo., C. Muzzi., R. Pagani., E. Marinello.

15.48 LACTOFERRIN INHIBITS PAPAIN-LIKE CYSTEINE PROTEASES.
Leboffe L., Antonini G.

15.49 ISOFORM SPECIFIC PHOSPHORYLATION OF THE N-TERMINAL DOMAIN OF p53 BY PROTEIN KINASE CK1 - AN IN VITRO ANALYSIS
A. Venerando, O. Marin, L.A. Pinna

15.50 PRODUCTION AND CHARACTERISATION OF THERAPEUTIC PROTEINS IN *NICOTIANA BENTHAMIANA*.
F. Maset, V. De Filippis, F. Vantini, M. Gastaldelli, A. Olivieri and S. Boninsegna

15.51 THE HUMAN KERATOEPITHELIN FAS1-4 DOMAIN AS A MODEL FOR THE STUDY OF PROTEIN MISFOLDING AND AMYLOIDOGENESIS: RECOMBINANT EXPRESSION AND PRELIMINARY CHARACTERISATION.
S. Morlacchi, F. Sciandra, MG. Bigotti, AR. Clarke, A. Cameron, SB. Burston, M. Bozzi & A. Brancaccio.

15.52 IDENTIFICATION OF THE MMP-9 CLEAVAGE SITE ON THE RECOMBINANT BETA-DYSTROGLYCAN ECTODOMAIN.
S. Morlacchi, M. Bozzi, E. Pavoni, R. Inzitari, S. Monaco, M. Gioia, S. Marini, F. Sciandra, M. Castagnola, B. Giardina, M. Coletta, A. Brancaccio

15.53 AMYLOID CONFORMATIONS OF HUMAN LYSOZYME
M.F. Mossuto, A. Dhulesia, P. Polverino de Laureto, X. Salvatella, C.M. Dobson

15.54 NOVEL ANTIBACTERIAL PEPTIDES ISOLATED FROM THE EUROPEAN PAPER WASP, *POLISTES DOMINULUS* (HYMENOPTERA, VESPOIDEA)
E. Pagni, B. Pantera, L. Carresi, G. Mastrobuoni, S. Turillazzi, G. Cappugi and L. Pazzagli

15.55 STRUCTURAL INSIGHTS OF HEPARIN-BINDING HAEMAGGLUTININ FROM *M. TUBERCULOSIS*
E. Pedone C.Esposito, A.Ruggiero, M.V.Pethoukov, D. I.Svergun, C. Pedone and R. Berisio

15.56 STRUCTURAL AND FUNCTIONAL CHARACTERIZATION OF TWO RIBONUCLEOSIDE HYDROLASES FROM THE ARCHAEON SULFOLOBUS SOLFATARICUS.
I. Peluso, A. Marabotti, A. Facchiano, D. Napoli, C. Cacciapuoti, M. Porcelli

15.57 CATALYTIC FIBRILS, A NANOSCALE BIOMATERIAL GENERATED BY AMYLOID FIBRILS FUNCTIONALIZED WITH AN ENZYME
F. Guglielmi, D.M. Monti, A. Arciello, S. Torrassa, F. Cozzolino, M. Monti, A. Relini and R. Piccoli

15.58 IN VITRO AND IN VIVO ANTIMICROBIAL ACTIVITY OF A BRANCHED PEPTIDE SPECIFIC FOR GRAM-NEGATIVE BACTERIA.
A. Pini, C. Falciani, S. Bindi, S. Iozzi, J. Brunetti, E. Marcelli, F. Bonelli, E. Mantengoli, G.M. Rossolini, L. Bracci

15.59 ON THE OXYGEN REACTIVITY OF FLAVOOXIDASES
L. Piubelli, M. Pedotti, G. Molla, S. Ghisla, M.S. Pilone, L. Pollegioni

15.60 CHARACTERIZATION OF OLIGOMERIC SPECIES ON THE AGGREGATION PATHWAY OF HUMAN LYSOZYME
P. Polverino de Laureto, E. Frare, M.F. Mossuto, S. Tolin, M. Dumoulin, C. M. Dobson and A. Fontana

15.61 BETA-2 GLYCOPROTEIN-I INHIBITS THROMBIN-MEDIATED PLATELET AGGREGATION AND PAR-1 HYDROLYSIS
N. Pozzi, R. De Cristofaro, D. Zaramella, V.Pengo and V. De Filippis

15.62 STRUCTURAL EFFECTS OF MUTATING THE CATALYTIC ASP102 IN HUMAN ALPHA-THROMBIN
N. Pozzi, E. Di Cera and V. De Filippis

15.63 UNRAVELLING AF4 CROSS-TALK MAY GIVE HINTS INTO THE MOLECULAR PATHOGENESIS OF ACUTE LYMPHOBLASTIC LEUKAEMIA
D. Sabbatino, A. Cuomo, T. Fioretti, A. Cevenini, G. Esposito, M. Ruoppolo, F. Salvatore

15.64 A PROTEOMIC AND RT-QPCR INTEGRATED APPROACH FOR THE IDENTIFICATION AND CHARACTERIZATION OF DEFENSE PROTEINS EXPRESSED IN GERMINATING LUPIN SEEDS.
A. Scarafoni, A. Ronchi, B. Prinsi, L. Espen, M. Duranti

15.65 CACO-2 CELL MONOLAYERS TO STUDY THE TRANSPORT OF LUPIN CONGLUTIN γ ACROSS HUMAN INTESTINAL EPITHELIUM.
A. Scarafoni, J. Capraro, C. Magni, A. Clemente, M. Duranti

15.66 STRUCTURAL FEATURES OF TREHALOSE-MALTOSE BINDING PROTEIN FROM THERMUS THERMOPHILUS: STRATEGIES OF THERMAL ADAPTATION AT HIGH TEMPERATURES.
A. Scirè, A. Marabotti, V. Aurilia, M. Staiano, P. Ringhieri, L. Iozzino, R. Crescenzo, S. D'Auria, E. Bertoli, F. Tanfani

15.67 THE DEGREE OF STRUCTURAL PROTECTION AT THE EDGE β-STRANDS DETERMINES THE PATHWAY OF AMYLOID FORMATION IN GLOBULAR PROTEINS.
G. Soldi, F. Bemporad, F. Chiti

15.68 PROTEOMIC APPROACH TO ANALYSE PLASMA PROTEIN CHANGES EXERCISE-INDUCED IN HORSES: A PRELIMINARY STUDY.
E. Chiaradia, L. Avellini, M. Tartaglia, A. Gaiti

15.69 ANALYSIS OF HORSE SYNOVIAL FLUID BY TWO-DIMENSIONAL GEL ELECTROPHORESIS: A PILOT STUDY.
M. Tartaglia, L. Avellini, V.M. Masi, M.Pepe, A. Gaiti, E. Chiaradia

15.70 STRUCTURAL AND FUNCTIONAL PROPERTIES OF "ZERO-LENGTH" RIBONUCLEASE A OLIGOMERS
F. Vottariello, C. Costanzo, G. Gotte, and M. Libonati

15.71 ON THE MECHANISM OF L-LACTATE OXIDATION CATALYZED BY FLAVOCYTOCHROME B2: A COMBINED FIRST PRINCIPLE MOLECULAR DYNAMICS AND SITE DIRECTED MUTAGENESIS STUDY.
D. Zucchini, G. Caprini, G. Tabacchi, E. Fois and M. A. Vanoni

15.72 FUNCTIONAL CHARACTERIZATION OF A PSYCHROPHILIC THIOREDOXIN SYSTEM
G. Evangelista, P. Falasca, R. Cotugno, M. Masullo, E. De Vendittis, G. Raimo

16.1 RETINOIC ACID BIOSYNTHESIS IS INHIBITED BY ESTROGEN
G. Taibi, G. Carruba, L. Cocciadiferro, C.M. A. Nicotra

16.2 REDOX REGULATION OF ANOIKIS: REACTIVE OXYGEN SPECIES AS ESSENTIAL MEDIATORS OF CELL SURVIVAL
E. Giannoni, F. Buricchi, G. Grimaldi, M. Parri, F. Cialdai, G. Raugei, G. Ramponi and P. Chiarugi

16.3 S1P1 RECEPTOR NEGATIVELY REGULATES PDGF-DEPENDENT PROLIFERATION OF C2C12 MYOBLASTS.
C. Donati, P. Nincheri, F. Cencetti, C. Bernacchioni, E. Rapizzi, P. Bruni

16.4 TARGETING THE X-LINKED INHIBITOR OF APOPTOSIS PROTEIN (XIAP) THROUGH 4-SUBSTITUTED AZABICYCLO[5.3.0]ALKANE SMAC-MIMETICS. STRUCTURE, ACTIVITY AND RECOGNITION PRINCIPLES.
F. Cossu , E. Mastrangelo, M. Milani, G. Sorrentino, D. Lecis, D. Delia, P.Seneci, C. Scolastico and M. Bolognesi

16.5 TRANSFORMING GROWTH FACTOR-β ELICITS MYOFIBROBLASTIC DIFFERENTIATION OF MYOBLASTS VIA SPHINGOSINE KINASE/ SPHINGOSINE 1-PHOSPHATE AXIS.
F. Cencetti, C. Donati, C. Bernacchioni, E. Rapizzi, P. Nincheri, P. Bruni

16.6 PRO-APOPTOTIC AND CYTOSTATIC ACTIVITY OF NATURALLY OCCURRING CARDENOLIDES.
E. Bloise, P. Torre, N. De Tommasi, M. A. Belisario

16.7 CAMPTOTHECIN INDUCES S-PHASE ARREST AND APOPTOSIS IN MG63 OSTEOSARCOMA CELLS
A. De Blasio, R. Di Fiore, C. Messina, P. Di Fazio, G. Costa, A. Attanzio and R. Vento

16.8 PROLONGED TREATMENT OF HUMAN OSTEOSARCOMA MG63 CELLS WITH 3AB INDUCES THE PRODUCTION OF TUMOR INITIATING CELLS
R. Di Fiore, R. Drago-Ferrante, P. Carta, G. Augello,A. Santulli, R. Vento

16.9 THE MECHANISM BY WHICH HISTONE DEACETYLASE INHIBITORS SENSITIZE HEPATOMA AND COLON CANCER CELLS TO TRAIL-INDUCED APOPTOSIS
D. Carlisi, A. D'Anneo, M. Lauricella, S. Emanuele, P. Di Fazio,L. Angileri, T. Russo, R. Montalbano, R. Vento and G. Tesoriere

16.10 THE SYNTHETIC CANNABINOID WIN55,212-2 SYNERGIZES WITH THE DEATH RECEPTOR LIGAND TRAIL TO INDUCE APOPTOTIC EFFECTS IN HEPG2 HEPATOMA CELLS.
O. Pellerito, P. Portanova, M. Giuliano, G. Calvaruso, R. Vento and G. Tesoriere

16.11 REVERSINE SELECTIVELY INDUCES CELL DEATH IN TUMOR CELLS
M. Piccoli, G. Palazzolo, N. Papini, L. Dileo, C. Sitzia, E. Conforti, G. Tettamanti, B. Venerando, and L. Anastasia

16.12 UPSTREAM STIMULATING FACTOR 1 (USF1) IS INVOLVED IN THE TRANSCRIPTIONAL ACTIVATION OF THE HUMAN ALDOLASE C GENE IN PC12 CELLS DURING NEURONAL DIFFERENTIATION INDUCED BY NGF
A. Mancini, L. Serio, A. D'Angelo, P. Buono and F. Salvatore

16.13 ABNORMAL PROLIFERATION AND DIFFERENTIATION POTENTIALITY OF MESENCHYMAL STEM CELLS IN A MURINE MODEL OF OSTEOGENESIS IMPERFECTA.
A. Lupi, R. Gioia, A. Rossi, R. Tenni, C. Panaroni, G. Cetta, J.C. Marini, A. Forlino

16.14 ROLE OF INTRACELLULAR CALCIUM IN CELL DEATH INDUCED BY A MIXTURE OF ISOTHIAZOLINONES IN HL60 CELLS.
S. Frosali, A. Leonini, A. Ettorre, G. Di Maio, S. Nuti, S. Tavarini, P. Di Simplicio, A. Di Stefano

16.15 GRAFTING MESENCHYMAL STEM CELLS IN CARDIAC INFARCTED AREA USING A HYALURONAN-BASED SCAFFOLD IMPROVES CARDIAC FUNCTION AND SCAR REVASCULARIZATION.
E.Fiumana, L.Foroni, M.Tsivian, M.Carboni, C.Orrico, F.Neri, G.Arpesella, B.Nardo, G.Pasquinelli, C.Muscari, C.Guarnieri, C.M.Caldarera

16.16 REACTIVE OXIGEN SPECIES MEDIATE HIF-1α –INDUCED SURVIVAL IN METASTATIC MELANOMA
M. Calvani, M. Parri, E Giannoni, G. Comito, and P. Chiarugi

16.17 THE ACTIVITY OF CYTOSOLIC NADH/CYTO-C ELECTRON TRANSPORT SYSTEM IN APOPTOTIC HeLa CELLS.
V. Gorgoglione, D. D. Lofrumento, G. La Piana, D. Marzulli, D. Laraspata, V. Palmitessa, M. L. Romano, N. E. Lofrumento

SCI.1 EXOGENOUS DELIVERY AND MOLECULAR EVOLUTION: PEPTIDES BASED ON C^{α}-METHYLATED α-AMINO ACIDS AS ASYMMETRIC CATALYSTS IN THE SYNTHESES OF SIMPLE SUGARS
F. Formaggio, A. Moretto, C. Toniolo, Q. B. Broxterman, A. L. Weber, S. Pizzarello

SCI.2 BACKBONE NMR ASSIGNMENT AND PRELIMINARY STRUCTURAL ANALYSIS OF *T. BRUCEI* GRX3
M. Bellanda, K. Hellberg, J. Melchers, C. Andrésen, R.L. Krauth-Siegel, S. Mammi, M. Sunnerhagen

SCI.3 REACTIVE OXYGEN SPECIES HIF-1α INDUCED SURVIVAL IN METASTATIC MELANOMA
M.Calvani , M. Parri, E. Giannoni, G. Comito, P. Chiarugi

SCI.4 POSSIBLE ROLE OF OXIDATIVE STRESS IN THE PATHOGENESIS OF CHRONIC VENOUS INSUFFICIENCY. PRELIMINARY RESULTS OF A POPULATION STUDY
C. Cervellati, E. Franzoni, E. Masotti, E. Cremonini, A. Guariento, C.M. Bergamini

SCI.5 SYNTHESIS AND ACTIVITY IN PHOTODYNAMIC THERAPY OF POLY(ETHYLENE GLYCOL) CONJUGATED PHEOPHORBIDE.
S. Drioli, G.M. Bonora, L. Xodo, V.Rapozzi

SCI.6 THE CHIRAL SEQUENCE OF A NATURAL PEPTIDE INHIBITOR OF HIV-1 INTEGRASE ELUCIDATED
F. Formaggio, M. De Zotti, C. Toniolo, B. Kaptein, Q. B. Broxterman, P. J. Felock, D. J. Hazuda, S. B. Singh, and H. Brückner

SCI.7 ANTIBACTERIAL ACTIVITY AND RESISTANCE TO PROTEOLYTIC DEGRADATION OF TRICHOGIN GA IV AND SELECTED ANALOGUES
F. Formaggio, C. Toniolo, M. De Zotti, L. Stella, M.-H. Kim, Yoonkyung Park, Kyung-Soo Hahm

SCI.8 ARE LYMPHOCYTES DOPAMINERGIC CELLS? QUESTIONING THE PROTEOMICS RESPONSE OF A T-CELL MODEL TO DOPAMINE TREATMENT.
T. Alberio, G. Gentile, C. Anchieri, M. Simmaco, M. Fasano

SCI.9 INHIBITION OF ALPHA CRYSTALLIN AMYLOID FIBRILS FORMATION BY CARNOSINE
V. G. Nicoletti, F. Attanasio, A. Cataldo, S. Fisichella, S. Nicoletti, B. Pignataro, A. Savarino, E. Rizzarelli

SCI.10 ANTIOXIDANT AND ANTIPROLIFERATIVE ACTIVITY OF SEEDS FROM BETA VULGARIS CICLA
P. Ninfali, M. Blasa, L. Gennari, D. Angelino, A. Croce, M. Felletti, C. Buondelmonte, S. Dominici, L. Giorgi, A. Corallini

SCI.11 STUDIES ON MYCOBACTERIUM TUBERCULOSIS GLUTAMYL-tRNA SYNTHETASE
S. Paravisi, M. A. Vanoni

SCI.12 STRUCTURE, STABILITY AND DYNAMICS OF A MESOPHILIC-PSYCROPHILIC PAIR
I. Russo Krauss, A. Merlino, I. Castellano, E. De Vendittis, M. Conte, A. Vergara, F. Sica

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