SIB 2017

59th CONGRESS

Italian Society of Biochemistry and Molecular Biology

Caserta, September 20 – 22, 2017

BOOK OF ABSTRACT

A.A.V.V.
SIB 2017 – 59th Congress – Italian Society of Biochemistry and Molecular Biology
Book of Abstract

9 8 7 6 5 4 3 2 1
2021 2020 2019 2018 2017

Le cifre sulla destra indicano il numero e l'anno dell'ultima ristampa effettuata

EdiSES srl – Piazza Dante 89 – 80135 Napoli
Tel. 081/7441706-07 Fax. 081/7441705

http://www.edises.it E-mail: info@edises.it

ISBN 978 88 7959 9757

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PROGRAM OVERVIEW

Wednesday, September 20

12.00 Opening of the Secretariat & Registration

Auditorium-Scuola Specialisti dell'Aeronautica Militare Viale Ellittico 33, CE

14.00 Congress Opening & Welcome

Auditorium-Scuola Specialisti dell'Aeronautica Militare Viale Ellittico 33, CE

14.30 "E. Antonini" Lecture

"NMR-from Basic Research to Daily Human Life"

Kurt Wüthrich, Nobel Prize in Chemistry

Plenary Symposium

Protein Structure, Dynamics and Interactions

Auditorium-Scuola Specialisti dell'Aeronautica Militare Viale Ellittico 33, CE

17.00 **Visit to the Caserta Royal Palace and Welcome Cocktail**

Thursday, September 21

Plenary Symposium

9.00 **Signals driving cell proliferation, commitment, and self-renewal**

Auditorium-Scuola Specialisti dell'Aeronautica Militare Viale Ellittico 33, CE

11.00 **Coffee Break**

11.30 **Signals driving cell proliferation, commitment, and self-renewal**

Auditorium-Scuola Specialisti dell'Aeronautica Militare Viale Ellittico 33, CE

12.30 **Lunch**

Parallel Symposia

14.00 **Mitochondrial plasticity: from energetic metabolism to ROS signalling**

Auditorium-Scuola Specialisti dell'Aeronautica Militare - Viale Ellittico 33, CE

14.00 **Enzymes and metabolic pathways: mechanisms and technologies for advancing the frontiers of enzymology**

AULA 1 - Univ. degli Studi della Campania "Luigi Vanvitelli" - Viale Ellittico, 31, CE

15.30 **Coffee Break and Poster Session - Exposition**

Biblioteca - Univ. degli Studi della Campania "Luigi Vanvitelli" Viale Ellittico 31, CE

16.30-18.00 **Parallel Sessions: Short talks selected from submitted abstracts**

Università degli Studi della Campania "Luigi Vanvitelli" Viale Ellittico 31, CE

18.30 **SIB Members Assembly**

Masseria GiòSole - Capua

20.30 **Social Dinner**

Masseria GiòSole - Capua

Friday, September 22

Plenary Symposium

9.00 **Transcriptional regulation, chromatin structure and epigenetic modifications**

Auditorium-Scuola Specialisti dell'Aeronautica Militare Viale Ellittico 33, CE

10.30 **Coffee Break**

Parallel Symposia

11.00 **Proteomics in Cell Biology and Disease Mechanisms**

Auditorium-Scuola Specialisti dell'Aeronautica Militare - Viale Ellittico 33, CE

11.00 **Novel Frontiers of Nutritional and Environmental Biochemistry**

AULA 1 - Univ. degli Studi della Campania "Luigi Vanvitelli" - Viale Ellittico, 31, CE

13.00 **Lunch**

Plenary Symposium

Industrial and Molecular Biotechnologies

Auditorium-Scuola Specialisti dell'Aeronautica Militare Viale Ellittico 33, CE

14.30 **Session I: Future perspectives in biotechnology research**

15.20 **Session II: Mechanisms, processes and technologies for innovative applications**

16.20 **Session III: Molecular targeting for drug discovery**

17.00 **Closing remarks**

“E. Antonini” Lecture

NMR — from Basic Research to Daily Human Life

Kurt Wüthrich

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For the direct observation of transitions between Zeeman levels (Nobel Prize in Physics 1902 to Pieter Zeeman) by nuclear magnetic resonance (NMR) spectroscopy, Felix Bloch and Edward Purcell were awarded the Nobel Prize in Physics 1952. NMR has then been used in a wide range of fundamental studies in physics, and in the 1960s it became an important analytical tool in chemistry. Based on novel concepts and advances in NMR instrumentation and informatics tools, exciting developments in the early 1970s laid the foundations for magnetic resonance imaging (MRI), which is today a key technique in medical diagnosis (Nobel Prize in Physiology or Medicine 2003 to Paul Lauterbur and Peter Mansfield), and for the use of NMR spectroscopy in structural chemistry and biology (Nobel Prizes in Chemistry to Richard R. Ernst 1991 and to KW 2002). Fundamental understanding of these advances was greatly helped by Albert Einstein’s 1905 treatise of the Brownian motion, which was first described by the English botanist Robert Brown in 1827. NMR is thus one of the research areas where the results of basic research during the 19th and the first half of the 20th century provided the basis for technological breakthroughs in the second half of the 20th and into the 21st century. The ensuing methods now support research in chemistry, structural biology and drug discovery, as well as providing novel, non-invasive approaches in medical diagnosis, which all contributes to improved quality of daily human life.

Plenary Symposium

Protein Structure, Dynamics and Interactions

The RNA exosome - ribosome connection: coupling the RNA degradation and translation machineries

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The RNA exosome is a conserved macromolecular machine that degrades a wide variety of RNAs from their 3' end. Exosome-mediated RNA degradation leads to the complete elimination of nuclear and cytoplasmic transcripts in turnover and quality control pathways, and to the partial trimming of RNA precursors in nuclear processing pathways. How the exosome combines specificity and versatility to either eliminate or process RNAs is not well understood, but an important aspect lies in the association with cofactors that appear to modulate the activity of the complex and target it to different transcripts in different cellular compartments.

Over the years, we have used biochemical and structural studies to understand how the core complex of the yeast exosome recognizes and degrades RNA substrates. We have now proceeded to study how the exosome core complex associates and functions with its nuclear and cytoplasmic cofactors. Increasing evidence indicates that the regulatory complexes of the exosome interact with pre-ribosomes in the nucleus (for processing) and mature ribosomes in the cytoplasm (for turnover and quality control). The results pave the way to understand the crosstalk between exosome and ribosomes and more generally to understand the integration of different gene expression machines that have been so far studied individually.

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STRUCTURAL DYNAMICS AND MOLECULAR RECOGNITION

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Characterising the structural dynamics of biomolecules is an extremely difficult task given the limitation in the resolution of experimental techniques and in the time resolution and accuracy of computational methods. Hybrid methods are emerging that allows combining multiple sources of experimental data (NMR, SAXS, FRET, CRYO-EM, Mass-Spec, cross-linking, etc) with molecular simulations in such a way to increase the resolution of all single techniques. The resulting structural ensembles can then be used to shed light on the link between biomolecules motion and function. I will introduce some of the recent advancements in hybrid methods for ensemble determination and show how these can be used to probe molecular recognition of dynamic systems as for example intrinsically disordered peptides and their assembly into large dynamic oligomers and multi-domain proteins.

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Competition between a toxic tRNase and the biosynthetic enzyme CysE for binding to *O*-acetylserine sulfhydrylase

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In bacteria and plants, serine acetyltransferase (CysE) and *O*-acetylserine sulfhydrylase-A (CysK) collaborate to synthesize L-Cys from L-Ser. CysE and CysK form a high-affinity complex, called cysteine synthase where the C-terminus of CsyE occupies CysK active site and inhibits its activity (1). Conversely, CysE active site is allosterically altered by CysK to alleviate substrate and feedback inhibition by L-Cys (2). Intriguingly, some CysK homologs have been co-opted to regulate diverse biological activities, like for example gene expression and response to hypoxia, thus CysK has been classified as a moonlighting protein (3). One interesting example of moonlighting activity of CysK is the activation of a latent tRNase (CdiA-CT) involved in contact-dependent growth inhibition in uropathogenic *E. coli* 536. CdiA-CT anchors to CysK active site through a mechanism very similar to that exploited by CysE, and is activated upon binding (4-5). CysK:CdiA-CT and cysteine synthase complexes form through a two-step mechanism with a slow isomerization phase after the initial encounter, leading to very stable complexes of comparable nanomolar affinity. However, the second-order rate constant for cysteine synthase complex formation is two orders of magnitude larger than that of the CysK:CdiA-CT complex, that forms but also dissociates slowly. Slow-binding ensures robust nuclease activity in target bacteria even in the presence of excess competing CysE.

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3D of human lysosomal acid-alpha-glucosidase provides molecular bases of pharmacological chaperones interactions for the treatment of Pompe disease

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Pompe disease (PD) is a rare metabolic disorder caused by mutations in the gene that encodes the hydrolase acid α-glucosidase (GAA) involved in the lysosomal breakdown of glycogen. Functional deficiency of GAA results in lysosomal accumulation of glycogen and cellular damage in all tissues, particularly cardiac and skeletal muscle. The only approved treatment for PD, enzyme replacement therapy with recombinant human GAA (rhGAA), has shown limited therapeutic efficacy, thus further innovative approaches would be desirable. Pharmacological chaperone therapy represents a promising strategy, showing significant advantages in terms of the bioavailability of drugs, oral administration and positive impact on the quality of patients' lives [1]. Recently, we identified N-acetyl-cysteine (NAC) and other allosteric chaperons that improved the stability of rhGAA toward pH and temperature without inhibiting its catalytic activity, and enhanced rhGAA efficacy in PD fibroblasts and in mice knocked-out in GAA [2]. Unlike the known chaperones for GAA, they are not competitive inhibitors and interact with an allosteric site.

We report here on the recently solved 3D of rhGAA that allows mapping of disease mutations, provides molecular bases of pharmacological chaperones interactions, and supply an accurate molecular scaffold for *in silico* screening to map novel druggable sites and identify novel pharmacological chaperones [3].

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Function from structure: Lpp20 from the human pathogen *Helicobacter pylori*

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Helicobacter pylori is a bacterium that affects about 50% of the world population and, despite being often asymptomatic, it is responsible of several gastric diseases, from simple peptic ulcer to gastric cancer [1]. Altough it lives with humans since ancestral times, their reciprocal influence is still not fully understood and deserves more in depth investigation. The antigenic properties of several *H. pylori* proteins inspired the development of a vaccine for a long time. The protein Lpp20 (HP1456), corresponding to the product of gene hp1456 [2], plays an important role in bacterium development and survival, but its physiological role in the host infection has not beenclarified yet.

Lpp20 is a lipoprotein bound to the external membrane of the bacterium as well as in secreted vesicles along with other two proteins of the same operon, HP1454 and HP1457. The crystal structure, determined by SAD using a Se-Met derivative at 1.9 Å resolution, shows a alpha1-beta1-alpha2-alpha3-beta2-beta3-alpha4 topology, with a bent shape. Its fold results to be similar to that of Tip-alpha, a carcinogenic factor present in *H. pylor*i. Recombinant Tip-alpha was shown to stimulate the production of IL-1alpha and TNF-alpha from gastric cells in vitro and is assumed to be associated with the development of gastritis and gastric cancer [3]. The fact that both proteins are secreted and share a similar architecture prompted us to test the effects of Lpp20 on gastric human cells in order to gain novel insights on its function. Treatment of MKN-28 and AGS cells with recombinant purified Lpp20 induced the formation of filopodia, suggesting invasive morphological changes. Additionally, downregulation of E-cadherin, a significant marker of the epithelial–mesenchymal transition (EMT), is also observed.

To the best of our knowledge this is the first report showing that Lpp20, analogously to what observed for Tip-alpha, could act as a new inducer of epithelial-mesenchymal transition.

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Plenary Symposium

Signals driving cell proliferation, commitment, and self-renewal (session I)

Functionalized nanofibers for regenerative medicine

Evzen Amler

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Tissue engineering is a rapidly developing field characterized with a high expectation from human and veterinary applications. Its advantage is mimicking of natural healing with formation of native tissues with a better integration to organism and a limited danger of carcinogenesis, An evident disadvantage seems to be necessity of cell processing in clean rooms which is connected with costly processes and large obstacles from medical authorities prior any broader clinical application. A plausible alternative offers regenerative medicine which drives all healing processes directly in the patient´s body. This approach, however, need rather a complex scaffolding material.

This presentation deals with functionalized nanofibers used as scaffolds for regenerative medicine characterized with regulated drug delivery of bioactive substances. It focuses on core/shell nanofibers with encapsulated liposomes and platelet derivatives. Specific microparticles derived from nanofibersn and novel technologies for nanofiber production belong also among main interests. Functionalized nanofibers are characterized with suitable properties needed for a drug delivery platform, biocompatibility, the ability to carry a broad range of therapeutics and dispersability in water. Nanofibers are characterized with a small size, stable core and low cytotoxicity. Nanodiamond monolayers have been shown to act as a platform for neuronal growth similar to protein-coated materials. They are suitable for wound dressing: controlled release of drugs.

One carbon and sulfur amino acid metabolism: two biochemical pathways connected to new signaling molecules of medical relevance

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Sulfur amino acid metabolism is characterized by strict interconnections among the methionine-homocysteine cycle, the transsulfuration pathway and one carbon metabolism. Two compounds, strictly related to this metabolism, have been involved as effectors on the vasculature: homocysteine and, more recently, hydrogen sulfide. The transsulfuration pathway, in particular, is the major pathway for both homocysteine metabolism and hydrogen sulfide biosynthesis. Hyperhomocysteinemia is a powerful cardiovascular risk factor, while hydrogen sulfide has been recently assessed as the third gaseous vasodilator, after nitric oxide and carbon monoxide. It has been shown that homocysteine toxicity on the vasculature is mainly indirect, being mediated by several of its own derivatives, acting through various mechanisms, including an impairment of methyl transfer reactions - particularly protein and DNA methylation and relevant epigenetic modifications (1) - and release of mediators of the inflammatory response. The latter has been extensively studied also using cell models. A number of chemokines and cytokines as well as "A Disintegrin And Metalloproteinase 17" (ADAM17) have been shown to play a role in endothelial activation and damage (2).

The vasodilator gas hydrogen sulfide has drawn scientists' attention in cardiovascular medicine because of its protective effects on the vasculature. Its metabolic derangement and, hence, potential involvement in the pathogenesis of hypertension and vascular damage, particularly in chronic kidney failure patients, does occur through mechanisms partly related to inflammation (3, 4). On the other hand, kidney failure represents a growing sanitary emergency throughout the world and a suitable model to study cardiovascular risk factors and their mechanisms of action. Association of both traditional and kidney failure specific risk factors - also including hyperhomocysteinemia - characterizes this complex disease. Several differences in the metabolism of sulfur amino acids and their derivatives, in kidney failure disease model vs normal individuals, have been so far characterized. The relevant compounds altered include homocysteine and hydrogen sulfide, and a number of other sulfur amino acid derivatives, which display the typical behavior of retention compounds, thus exerting the role of prospective uremic toxin, such as lanthionine (5, 6).

Present findings, as a whole, support (a) the interpretation that hyperhomocysteinemia is a red flag in a disrupted circuit; (b) the role of biochemical derangements of sulfur amino acid and one carbon metabolisms in the genesis of various compounds, which may contribute to cardiovascular damage for their potential ability to trigger inflammatory response.

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PHOTOTRANSDUCTION IN NORMAL AND ALTERED CONDITIONS: FROM MOLECULES TO NETWORKS

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Light sensitivity in photoreceptor cells is finely regulated by negative feedback loops that involve Ca^{2+} and guanosine 3´,5´-cyclic monophosphate (cGMP) as second messengers. The drop of cytoplasmic [Ca^{2+}] following rod illumination is detected by guanylate cyclase-activating proteins (GCAPs), a family of neuronal Ca^{2+}/Mg^{2+} sensors that control the activity of membrane bound guanylate cyclases (GC) in a Ca^{2+}-dependent manner. GCAPs are quite unique regulators, in that they act as inhibitors of the GC at high (250–800 nM) intracellular [Ca^{2+}], while they switch to an activator of the enzyme at low (20–100 nM) [Ca^{2+}], thereby contributing to the switch-off of the phototransduction cascade.

To date, up to 15 single point mutations in GCAP1 have been associated to cone, cone-rod and macular dystrophies. We present a thorough characterization of altered structure/function relationships in GCAP1 in the presence of disease-associated missense mutations. Interestingly, some of the GCAP1 variants showed a severe disturbance of Ca^{2+}/Mg^{2+} sensing properties, while some other mutants, including the L176F and L84F variants showed unaltered affinity for Ca^{2+}. However, all the disease-associated GCAP1 variants analysed so far showed a remarkable dysregulation of the GC-activity, leading to constitutive activation of the target enzyme at physiological [Ca^{2+}]. A similar aberrant regulation of the target enzyme must therefore result from a similar perturbation of the GCAP1-GC interaction, which may eventually cause dysregulation of both Ca^{2+} and cGMP homeostasis, and result in retinal degeneration. We corroborate biochemical experimental data with a computational approach based on exhaustive molecular dynamics simulations of GCAP1 in its Mg^{2+} and Ca^{2+} loaded states. By applying a Protein Structure Network (PSN) paradigm we could determine the intramolecular communication pathways involved in the specific GC activator/inhibitor switch, which is likely to be altered in disease-associated conditions.

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Sphingosine 1-phosphate signaling axis as mediator of vital skeletal muscle functions

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Sphingosine 1-phosphate (S1P) is a powerful bioactive lipid recognized to be highly versatile not only for the variety of transmitted biological actions but also for the involved molecular mechanisms. Initially identified as by-product of sphingolipid breakdown, indeed S1P is physiologically present in serum, and acts as ubiquitous regulator of multiple key cellular processes, including proliferation and survival, cell motility and differentiation (15). S1P is synthesized via the ATP-dependent phosphorylation of sphingosine catalysed by two distinct isoenzymes sphingosine kinase-1 and 2 whereas it is reversibly degraded by two specific phosphatases or irreversibly cleaved by S1P lyase (SPL). The sphingolipid can exert its functions as intracellular messenger, as well as extracellular ligand of a family of five specific G protein coupled receptors (S1P1-5), being its cellular export mediated by specific and unspecific transporters, such as spinster2 (Spns2) and ABC family members.

Since many years we have been focused on dissecting the role of S1P in skeletal muscle, highlighting how its metabolism and signaling crucially regulate vital skeletal muscle functions. In particular, S1P has been shown to stimulate proliferation of satellite cells, the skeletal muscle resident stem cells, and differentiation of myoblasts towards myotubes, thus contributing to skeletal muscle regeneration. Interestingly we have established that S1P metabolism in skeletal muscle precursor cells is under the control of multiple growth factors and cytokines responsible not only for skeletal muscle repair but also for fibrotic degeneration and we have identified the specific S1P receptor subtypes implicated in the observed divergent actions.

On the whole these studies point at modulators of S1P metabolism and S1P receptors agonists/antagonists as new molecular tools for improving skeletal muscle regeneration attenuating tissue fibrosis and possibly ameliorate age-related muscle wasting.

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Crosstalk between insulin and mTOR signaling in Down Syndrome and Alzheimer diseas

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Down Syndrome (DS) or trisomy 21 is the most common genetic cause of intellectual disability. Virtually all people with DS have sufficient neuropathology for a diagnosis of Alzheimer disease (AD) by 40 years [1-2], including senile plaques and neurofibrillary tangles. Common pathways of neurodegeneration include mitochondrial dysfunction, increased oxidative stress (OS), altered mTOR axis and reduced glucose metabolism. Epidemiological studies showed that hallmarks of peripheral metabolic disorders, such as glucose intolerance and/or impairment of insulin secretion, are associated with a higher risk to develop dementia or AD. Interestingly, human post-mortem studies have convincingly shown that a dysregulation of brain insulin signaling with reduced downstream neuronal survival and plasticity mechanisms are consistent and fundamental abnormalities in AD. In particular, AD brain is characterized by a phenomenon known as brain insulin resistance (BIR) – broadly defined as the inadequate response to insulin by target cells – due to reduced IR activation and increased levels of inhibitory phosphorylation of IRS1.

The present study aims to analyze the crosstalk between insulin and mTOR signaling as possible contributing factors to the neurodegenerative process in a transgenic mouse model of DS (Ts65Dn) and AD (3xTgAD) as function of aging. Further, we will investigate the role of biliverdin reductase A (BVR-A) as a novel regulator of insulin signalling cascade and how its impairment might contribute to the onset of BIR.

Our results show that OS-induced impairment of BVR-A kinase activity is an early event, prior the accumulation of Aβ and tau pathology, and that this alteration contributes to the onset of brain insulin resistance along the progression of AD pathology in 3xTg-AD and Ts65Dn mice.

We propose a new paradigm for which: OS-induced impairment of BVR-A is responsible for a sustained activation of IRS1, which then causes the stimulation of negative feedback mechanisms aimed to turn-off IRS1 hyper-activity and thus BIR.

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Natural products as a source of antiaging molecules

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Oxidative stress is the imbalance between the production of free radicals and antioxidant defenses. It is mainly initiated by reactive oxygen species (ROS), which can damage cell structures and advance skin aging. Skin aging is mainly due to intrinsic and extrinsic aging factors (photo-aging). Photo-aging is a consequence of exposure to ultraviolet radiations, which may account for up to 80% of visible aging signs in the skin and correlates with cancer risk. Natural antioxidants are known to prevent or delay oxidative stress-related damage, thus, we propose the use of natural antioxidants as an effective approach to prevent symptoms related to photo-induced aging of the skin. In particular, we analyzed the protective effects of different antioxidants in protecting cells from UVA damage. We used: a new tomato hybrid (DHO) [1], enriched in ascorbic acid; a blue berry (açai), rich in malvidin and cyanidin [2]; Opuntia ficus indica nopal, rich in eucomic and piscidic acids [3]. Our results indicate that all the natural extracts analyzed are able to protect cells from UVA-induced ROS production, GSH depletion, lipid peroxidation and MAPK cascade activation; DHO and Opuntia can protect cells from apoptosis. In conclusion, the extracts obtained from these natural sources are able to reduce the negative effects induced by UVA radiations.

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Plenary Symposium

Signals driving cell proliferation, commitment, and self-renewal (session II)

Targeting breast Cancer cell survival and migration through inhibition of hexosamine biosynthetic pathway

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Aberrant N- and O-linked protein glycosylation, frequently observed in cancer, play several roles in different steps of tumor progression. It is a consequence of a number of factors among which a boost of HBP flux leading to an increased intracellular concentration of uridine diphosphate N-acetylglucosamine (UDP-GlcNAc), the terminal metabolite of HBP and the main donor substrate for protein glycosylation. Although the cause of the increased flux through the HBP is not yet completely clear, it is likely to occur as a result of an enhanced glucose and glutamine uptake and of an increased expression and activity of HBP specific enzymes regulated by oncogenes as Ras and Myc. Therefore, we hypothesized that compounds attenuating HBP flux may be valuable targets for cancer therapy. Here we report the validation of a novel modulator of the HBP pathway, namely FR051, in different type of breast cancer cells. We show that FR051, attenuating HBP flux leads to a dramatic decrease of cell proliferation, survival and migration by directly interfering with N- and O-glycosylation protein levels. In particular, FR051 treatment provokes a sustained activation of the Unfolded Protein Response and a significant accumulation of intracellular ROS both actively participating to cancer cell death. Furthermore, FR051 suppresses also cancer growth in MDA-MB- 231 xenograft mice.

Supported by the Italian Association Cancer Research (grant No. IG-15364) and by University of Milano Bicocca (FAR 2016).

Impact of cancer cells metabolic reprogramming on tumor progression and drug resistance: potential implications for cancer treatment

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Tumour cells have long been considered defective in mitochondrial respiration and mostly dependent on glycolytic metabolism. However, this assumption is currently challenged by several lines of evidence in a growing number of tumours. Ovarian cancer (OC) is one of the most lethal cancers worldwide, but it continues to be a poorly understood disease and its metabolic features are far to be elucidated. In this context, we investigated the role of tumour necrosis factor receptor-associated protein 1 (TRAP1), an antiapoptotic chaperone involved in stress-adaptive response of cancer cells, which is found upregulated in several cancer types and is a key modulator of tumour cell metabolism with a crucial role in the "oncogenic" switch towards glycolytic phenotype (1). Surprisingly, we found that TRAP1 expression inversely correlated with grade, stage and lower survival in a large cohort of OC patients. Accordingly, TRAP1 silencing induced resistance to cisplatin, resistant cells showed increased oxidative metabolism compared with their sensitive counterpart, and the bioenergetic of higher grade tumours indicated increased mitochondrial respiration. Strikingly, cisplatin resistance was reversible upon pharmacological inhibition of mitochondrial oxidative phosphorylation by metformin/oligomycin. At molecular level, increased oxidative metabolism in low TRAP1-expressing OC cells and tissues enhanced production of inflammatory mediators such as interleukin (IL)-6 and IL-8. Mechanistically, we identified members of the multidrug resistance complex as key mediators of such metabolism-driven, inflammation-induced process (2). Furthermore, downregulation of TRAP1 associates with higher expression of p70S6K, a kinase frequently active in OC with emerging roles in cell migration and tumor metastasis. Indeed, TRAP1 silencing in different OC cells induces upregulation of p70S6K expression and activity and enhancement of cell motility and epithelial-mesenchymal transition. Strikingly, pharmacological inhibition of p70S6K reverts the high motility phenotype of TRAP1 knock-down cells (3). Altogether, our results candidate TRAP1 pathway and protein partner "signature" as promising biomarkers

for OC, opening new scenarios about its role in linking chemoresistance to metastatic potential and metabolic remodelling.

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TGFbeta-induced epithelial-mesenchymal transition: negative feedback regulation by vitamin D

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TGFβ is a key modulator of the epithelial-mesenchymal transition (EMT). Due to its central role in cancer progression and spreading, its signaling is targeted by many therapies aimed at preventing metastasis formation. We unveil a novel mechanism of action of TGFβ; in a model of human epithelial-mesenchymal transition we demonstrate for the first time that TGFβ strongly induces the expression of vitamin D receptor (VDR) and that $1,25(OH)_2D_3$ (the active form of vitamin D) is able to contrast the TGFβ-driven transition by a transcriptional and metabolic modulation, with a time-dependent efficacy. TGFβ induces the mitochondrial respiratory activity, evaluated as transcriptional induction of respiratory complex, enhanced mitochondrial membrane potential and increased levels of mitochondrial ATP; $1,25(OH)_2D_3$ abrogates this mitochondrial stimulation and decreases the production of reactive oxygen species triggered by TGFβ. We describe for the first time in human bronchial epithelial cells a metabolic effect of TGFβ that supports the cellular invasion seen in transition. We identified a novel signaling pathway by which $1,25(OH)_2D_3$ opposes the effect of TGFβ in EMT: in addition to the previously described transcriptional activity such as the induction of E-Cadherin, $1,25(OH)_2D_3$ curbs mitochondrial respiration and reduces the production of energy required for cell motility. Taken together our observations suggest that the increased expression of VDR might represent a regulatory negative feedback exerted by TGFβ on its own signaling. The negative feedback exerted by $1,25(OH)_2D_3$ could be necessary to maintain a balance in metabolism and avoid the excessive production of ROS; this could be optimal for cancer cell survival and spreading. Intriguingly, this mechanism could be exploited for therapeutic benefit, because the elevated levels of $1,25(OH)_2D_3$ could restrain the metabolic shift evoked by TGFβ and could limit or even prevent cancer migration and metastasis.

Sphingosine-1-P and its plasma membrane receptors in human glioma cells

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Glioblastoma multiforme (GBMs), the most frequent and deadly brain tumors in humans, are characterized by extended invasiveness and cell growth. Different sphingolipid metabolites, such as sphingosine-1-phosphate (S1P), have emerged as active mediators in the complex network of signaling pathways involved in the control of physiological and pathological cell behavior (1). Increasing evidence supports that S1P is implicated in sustaining cell invasiveness. On the other side, various growth factor receptors, such as EGFR, are frequently mutated and/or overexpressed in GBMs (2). Very importantly, S1P is able to regulate EGFR expression in lung adenocarcinoma and rat vascular smooth muscle cells suggesting the existence of a cross-talk between the S1P axis and growth factor signaling pathways induced by EGF in tumors. We investigated the crosstalk between S1P and the EGF/EGFR pathways, focusing on its role in glioma invasiveness.

We used U87MG human GBMs cell line overexpressing EGF receptor (EGFR+). EGFR+ cells are characterized by increased levels of extracellular S1P and an higher expression of the active SK-1 form (phosphorylated SK-1). These cells showed increased ability to invade into Matrigel. The high chemioinvasion ability as well as spheroids sprouting were significantly inhibited in EGFR+ cells treated with SK inhibitors, or with S-FTY720-vinylphosphonate, the antagonist of S1P receptors. Moreover, we found that S1P added to the cell medium maintained the ability to drastically increase invasion in EGFR+ cells treated with SK inhibitors. At variance, S1P did not induced invasion over the basal values when glioma cells were treated with FTY720. Altogether our data strongly suggest that increased S1P secretion and signalling associated with EGFR overexpression/iperactivation play an important role in EGFR+ glioblastoma invasiveness by enhancing the invasion potential of GBM.

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Parallel Symposia

Mitochondrial plasticity: from energetic metabolism to ROS signaling

Mitochondrial Stress Signalling in Disease, Aging and Immunity

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Mitochondria are multi-faceted organelles in eukaryotic cells that function at the nexus of energy metabolism, oxidative stress, and apoptosis. Consequently, circumstances (genetics, environmental factors, aging) that result in mitochondrial dysfunction disrupt a multitude of cellular processes that can cause human disease pathology. Changes in redox balance due to altered mitochondria reactive oxygen species production, decline or rewiring of cellular energy metabolism, and cell death are some of the major downstream cellular consequences leading to mitochondrial-based pathology, ranging from heart, skeletal muscle and nerve dysfunction to metabolic diseases, blindness, and deafness. I will discuss our latest interrogations of mitochondria-to-nucleus signaling pathways and some novel connection to disease, aging and immunity.

Supported by NIH grants R01 AG047632, R01 AR069876, and R33 ES025636

The cristae modulator Optic atrophy 1 requires mitochondrial ATP synthase dimerization to safeguard mitochondrial function

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It is unclear how the mitochondrial fusion protein Optic atrophy 1 (OPA1), which inhibits cristae remodeling, protects from mitochondrial dysfunction. Here we identify the F1Fo-ATP synthase (ATPase) as the effector of OPA1 in mitochondrial protection. ATPase dimerization and activity was influenced by apoptotic and genetic manipulations of cristae shape. Increased OPA1 dosage stimulated ATPase dimerization and could even correct the ultrastructural defects of mitochondria where ATPase dimerization was genetically impaired. Conversely, OPA1 required ATPase dimers to protect mitochondrial function and cell viability from respiratory chain inhibition. Thus, OPA1 protects mitochondria by stimulating ATPase dimerization.

Mitochondria and clock-genes interplay: a dynamic timekeeping of cell metabolism.

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Mounting evidences are disclosing the tight correlation between circadian rhythms and cell metabolism to align bioenergetic demand to environmental variants. Although at the organismal level the central timekeeper is the hypothalamic suprachiasmatic nuclei practically all the peripheral tissues are equipped with autonomous oscillators made up by common molecular clockworks (mastered by the BMAL1-CLOCK transcription factors) constituted by circuits of gene expression interconnected with positive and negative feed-back loops. In the last few years we focused on the interplay between the clock gene machinery and the mitochondrial physiology. Using well-established *in-vitro*-synchronized cultured cells, we demonstrated a BMAL1-dependent ultradian oscillation of the mitochondrial respiratory activity [1] as well as of the glycolytic activity. This translated in a rhythmic change of the cellular energy charge. The rhythmic respiratory activity was associated with: i) oscillation in cellular NAD content; ii) clock-genes-dependent expression of NAMPT and Sirtuins 1/3; iii) reversible acetylation of a single subunit of the mitochondrial respiratory chain Complex I; iv) reversible phosphorylation state of the pyruvate dehydrogenase (PDH). In this contest the mitochondrial-endoplasmic reticulum (ER) calcium homeostasis appears to be involved as inhibition of either of the mitochondrial calcium uniport, the ER Ca^{2+}-channel(s), the cyclic ADP-ribose (cADPR) synthesis resulted in alteration of the rhythmic respiratory activity. Notably, pharmacological inhibition of the mitochondrial OxPhos system resulted in dramatic deregulation of the clock-gene expression in synchronized cells and a similar result was attained with mtDNA depleted (Rho0) cells [2].

All together our findings provide novel levels of complexity in the interlocked feedback loop controlling the interplay between cellular bioenergetics and the molecular clockwork.

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Understanding the signalling between respiratory Complex I and hypoxia-inducible factor-1α in tumorigenesis

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Metabolic plasticity is a hallmark of neoplastic cells required to sustain continuous proliferation and to adapt to adverse environmental conditions. Interestingly, mitochondria-defective cancer cells have been shown to prefer reductive carboxylation of glutamine as a major source of carbon for the biosynthesis of building blocks [1-2]. We have previously demonstrated that severe respiratory Complex I (CI) dysfunction triggers the unbalance of NADH/NAD^+ and α-ketoglutarate/succinate ratios, and chronic hypoxia-inducible factor-1α (HIF1α) destabilization, hampering the tumorigenic potential of cancer cells both *in vitro* and *in vivo* [3-4]. Based on these findings, targeting CI can be envisioned as a lethality target for potential anticancer strategies and, hence, it is urgent to understand the mechanism(s) linking CI deficiency to HIF1α destabilization, and in turn to tumor growth decrease.

To this aim, we generated a panel of CI^{KO} cancer cell lines *via* gene editing of the crucial NDUFS3 subunit, which displayed a dramatic reduction of functional assembled CI and a defective bioenergetic phenotype, rendering them unable to grow under metabolic stress conditions unlike their CI-competent counterparts. Moreover, increased glucose consumption and lactate production were observed. Metabolic profiling of CI^{KO} cells revealed an unbalance in key TCA cycle metabolites known to regulate the activity of prolyl hydroxylases (PHDs). We thereby showed that this triggers PHD activity, leading to chronic HIF1α degradation and decrease of tumorigenic potential of CI^{KO} cells. Interestingly, we show

that CI^{KO} cells are able to form, albeit in a much longer time lap than their wild-type counterparts, xenografts in mice, although their histologic architecture is strikingly different and suggestive of a tight dependence on the murine stroma for survival. By allowing therefore a wider window for intervention, CI may represent decisive marker in orienting therapeutic choices or predicting outcome, and their peculiarities add up to constitute their *oncojanus* features.

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Parallel Symposia

Enzymes and metabolic pathways: mechanisms and technologies for advancing the frontiers of enzymology

Human Aromatase: Catalytic Mechanism And Role On The Emerging Problem Of Endocrine Disruptors

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Human aromatase is the cytochrome P450 catalysing the conversion of androgens into estrogens playing a key role in the endocrine system. X-ray crystallography, mutagenesis and bioelectrochemical studies have provided key information on the finely tuned steps required for its catalytic mechanism where molecular oxygen is activated and introduced in the substrate via a three cycles mechanism, each of eight different steps. Due to this role, it is likely to be a target of the so-called endocrine disrupting chemicals, a series of compounds able to interfere with the hormone system with toxic effects. If on one side the toxicity of some compounds such as bisphenol A is well known, on the other side the toxic concentrations of such compounds as well as the effect of the many other molecules that are in contact with us in everyday life still need a deep investigation. The availability of biological assays able to detect the interaction of chemicals with key molecular targets of the endocrine system represent a possible solution to identify potential endocrine disrupting chemicals. Here the so-called alkali assay is applied and validated using anastrozole and sildenafil as known aromatase inhibitors. Out of the small library of compounds tested, resveratrol and ketoconazole resulted to inhibit aromatase activity, while bisphenol A and nicotine were found to exert an inhibitory effect at relatively high concentrations (100 µM), and other molecules such as lindane and four plasticizers did not show any significant effect. These data are confirmed by quantification of the product estrone in the same reaction mixtures through ELISA. Overall, the results show that the alkali assay is suitable to screen for molecules that interfere with aromatase activity. As a consequence it can also be applied to other molecular targets of EDCs that use NAD(P)H for catalysis in a high throughput format for the fast screening of many different compounds as endocrine disrupting chemicals.

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AMINO ACID OXIDASES: METABOLIC COMPLEXITY FROM STRUCTURAL SIMPLICITY

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Amino acid oxidases/deaminases (AAOs/AADs) are flavoproteins which catalyse the O_2-dependent oxidative deamination of D- or L-amino acids (AAs) to yield the corresponding α-keto acids and ammonia.

These enzymes share a very simple active site architecture (no residues are directly involved in catalysis) and an identical catalytic mechanism. Although AAOs/AADs share a common ancestor, during evolution they acquired a large substrate promiscuity: this allowed the integration of these enzymes into different metabolic pathways to fulfil distinct physiological functions in different organisms [1]. In microorganisms AAOs which possess a narrow substrate specificity can be involved into the synthesis of cofactors: e.g., *Bacillus subtilis* glycine oxidase and *Sulfolobus tokodaii* L-aspartate oxidase catalyse the first step of the biosynthesis of thiamine and NAD^+, respectively. The former enzyme was *in vitro* evolved and introduced in *M. sativa* to generate a novel metabolic pathway for the degradation of the herbicide glyphosate [2]. On the other hand, AAOs/AADs possessing a wide substrate specificity are fundamental for the catabolic utilization of amino acids as carbon or nitrogen source. Notably, only in *Proteus* a peculiar membrane-bound L-amino acid deaminase evolved which does not employs O_2 but transfers electrons to the membrane electron transport chain [3]. In mammals, D-amino acid oxidases acquired unique structural and biochemical properties that allowed them to fulfil specific physiological functions: as a matter of fact, D-amino acid oxidase and D-aspartate oxidase play a main role in the regulation of the neuromodulators D-serine and D-aspartate in the brain [1].

Amino acid oxidases represent an inspiring example of how Nature recycled the same structural scaffold to accomplish several different physiological functions throughout all taxonomic kingdoms.

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METABOLIC ENGINEERING IN E.COLI K4 TOWARDS CHONDROITIN PRODUCTION: AN INSIGHT IN THE BIOCHEMICAL MACHINERY OF CAPSULAR POLYSACCHARIDE BIOSYNTHESIS

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Chondroitin sulfates, is widely used in the treatment of arthritis, up to date it is produced through extractive procedures from animal tissues. Semi synthetic approach were accomplished by our group obtaining from the capsular polysaccharide (CPS) of *Escherichia coli* K4 a natural identical heteropolysaccharide (Bedini et al.,2011). To improve fermentative production of chondroitin, recombinant strains based on the overexpression of the anti-terminator RfAH were obtained and DO-stat fed- batch processes were developed to obtain up to 5 g/L of chondroitin in the fermentation broth (Cimini et al. 2013). Specific downstream processing permitted to obtain pharmaceutical grade chondroitin, that tested *in vitro* demonstrated to better preserve the morphology and phenotype of primary human chondrocytes (Stellavato et. al, 2016). The new opportunity to use as active principle directly unsulfated chondroitin prompted the more recent research activities to exploit metabolic engineering strategies to improve the biosynthesis of K4 CPS(Cimini et al.,2015). The genes responsible for CPS biosynthesis and transport in *E. coli* K4 are clustered and organized in three regions. The central part, region 2, is serotype specific and contains 7 genes that are directly involved in activated precursors biosynthesis, namely UDP-sugars, (*kfoA*, *kfo*F), polymer assembly (*kfo*C), and fructosylation (*kfo*E). The engineering of the crucial enzymes of the biosynthetic pathways was studied in order to increase the specific yield of chondroitin on biomass (Yp/x). Experiments are ongoing on a number of engineered strains obtained in the attempt of optimizing production while unravelling the complex biosynthetic machinery of CPS biosynthesis. Improved yields will shorten the gap towards industrial production of chondroitin for the application in medical devices and possibly in novel pharmaceutical preparations.

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Enzymes that make and enzymes that fix mistakes: Nit1 is a 'repair' amidase that hydrolyzes deaminated glutathione

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Enzymes of intermediary metabolism are not perfectly specific and tend to act on intracellular compounds resembling their true substrate. The products of such side reactions are non-classical metabolites, which in several cases need to be eliminated or recycled by specific enzymes, called metabolite repair enzymes [1, 2]. The increasing rate at which repair enzymes are being discovered suggests that a substantial fraction of currently 'unclassified' enzymes, encoded in eukaryotic and prokaryotic genomes, might in fact be involved in metabolite repair.

Here I will describe a study on the mammalian protein Nit1, whose enzymatic function has long remained a puzzle. Nit1 is highly conserved in eukaryotes and is thought to act as tumor suppressor. Despite being ~35% sequence identical to ω-amidase (Nit2), it had been shown that Nit1 does not hydrolyze efficiently α-ketoglutaramate (the known physiological substrate of Nit2).

However, we demonstrated that both the mammalian Nit1 and its yeast ortholog can very efficiently hydrolize deaminated glutathione (dGSH), i.e., a form of glutathione in which the free amino group has been replaced by a carbonyl group. We further showed that *Nit1*-KO mutants of both human and yeast cells accumulate dGSH, and that the same compound is excreted in large amounts in the urine of *Nit1*-KO mice. Finally, we showed that several mammalian aminotransferases can form dGSH *via* a common (if slow) side-reaction, and provided indirect evidence that transaminases are mainly responsible for dGSH formation in cultured mammalian cells.

Altogether, these findings delineate a typical instance of metabolite repair, whereby the promiscuous activity of some abundant enzyme(s) of primary metabolism leads to the formation of a useless and

potentially harmful compound, which needs a suitable 'repair' enzyme to be destroyed or reconverted into a useful metabolite. The need for a dGSH repair reaction does not seem limited to eukaryotes: we demonstrated that Nit1 homologs acting as excellent dGSH amidases also occur in *Escherichia coli* and other glutathione-producing bacteria [3].

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Plenary Symposium
Transcriptional regulation, chromatin structure and epigenetic modifications

TRANSCRIPTION FACTORS CONTROL IN PLURIPOTENCY

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Pluripotency is the capability of a single cell to generate all embryonic and adult cell types. *In vivo* this ability is exhibited by the epiblast and *in vitro* by pluripotent stem cells (PSCs). Epiblast cells of the early blastocyst exhibit naïve pluripotency characterized by ubiquitous expression of Nanog, Klf2/4 and Esrrb; while epiblast cells of late blastocyst and post-implantation embryos show primed pluripotency, which, in turn, exhibits down-regulation of naïve factors and activation of specific markers such as Otx2, Fgf5, and Oct6. *In vitro* PSCs may convert into a naïve state of pluripotency if provided with the two inhibitor molecules (2i), which respectively inhibit FGF signalling and activate WNT signalling; alternatively PSCs may also convert to a primed state of pluripotency if LIF is replaced with FGF. Mouse embryonic stem cells (ESCs) are PSCs derived from both the inner cell mass (ICM) and early preimplantation epiblast; they can be indefinitely propagated in culture by ensuring provision of LIF+FBS and may efficiently integrate into host blastocysts and contribute to all body tissues. ESCs cultured in LIF+FBS are characterized by cell heterogeneity in both expression of specific transcription factors and sensitivity to signalling molecules, which together define a state ensuring self-renewal and ability to convert into naïve or primed pluripotency. This cell heterogeneity is exemplified by the detection of naïve and primed markers in specific ESC sub-type compartments. A similar heterogeneity exists in preimplantation mouse embryos at E4.5-E4.7 when the epiblast gradually loses naïve identity and begins to induce early primed pluripotency. Signalling pathways-mediated committment to naïve or primed pluripotency is associated to a response in the expression of specific genes which ultimately determine the state of pluripotency. For example Nanog overexpression or loss of Otx2 are sufficient to drive LIF-independent self-renewal. Moreover, Otx2 is required for transition into early primed pluripotency. We hypothesize that integration of the Otx2 and Nanog antagonistic networks specifies the heterogeneous identity of ESCs cultured in LIF+FBS. To gain insights into this mechanism, we studied whether different dosage of Otx2 and/or Nanog may intrinsically modify identity and pluripotent state of ESCs cultured in LIF+FBS without the contribution of signalling effectors. Our data suggest that Otx2 and Nanog cooperate to deliver from their antagonistic regulatory networks the heterogeneous and flexible identity of ESCs cultured in LIF+FBS. Without integration of this mutual antagonism, the plasticity of ESCs is lost and the capacity for bidirectional conversion into naïve or primed pluripotency is forced in a more unidirectional manner either forward (primed) or reverse (naïve), which are respectively established by the dominance of the Otx2

regulatory network over that controlled by Nanog or *vice versa*. When both networks are abolished, ESCs retained a degree of plasticity sufficient to accomplish bidirectional conversion.

MECHANISMS OF CONTROL OF CELLULAR IDENTITY BY POLYCOMB CHROMATIN MODIFIERS

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Establishing and then maintaining cellular identity during differentiation requires signalling events to be transmitted to the chromatin level; transcription factors (TFs) and chromatin-remodelling activities work together to orchestrate the transcription programs underlying this transmission. It is now clear that chromatin remodelers play a major role in regulating cellular identity, resulting one of the most mutated pathways among all type of human cancers. In this context, Polycomb proteins (PcG) play a crucial role as regulators in development and differentiation and are frequently mutated or altered in their activity in numerous types of human cancers, via molecular mechanisms that are still poorly understood.

At the meeting will be presented the recent advances of our laboratory aimed to dissect the molecular mechanisms underling the activity of distinct PcG activities in establishing and maintaining cell type specific transcriptional identity during both normal homeostasis and pathological conditions.

RNA as a carrier of epigenetic information

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Both prokaryotic and eukaryotic cells release into the extracellular matrix membrane-bound structures of different sizes, origin and composition, collectively called extracellular vesicles (EVs) [1]. Tumor cells, in particular, use EVs to transfer both nucleic acids and proteins to the surrounding normal cells, thus inducing in them transformed behaviours or killing them. G26/24 oligodendroglioma cells, for example, transfer by EVs pro-apoptotic proteins, such as TRAIL and Fas-Ligand [2], extracellular matrix remodelling proteases (such as ADAMTS) [3], and even the H1.0 histone protein [4].

Another tumour cell line, with a different tissue origin (A375 melanoma cells) releases into the medium EVs containing a sumoylated form of H1.0 histone and even H1.0 mRNA [5]. By a T1 RNase-protection assay, we evidenced, in these EVs, three main H1.0 RNA-protein complexes, the most abundant of which had an apparent molecular mass of about 65 kDa. By affinity chromatography on biotinylated H1.0 RNA, we isolated a group of proteins, then analysed by mass spectrometry. One of these proteins is the myelin expression factor-2 (MYEF2). The presence of this protein in EVs was also confirmed by Western blot analysis [5]. MYEF2 was already known as a transcription factor with putative RNA-binding domains. Our demonstration of its actual ability to bind RNA, together with the well accepted ability of EVs to transfer microRNAs (miRNAs) and long non coding RNAs (lncRNAs), rises the possibility that different classes of RNA can function as carriers of factors that, once in receiving cells, epigenetically change their transcriptional potential.

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Multifunctional role of the ZNF224 transcription factor

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ZNF224 is a member of the zinc finger proteins family containing the Kruppel-associated box (KRAB-ZFPs). It was originally identified as a transcriptional repressor, involved in gene-specific silencing through the recruitment of the corepressor KAP1 and chromatin-modifying activities on the promoter of its target genes (1). Moreover, the arginine methyltransferase PRMT5 was identified as an additional component of the ZNF224 repression complex and it was demonstrated that histone arginine methylation is required to elicit ZNF224-mediated transcriptional repression, thus providing new information on chromatin modification required for repression of gene transcription by KRAB-ZFPs (2).

Recent findings indicate that ZNF224 can behave both as a tumor suppressor or an oncogene in different human cancers. The transcriptional regulatory properties of ZNF224 appear to be complex and influenced by specific sets of interactors. In chronic myelogenous leukemia (CML), ZNF224 plays a key pro-apoptotic and antiproliferative role. To exert these effects, ZNF224 works in a DNA-binding-independent mode of transcriptional regulation, acting as a transcriptional cofactor of Wilms' tumor protein 1, WT1 (3,4). More recently, it was demonstrated that ZNF224 acts as an oncosuppressor in CML also by operating as a DNA-binding transcriptional repressor. Indeed, we identified the oncogene c-myc as a direct target of ZNF224 transcriptional repression activity in CML. On the other hand, we demonstrated that ZNF224 plays an important oncogenic role in Chronic Lymphocytic leukemia (CLL). ZNF224 expression is associated with survival advantages and drug-resistance in CLL. Furthermore, ZNF224 positively modulates cyclin D3 gene expression, thus likely contributing, besides apoptosis resistance, also to impaired growth of CLL cells (5).

Other authors showed that ZNF224 affects cell growth and suppression of apoptosis in bladder and breast cancer cells (6,7). All together, these findings highlight a dual role for ZNF224 in cancer. The characterization of the multiprotein complexes which ZNF224 is involved in will contribute to elucidate how alterations of these complexes could affect apoptosis and cell growth in cancer.

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Mitochondrial Transcription in Animal Systems: Recent Advances and Challenges

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Animal cells contain two separate genetic systems localized in the nucleus and in mitochondria.

The most studied example of mitochondrial genome is the mitochondrial DNA (mtDNA) from mammals. With its double-stranded molecule, densely packed with 37 genes in only about 16.5 kbp, mammalian mtDNA represents an exceptional example of genetic economy. It encodes 22 tRNAs, two ribosomal RNAs and 13 proteins that are core components of Complexes I, III, IV and V of the respiratory chain. The remaining proteins needed for the proper function of the organelle are nuclear-encoded and imported into mitochondria (1).

All genes in mammalian mtDNA lack introns, there are almost no intergenic sequences and there is only one longer noncoding region that functions as regulatory region. A basic and simplified transcription mechanism was proposed in the mid-80s; it is based on the existence of three transcription units and the punctuation model for the mitochondrial RNAs processing. The genome is transcribed by a dedicated protein machinery, which includes the mitochondrial RNA polymerase (POLRMT) and some auxiliary factors, the concerted action of which ensures the fine-tuning of transcription.

Mitochondrial genomes in metazoans show a basic invariance in the gene content but remarkable differences in gene organization. Our studies in sea urchin and Drosophila showed that in invertebrates, despite the conservation of the basal transcription apparatus, the transcription mechanism is different from that of mammals. This suggests that, during evolution, the differences in the mitochondrial gene organization probably triggered changes in the transcription mechanisms with unexpected implications (2).

Recently, the resolution of the atomic structure of most components of the mammalian transcription machinery and the use of conditional mouse knockouts has increased the understanding of the transcription process at both levels of initiation and termination, and their regulatory pathways (1).

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Parallel Symposia

Proteomics in Cell Biology and Disease Mechanisms

Coupling proteomics and genetics to study cilia: a disease relevant organelle

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The availability of thousands of genetic variants means that it is now possible to discover the genetic basis of biological variation and disease. However, this quest is hampered by the complexity and quantity of the genetic data generated. A major problem when interrogating these data is the difficulty in understanding the potential functional consequences of what are typically thousands of variants possibly responsible for a particular disease or trait. Over the last decade, my group has developed numerous computational tools to study and predict molecular mechanism by integrating proteomics, three-dimensional structures and information related to protein function. I will discuss our approaches applied to ciliopathies, a specific class of genetic diseases (those related to ciliary dysfunction), where we identified new proteins, genes and variants of functional and clinical significance. Our approach addresses a current large gulf between those determining genetic data and those most specialized in understanding molecular mechanism.

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Proteolysis and amyloid formation: a new way of reading an old story

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The chemical characterization of natural amyloid fibrils in systemic amyloidosis suggest that most of the constitutive culprit proteins are cleaved (1) . The role of selective proteolytic cleavage on the amyloidogenesis of globular protein is an old but still mysterious phenomenon generating two major question: 1. Does the cleavage occur before or after the deposition of amyloid? 2. Does the cleavage has any role on the fibrillar conversion of globular proteins? The long-term research story of a putative role of protein fragmentation in Transthyretin (TTR) amyloidosis illuminates on the slow and continuous progress made in the last 20 years to better understand how the structural dynamics of amyloidogenic proteins could offer a special susceptibility to proteolytic cleavage. A region of local instability is well tolerated in the TTR assembled homotetramer, but a selective proteolytic cleavage has a catastrophic effect on tetramer stability (2). The transthyretin model reveals how local unfolding and proteolytic cleavage might represent two obligate partners equally responsible for the early pathogenic event of the amyloid cascade.

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Not all roads lead GAA to lysosomes

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Pompe Disease (PD) belongs to the Lysosomal Storage Diseases (LSDs) and it is originated by mutations in the GAA gene coding for acid α-glucosidase (GAA), an enzyme active in lysosomes where it breaks down glycogen into glucose. Impaired glycogen degradation and accumulation within the lysosomes leads to enlargement of cardiac and skeletal muscles, with irreversible damages and premature death. In the last years, several therapeutic approaches have been proposed for PD treatment. Among these, the Enzyme Replacement Therapy (ERT), based on the administration of recombinant human GAA (rhGAA, Myozyme) to PD patients, resulted one of the most effective.

GAA reaches lysosomes directly via the trans-Golgi network (TGN) and the endosome-lysosome pathway. Although endowed with normal enzymatic activity, some GAA mutants are impaired in following the normal route to lysosome thus originating the pathology. Moreover, ERT is less effective than expected since only a portion of the injected enzyme was demonstrated to be available for catalysis. In order to go deeper in the molecular basis of PD, we investigated the intracellular traffic pathways followed by wild-type GAA to move from ER to lysosomes by a functional proteomic approach. Endogenous GAA protein partners were isolated by immunoprecipitation and identified by LCMSMS based strategies. Several putative interactions were also investigated for L552P GAA mutant, in order to determine their role in the PD onset.

An analogous approach was employed to unravel the uptake pathway of the recombinant GAA (rhGAA, Myozyme), in order to define the fate of recombinant protein once internalised into the cell.

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Human saliva: a precious bodily fluid rich of information

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Saliva is a very attractive bodily fluid for the diagnosis and prognosis of diseases for many reasons: i) collection of saliva is usually economical, "safe", "easy" and can be performed without the help of health care workers (it allows for home-based sampling); ii) it is considered an acceptable and non-invasive process by patients because it does not provoke any pain and so can be easily collected from patients in the pediatric age range[1].

Our group started more than fifteen years ago a study on this bodily fluid with an integrated top-down, bottom-up proteomic platform. The lecture will describe the proteoforms of the main families of human salivary proteins, i.e. proline-rich proteins, histatins, statherins, cystatins, α-defensins and β-thymosins, characterized by this strategy as well their post-translational modifications (phosphorylation, sulfation, glycosylation, cyclization, fragmentation) which are due to enzymes common to other exocrine and endocrine glands and tissues[2,3].

Age related trends will be discussed with a particular concern to the physiological variations observed in pre-term newborns and in the pediatric age range[4]. Some examples of variations of the human salivary proteome observed in multi-factorial diseases will be therefore reported[5].

The putative role in the oral cavity of some salivary proteoforms detected and the demanding issues arising from the proteomic results until now obtained will be finally pointed out.

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Deciphering the SECRET language of cellular messengers by OMICS approaches

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Over the last decades, the investigation of extracellular molecules involved in cell signalling has opened new avenues in proteomic research (1). The elucidation of cellular secretomes, i.e. the sub-populations of a proteome secreted by cells, tissues or organisms, is eliciting a growing interest mainly related to potential implications in biomarker discovery of diagnostic and/or prognostic significance. Secreted proteins (e.g. growth factors, cytokines, chemokines, enzymes, proteases) finely coordinate and modulate signaling pathways, thus regulating most cell functions through paracrine and/or autocrine mechanisms. Furthermore, extracellular signaling molecules also affect local niche biology and influence the cross-talking with the surrounding tissues. The understanding of this molecular language may provide an integrated and broader view of cellular regulatory networks under physiological and pathological conditions. Nevertheless, the analysis of secreted proteins is still a challenging task due to the low amounts of secreted factors and technical difficulties that may hamper the subsequent mass spectrometry analysis.

We present an optimized workflow for the profiling of conditioned media of cultured cell lines together with crucial issues related to the collection of conditioned media, secretome preparation and mass spectrometry analysis. Furthermore, an overview of applications for the secretome characterization of model systems including stem cells, tumor cells and biological fluids is also presented.

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PROTEOMIC APPROACH TO STUDY PANCREATIC HUMAN ISLETS AFTER CYTOKINE EXPOSURE

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In the progression of type 1 diabetes (T1D), it is well known that pancreatic islet β cells are mainly destructed by autoimmune-mediated apoptosis, and inflammatory cytokines play critical roles in this process. Indeed, at the early stage of T1D, immune cells infiltrate into the islets and secrete inflammatory cytokines which amplify the autoimmune reaction. The chronic exposure of β cells to pro-inflammatory cytokines finally induces islet dysfunction and β cell apoptosis. The aim of the present work is to investigate if cytokines could trigger their pro-inflammatory effect through lysine acetylation deregulation. Indeed, recent evidence suggests that protein lysine acetylation may play a critical role in regulating metabolic and antioxidant pathways.

The study was performed on human pancreatic islets without treatment and islets treated with a cytokines cocktail with or without metformin. We used two-dimensional electrophoresis and western blot analysis to locate the preferentially acetylated proteins which were subsequently identified by LC-MS.

From the comparison, a global down-regulation of acetylation comes to light in human islets treated with cytokines respect to control, except for two proteins. One of these two proteins was mitochondrial superoxide dismutase and acetylation is reported to significantly inhibit its activity, resulting in an increase in ROS, thus inducing β cells apoptosis. On the other side, we detected the reduction of acetylation for the most proteins, which are mainly involved in metabolism. When metformin was added with cytokines, we observed an increased acetylation, but only along with the presence of cytokines as if it act in reaction to a defective system. Our results seem to support the hypothesis that the effect of cytokines could be mediated by an alteration in the acetylation balance. On the other hand, metformin has been shown to exert a protective effect against this alteration.

Parallel Symposia

Novel Frontiers of Nutritional and Environmental Biochemistry

Neuroprotective effect of flavonoids and their underlying molecular mechanisms

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Accumulating evidence suggests that diet and lifestyle can play an important role in delaying the onset or halting the progression of age-related health disorders and to improve cognitive function. A growing number of dietary intervention studies in humans and animals and in particular those using flavonoids, have been proposed to exert a multiplicity of neuroprotective actions within the brain, including a potential to protect neurons against injury induced by neurotoxins, an ability to suppress neuroinflammation and a potential to promote memory, learning, and cognitive functions. These effects appear to be underpinned by two common processes. First, they are capable of interactions with critical protein and lipid kinase signalling cascades in the brain, leading to an inhibition of apoptosis triggered by neurotoxic species and to a promotion of neuronal survival and synaptic plasticity. Second, they induce beneficial effects on the vascular system, leading to changes in cerebrovascular blood flow capable of causing enhance vascularisation and neurogenesis, two events important in the maintenance of cognitive performances. Together, these processes act to maintain brain homeostasis and play important roles in neuronal stress adaptation and thus flavonoids might have the potential to prevent the progression of neurodegenerative pathologies.

The use of trout erythrocytes as a model for the study of oxidative stress and damage associated to environmental pollutants exposure

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Erythrocytes are a routinely used model to study the toxicity of new chemicals, in particular fish erythrocytes are useful simple models because they posses nuclei, mitochondria and other organelles typical of somatic cells . These cells are not thus simple haemoglobin contenitors but they are also able to maintain complex cellular processes, including protein synthesis and oxidative metabolism. Contrary to mammals and birds, a multiplicity of hemoglobin components Is present in fish erythrocytes, in order to provide oxygen for different purposes, namely the metabolic demands and the operation of the swim bladder. These haemoglobins are prone to oxidation, either as purified proteins or in the whole cell. This property permits to follow the autoxidation process over a relatively short time and to investigate the relationship between met-Hb formation and impairment of cellular structures in erythrocytes(cell membrane, nucleus, mitochondria etc.). The nucleated trout erythrocyte represents also a stimulating cellular model to study oxidative damage associated with senescence processes. It is known that there is a correlation between the density of erythrocyte subpopulation and ageing. Keeping in account the upon described characteristics we used trout erythrocytes (and their subpopulations) to study physiological processes and the interaction of relevant environmental contaminants [1-6]. Hence, the nucleated trout erythrocyte represents a unique cellular model to study in vitro the antioxidant efficacy natural or synthetic compounds, the ageing process as well as ecotoxicological studies. The present communication will provide some examples of the application of the model to toxicological studies of metals and organometallic compounds relevant to anthropogenic pollution

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Pros and cons of polyphenols in cancer: the example of quercetin in chronic lymphocytic leukemia

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The concept that polyphenols, being antioxidant compounds, can fight cancer is deeply rooted in the general population. We recently reviewed clinical and pre-clinical studies on this topic suggesting that a clear cut must be done between the use of polyphenols in cancer treatment versus cancer prevention and that the experimental approaches to investigate one or the other should be significantly different, starting from adequate and specifically selected cellular models (1). A good example derives from the use of quercetin in chronic lymphocytic leukemia (CLL).

Quercetin, the most abundant flavonoid present in the diet, is able to modulate several hallmarks of cancer, including resistance to apoptosis. Previous studies from our group demonstrated the capacity of quercetin to synergistically sensitize several leukemia cell lines and B-cells isolated from CLL patients when associated with death ligand agonists (e.g., anti-CD95 and rTRAIL) and chemotherapeutic drugs (fludarabine, ABT-737, other BH3-mimetics). This effect is mediated by changes in the expression of Mcl-1, an anti-apoptotic proteins belonging to the Bcl-2 family, whose activity has been associated to apoptotic resistance in CLL. Using a new cell line, HG3 derived from primary B-cells immortalized with Epstein-Barr virus, we demonstrated that the association between BH3-mimetics and quercetin synergistically induces apoptosis through the inhibition of PI3K/Akt signaling pathway. We also identified the protein kinase CK2 as the direct and primary target of quercetin, since CK2 activity is inhibited by the flavonoid within one minute from the treatment. Considering the rapid uptake of quercetin and its low toxicity against normal peripheral blood cells, we recommend the design of clinical studies aimed to demonstrate the potential use of the molecule in the adjuvant chemotherapy against CLL.

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Contribution of type 2 transglutaminase to the celiac cellular phenotype

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Type 2 transglutaminase (TG2) has an important patogenetic role in celiac disease (CD), an inflammatory intestinal disease caused by ingestion of gluten-containing cereals. Indeed, TG2 deamidates specific gliadin peptide, thus enhancing their immunogenicity. Moreover, transamidating activity causes an autoimmune response, being TG2 the main autoantigen. Anti-TG2 antibodies may have a pathogenetic role by modulating TG2 biological functions. Autoantibodies also derange the uptake of the toxic alpha-gliadin peptide 31-43 (p31-43) in an intestinal cell line, thus partially protecting cells from p31-43 damaging effects. Recently, an *in vitro* model of primary dermal fibroblasts has been developed to study constitutive differences between celiac and control cells. Using this model, we investigated whether anti-TG2 also displayed a "protective role" towards p31-43. We found that antibodies specifically reduced p31-43 uptake by fibroblasts derived from healthy subjects. Surprisingly, antibodies did not affect p31-43 uptake by celiac fibroblasts. We investigated whether such observed behavior depended on differences in TG2 expression level, activity or subcellular distribution. Analyses of TG2 expression and enzymatic activity did not reveal any significant difference between fibroblasts from healthy and celiac subjects. However, we observed that TG2 was more abundant in membranes of celiac fibroblasts than in membranes of control cells, with a slight higher association with surface cell membrane in CDs than in controls. Moreover, confocal images showed that TG2 differently distributed into vesicular compartment, with a higher association with the early endosome compartment in celiac fibroblasts than in control ones. Our findings suggest that TG2 may contribute to define the "celiac cellular phenotype", *i.e.* a condition independent from gliadin exposition and also evident far from the main site of inflammation. Moreover, they suggest that autoimmune response to TG2, which may have *per se* a damaging effect on celiac mucosa, also fails in its "protective role" in celiac cells.

Effect of diet on peripheral tryptophan hydroxylase and plasma tryptophan levels in Goldfish

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As feeding behavior is tuned by a complex interplay of neurotransmitters, neuropeptides and hormones, a deeper understanding of the influence of starvation on peripheral serotoninergic pathway in a model organism as Goldfish (Carassius auratus) (1) would be interesting. Previous studies have shown that starvation induces an increase of both TPH-1 mRNA levels in some tissues and tryptophan plasma concentration, while undetectable plasma serotonin (5-HT) levels have been observed (2). The aim of study was to confirm above mentioned data in fish of different size, integrating results by Western-blot analysis and measurement of plasma serotonin and tryptophan (TRP). Animals were acclimatized for 7 days into 2 tanks of F.W. and divided into 4 groups 4 fish each (Group 1 and 2 b.w. 10±2 g; Group 3 and 4 b.w. 20±5 g). Group 1 and 3 remained fasted for 3 days; Groups 2 and 4 were fed for 3 days. Plasma samples (heparin) were collected from caudal vein; fish were sacrificed for tissue sampling (anterior, mid, posterior intestine, liver and gills) and total RNA and proteins were extracted from homogenized tissues. Total RNA was quantified, reverse-transcribed to cDNA and processed by Real Time PCR, while proteins were evaluated through Western-blot assay. Plasma was used both for 5-HT and TRP detection by HPLC and hematological assays. Results showed higher TPH-1 and plasma TRP levels in fasted than in fed fish tissues except in gills. Fasting conditions may have induced an increase of TRP amounts coming from protein catabolism, which in turn may be responsible of the increased mRNA expression in gut and liver. Plasma 5-HT levels were below 1 ng/ml in all fish. No differences in blood parameters were found.

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Influence on food digestion and glycemic index by enzymatic protein crosslinking

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Enzymatic crosslinking of proteins is most generally applied to tailor the technological properties of numerous proteins and subsequent food structures. Among the enzymes potentially able to crosslink proteins some oxidative enzymes and transglutaminase (EC 2.3.2.13) are the most promising biocatalysts. In particular, the microbial isoform of transglutaminase (mTG) has been object of special attention in the food sector. The enzyme catalyzes the polymerization of proteins, primarily through formation of intermolecular ε-(γ-glutamyl)-lysine isopeptide crosslinks. Its properties, such as the calcium independency and the broad substrate specificity for the acyl donor, make this molecular form of the enzyme an efficient tool to modify the technological and biological properties of different food-based proteins. In fact, it has been demonstrated to improve the firmness, viscosity, elasticity and water-binding capacity of many foods, including meat, fish, wheat as well as dairy products, often enhancing their texture and functional properties. Lately, mTG was also found to change the digestibility properties and the glycemic index of some food related proteins. In this context we have subjected several food mTG-treated proteins to *in vitro* digestion following a procedure mimicking physiological conditions and aimed to facilitate the rationale design of foods. In fact, understanding the impact of food processing on protein digestion is an important prerequisite for predicting its effects on protein metabolism. Although in many instances the modified proteins appear more resistant to gastric and duodenal digestion, we have found that foods containing mTG-modified proteins are characterized by a low glycemic index. These findings could have high impact on the development of novel healthy functional foods able to enhance satiety.

Plenary Symposium

Industrial and Molecular Biotechnologies

Session I: Future perspectives in biotechnology research

Personalized medicine in oncology: from target therapies to advanced molecular diagnostics to genome-based clinical trials

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In the last twenty years cancer therapy has significantly benefited from the introduction of target therapies and the use of companion diagnostics to stratify patients' treatments. In spite of this, overall cancer survival has only been modestly impacted by these therapeutic advances. The main reason for these shortfalls is to be attributed to tumour heterogeneity (both inter- and intra-patient) and to the selection of resistant clones. Increased understanding of molecular oncology, an expanding universe of novel therapeutics, and low cost next-generation sequencing (NGS) techniques are now creating new opportunities for further improving cancer therapy through the establishment of genomic-driven personalized cancer therapy (1). In order to implement genomically informed therapy it is necessary to switch from single gene mutational analysis to targeted sequencing of an expanding universe of driver mutations targetable either directly or indirectly with approved or investigational therapies and therefore potentially "actionable." Genomic alterations, including mutations, insertions/deletions, fusions, and copy number changes, need to be curated in terms of the likelihood that they alter the function of a "cancer gene" at the level of a specific variant in order to discriminate so-called "drivers" from "passengers" mutations. NGS technologies capable to simultaneously detect single nucleotide changes, structural rearrangements and copy number changes will have to be developed, standardized and implemented on a large scale. Also, we need accessible, comprehensive, and frequently updated knowledge bases that describe genomic changes and their clinical implications, as well as continued education of clinicians and patients. The genomic characterization of large number of patients will have to be exploited to enrol patients in innovative basket clinical trials aiming at bridging the gap between detection of molecular alterations and identification of the appropriate treatments. Finally, improvements in the sensitivity of detecting circulating tumour DNA (ctDNA) by liquid biopsies will have to be implemented to allow monitoring cancer patients longitudinally, in order to anticipate tumour recurrences and modulate therapy against emerging drug-resistant tumour clones (2). All

these aspects will be discussed in the context of current efforts by the Italian Alliance Against Cancer (www.alleanzacontroilcancro.it), the national network of 21 Clinical and Research Institutes supported by the Ministry of Health, to establish a Precision Medicine program having the goal to sequence a significantly high number of genes and standardize procedures over the entire Italian territory, without increasing the costs and to create the basis for the implementation of genomic medicine of cancer at a national scale.

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Marine biotechnology: new opportunities for Italian research and industrial development

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Marine biotechnologies refer to the knowledge-based use of marine biological resources for the wellbeing of human, animals and the environment and includes various sectors, which have direct social implications such as food, nutraceuticals, biomedical. The future of marine biotechnology includes the development of the innovative technologies for maritime and off-shore industries (including energy use and emission, waste treatment, bioremediation, new materials, antifouling solutions etc.). Marine biotech is at the crossroad of the three pillars: 1) Understanding the (life of) oceans, 2) Living with changing oceans, 3) developing an eco-sustainable marine/maritime economy. Scientific knowledge (including marine biogeography, the functioning of marine organisms, the role of metabolic pathways) is the start point for optimizing application of marine biotech products. The knowledge of the molecules enabling specific biological processes is a second step. The economic implications of marine biotech typology of the product, and deal with costs associated to large-scale production, and legal gaps (including those dealing with patents systems or the way access to marine genetic resources and share benefits deriving from their commercial use). For all the above statements, marine biotech builds up on cross-sector actions towards the improvement of ocean economy. Genome bioinformatics and computational biology, sequence and structure analysis, molecular evolution and –omics technologies are key enablers to make advances in the biotech field. There is an urgent need to both maintain and stimulate research on marine biology and taxonomy, which together with the biochemistry competences can provide access to biological models and marine bio-resources, providing at the same time the identification of the specific products that can be exploited. Ecological role, physiological features and molecular adaptation mechanisms of micro-organisms provide information on unexplored solution for a variety of purposes. With this aim, it is also important to develop identify, reproduce and maintain new organisms as a model for the comprehension of the secrets of marine life and to expand the discovery in biotechnology and biomedicine. Biotechnological developments can use proteins, fatty- and amino-acids, enzymes, biopolymers and other compounds. Advancing our knowledge in marine chemical ecology and marine system biology/ecology is crucial to exploit the potential of marine molecules, organism and material in biotechnology. Research is needed on the genetic and molecular mechanisms of organisms is also needed to expand the application of biotechnologies to marine biodiscovery. Exploration of extreme habitats and their extremophilic biodiversity biodiversity is can provide new opportunities in order to identify

novel molecules or products of commercial interest. Bio-prospecting requires joint research efforts, clear rules on access and benefit sharing, ownership and environmental ethic and responsibility. Biomarkers and biosensors technologies, based also on microbial organisms have been recently recognised to have a huge potential. Eco-sustainable use of living marine resources requires a long-term approach and important technological investments, but could provide major returns in term of industrial and socio-economic development. Marine biotechnology is one of the most promising research field of the future and can help recovering polluted habitats, improving human health and nutrition, for thus sustaining the human population growth. Yet this potential is almost completely non-exploited yet in Italy. This talk aim at providing elements to consider this potential in the future of Italian research and industrial development.

Industrial and Molecular Biotechnologies

Session II: Mechanisms, processes and technologies for innovative applications

"Cold Biotechnology": enzymes and antifreeze proteins from Antarctic organisms

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Earth is a cold place where the temperature of over 85% of soil and water environments is close to the freezing point of water. Under these conditions, challenges for Life are multifaceted, as temperature affects several key biological processes. Nevertheless, a rich variety of organisms widespread across the Nature kingdoms thrives in cold habitats. To cope with environmental constraints, the so-called *cold-adapted* or *psychrophilic* organisms have evolved different adaptive strategies, among these cold-active enzymes and ice binding proteins, that are of interest for biotechnological application [1].

Cold-active enzymes retain high specific activity at low temperature thanks to specific changes in their molecular structure that allow for high flexibility of the whole protein or of the active site regions thereof. In several cases flexibility is accompanied by reduced stability. Such features make psychrophilic enzymes suited for application in low temperature processes, i.e. detergency, food processing, transformation of heat labile compounds. We are studying different cold active enzymes in the perspective of identifying specific features related to temperature adaptation and will discuss in particular the specific case of an acyl amino peptidase that shows a unique mechanism of oligomerization [2]. Recently we focused on a microbial consortium of the Antarctic ciliate *Euplotes focardii* and associated bacteria. This system is used as a source of novel enzymes (superoxide dismutase, beta-galactosidase, lipase, chitinase) and of ice binding proteins (IBPs). We will describe recent results about a bacterial IBP identified by metagenomics, as for its properties of thermal hysteresis and inhibition of ice re-crystallization and structure.

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Cryptic antimicrobial peptides, a still unexploited source of bioactive peptides

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Cationic antimicrobial peptides (CAMPs) are the first line of defence against microbial invasion in higher eukaryotes. They combine broad spectrum antimicrobial activity with antibiofilm activity, anti-inflammatory/immunomodulatory properties and wound-healing enhancement. Despite differences in their size, sequence and secondary structure they share a net positive charge and a high content of hydrophobic residues which are at the basis of an unusual mechanism: after contact with bacterial surfaces they fold into amphipathic structures which drive their insertion into lipid membranes; this in turn damages membrane impairing membrane functions. Some of the best known examples are cathelicidins like human LL-37. Very intriguingly several proteins, including proteins apparently not involved in immunity, can behave as sources of "cryptic AMPs" hidden in their primary structures and released by the action of host and/or bacterial proteases. Recently we developed a bioinformatic tool allowing to identify such unusual AMPs [1]. This tool is based on the demonstration that it is possible to assign to each peptide an "absolute score" (AS), based on the peptide amino acid composition and two bacterial strain dependent variables, which is linearly correlated to the antimicrobial potency expressed as Log (1000/MIC) where MIC is the minimal inhibitory concentration. Analyzing a library of four thousands secreted human proteins, we have identified several novel human cryptic CAMPS including peptides from Apolipoproteins E and B, Pepsinogen A, Fibrinogens α, β, and γ and a 11-hydroxysteroid dehydrogenase-1 β-like protein. Further cryptic CAMPs were identified in an archaeal protein and in plant ribosome inactivating proteins. All these peptides not only show broad spectrum antimicrobial activity but also several additional biological activities typical of CAMPs thus demonstrating that cryptic CAMPs are a still unexploited source of bioactive peptides.

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Hydrocolloid materials: reinforcement and new plasticizers

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Hydrocolloid materials derived from proteins and/or polysaccharides are increasingly used to produce edible films and coatings for food and pharmaceutical applications. Biomaterials need to have good mechanical resistance, elasticity and flexibility, low brittleness, and sufficient toughness to prevent its cracking during handling and storage. Although the limited mechanical and barrier properties of hydrocolloid films, their use may be extended by adding some reinforcement agents to the film forming solutions (FFSs), such as nanoparticles, chemical or enzymatic cross-linkers, and plasticizers. The advancement of nanotechnology has boosted interest to new types of composite materials in which the filler has at least one dimension smaller than 100 nm. Furthermore, compared to chemical cross-linking agents, enzymes offer undoubted advantages, first of all the absence of toxicity. Among the biocatalysts, *Streptoverticillium mobaerense* transglutaminase is a powerful tool, being able to produce protein inter- ε-(γ-glutamyl)lysine isopeptide bonds, to modify soluble substrate proteins into high molecular weight polymers inside the FFSs, and to improve film water vapor barrier features to allow effective food coatings [1,2]. Finally, plasticizing compounds are generally added to hydrocolloid FFSs to obtain the desired deformability of biomaterials. Our recent studies demonstrated the effectiveness of polyamines as a valid alternative to the conventional plasticizers (glycerol, sorbitol, propylene glycol, polyethylene glycol, oligosaccharides), being able in preventing both polysaccharide [3] and protein [4] chains to be closer and allowing them to slip one over the other and, consequently, in increasing film elongation and flexibility.

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UNDERSTANDING PROTEINS: TAILOR-MADE APPROACHES FOR BIOTECHNOLOGY SOLUTIONS IN INDUSTRIAL PROCESSES

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IXTAL SRL IS AN INNOVATION-DRIVEN CONTRACT RESEARCH ORGANISATION THAT PROVIDES SOLUTIONS IN THE FIELD OF PROTEIN SCIENCE, STRUCTURAL BIOLOGY AND DRUG DESIGN. THE MAIN PURPOSE OF THE COMPANY IS TO BRIDGE THE GAP BETWEEN THE ACADEMIC CUTTING-EDGE RESEARCHES AND TECHNOLOGIES AND THE INDUSTRIAL INNOVATION DEMAND.

PROTEIN RESEARCH ACTIVITIES REQUIRE SIGNIFICANT INVESTMENTS IN HIGH-VALUED HUMAN RESOURCES AND IN THE ACQUISITION OF DEDICATED INSTRUMENTATION. HENCE, THE OUTSOURCING OF SUCH ACTIVITIES TO SPECIALIZED COMPANIES IS BECOMING AN INCREASINGLY MARKED TREND IN THE LIFE SCIENCES AND BIOTECH SECTORS.

SINCE ITS FOUNDATION IN JANUARY 2015, IXTAL SRL HAS BEEN INVOLVED IN SCIENTIFIC PROJECTS AIMED AT SUPPORTING THE RESEARCH PIPELINE OF DIFFERENT PARTNERS FROM THE PHARMA AND FOOD INDUSTRY. IN PARTICULAR, THE COMPANY IS NOW ACTIVE ON THREE DIFFERENT RESEARCH ENDEAVOURS: I) THE RECOMBINANT EXPRESSION AND THE PURIFICATION, FOR A MULTINATIONAL PHARMACEUTICAL COMPANY, OF ACTIVE DOMAINS OF TWO HUMAN RECEPTORS FOR PROTEIN-LIGAND INTERACTION STUDIES; II) THE ENGINEERING OF A BIOTECHNOLOGICAL TOOLS FOR A SPECIFIC MODIFICATION OF NATURAL EXTRACTS TO BE USED AS FOOD ADDITIVES IN HIGH-SCALE PRODUCTION PROCESSES; III) X-RAY CRYSTALLOGRAPHY STUDIES FOR THE ABSOLUTE STRUCTURE SOLUTION OF SMALL MOLECULES OF PHARMACEUTICAL INTEREST TO BE PRESENTED ALSO AS INTEGRATING DATA FOR THE MARKET ACCESS DOCUMENTATION REQUIRED FROM THE REGULATORY AGENCIES[1].

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Industrial and Molecular Biotechnologies

Session III: Molecular targeting for drug discovery

MODULATION OF NMDA RECEPTOR FUNCTIONALITY ACTING ON THE D-SERINE CATABOLISM: A NOVEL THERAPEUTIC APPROACH FOR VARIOUS HUMAN PATHOLOGIES

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D-Serine (D-Ser) is a main co-agonist of NMDA receptors by acting at the glycine-modulatory site. In the human brain its level is controlled by the PLP-dependent enzyme serine racemase and the flavoenzyme D-amino acid oxidase (DAAO). Alterations in D-serine signaling have been reported in various neurological diseases. E.g., an anomalous reduction in D-Ser, associated to an altered DAAO level/activity, is apparent in schizophrenia (SZ) and bipolar disorder; several association studies have linked DAAO (and its regulatory interactor pLG72) with SZ susceptibility (1). On the other hand, D-Ser levels are greatly increased in the spinal cord of patients affected by familial (R199W DAAO mutation) and sporadic amyotrophic lateral sclerosis (ALS) as well as in Alzheimer's disease.

In order to clarify the role of DAAO on D-Ser level in the brain, the main properties of wild-type and variants corresponding to SNPs of this flavoenzyme were investigated by a combination of structural, biochemical and cellular studies (2,3). The increased DAAO activity producing an excessive depletion of D-serine, and resulting in NMDA receptor hypofunction which predisposes individuals to SZ, provided the rationale to propose its inhibition as therapeutic approach: a number of novel inhibitors have been designed (2). The mode of DAAO-pLG72 interaction was also investigated, as well as the effect of pLG72 mutations or expression level on DAAO activity and cellular D-Ser concentration (4).

We propose that targeting D-Ser metabolism could unveil favorable effects in the treatment of various human pathologies.

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Non-conventional splicing modulation by glucocorticoids: the case of ATM

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Ataxia Telangiectasia (A-T) is a rare incurable genetic disease, caused by biallelic mutations in the Ataxia Telangiectasia-Mutated (ATM) gene. Treatment with glucocorticoid analogues has been shown to improve the neurological symptoms that characterize this syndrome. Nevertheless, the molecular mechanism underlying the glucocorticoid action in A-T patients is not yet understood.

Here we report one of the possible molecular mechanisms involved in the partial restoration of ATM activity by the glucocorticoid analogue, dexamethasone (dexa), in cell lines derived from A-T patients. A non-canonical splicing, mediated by short direct repeat (SDR), has been identified and resulted markedly induced by the drug. This non-canonical splicing event generates a new transcript (ATMdexa1) that overcomes most of the mutations so far described within this gene and promotes the production of a shortened ATM protein. This newly identified protein variant, named miniATM, retains the catalytic domain of the native kinase. The functionality of this miniATM variant was ascertained by yeast complementation and kinase assays and was shown able to cooperate with the cellular machinery to rescue some cellular signaling failed in A-T cells.

Interestedly the same SDR-mediated transcript, so far demonstrated *in vitro*, is present in the RNA expression pattern of patients subjected to long-term treatment with dexa delivered through autologous erythrocytes (IEDAT, EudraCT Number 2010-022315-19). Surprisingly, quantitative PCR showed higher level of ATMdexa1 transcript in responder patients. Transcriptomic and preliminary proteomic results are reported, in order to highlight the likely RNA/protein pattern that might somehow assist the function of the rescuing protein miniATM, or directly promote the A-T phenotype improvement in LCLs and patients.

Acknowledgements:

1. Luciana Chessa (Department of Clinical and Molecular Medicine), Vincenzo Leuzzi and Daniela D'Agnano (Department of Pediatrics and Child Neurology and Psychiatry) University "La Sapienza" Roma.
2. Annarosa Soresina and Roberto Micheli, Department of Clinical and Experimental Sciences Spedali Civili and University of Brescia.
3. Sparks Children's Medical Research Charity.
4. EryDel SpA, Urbino.
5. European Project H2020 #667946 IEDAT.

Use of polymer conjugates for the intraperoxisomal delivery of engineered human alanine:glyoxylate aminotransferase: a protein therapy for primary hyperoxaluria type I

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Alanine:glyoxylate aminotransferase (AGT) is a liver peroxisomal enzyme whose deficit causes the rare disorder Primary Hyperoxaluria Type I (PH1). In PH1 patients, high amounts of endogenous oxalate are formed, leading to an increased urinary concentration and to the consequent formation of calcium oxalate stones first in the kidneys, and then in the whole body. PH1 displays a loss-of-function pathogenesis because the absence of functional AGT prevents the detoxification of glyoxylate inside peroxisomes, leading to its cytosolic accumulation and oxidation to oxalate[1]. Thus, an enzyme administration strategy to replenish the liver with active AGT might relieve disease symptoms. We recently described the conjugation of poly(ethylene glycol)-poly(L-glutamic acid) (PEG-PGA) with AGT via formation of disulfide bonds between the polymer and solvent-exposed cysteine residues of the enzyme. PEG-PGA conjugation does not affect AGT structural/functional properties, but allows the enzyme to be internalized in a cellular model of PH1 and restore glyoxylate-detoxification. The insertion of the C387S/K390S amino acid substitutions, known to favour the interaction with the peroxisomal import machinery, reduces conjugation efficiency, but endows the conjugates with the ability to reach the peroxisomal compartment[2]. These results, along with the finding that conjugates are hemocompatible, stable in plasma and not immunogenic, hold promise for the development of polymer-conjugated AGT as a therapeutic option for PH1 patients and represent the base for the application to other diseases due to the deficit of peroxisomal proteins.

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Short talks selected from submitted abstracts

Session 1: Proteins session 1 (P1)

YFHJ is a modulator of the frataxin function in bacteria

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Abstract

YfhJ (or IscX) is a protein of unknown function, which takes part in the iron-sulfur cluster assembly machinery, a highly specialised and essential metabolic pathway. YfhJ binds to iron with low affinity and interacts with IscS, the desulfurase central to cluster assembly. The YfhJ surface of interaction with IscS is the same involved in iron binding. Previous studies have suggested a competition between YfhJ and CyaY, the bacterial ortholog of frataxin, for the same binding surface of IscS. This competition could suggest a link between the two proteins with functional significance. Using a hybrid approach, we show here that YfhJ is a modulator of the inhibitory properties of CyaY: by competing for the same site on IscS, the presence of YfhJ rescues the rates of enzymatic cluster formation, which are inhibited by CyaY. The effect is stronger at low iron concentrations, whereas it becomes negligible at high iron concentrations. These results strongly suggest that Fe-S cluster assembly is an exquisite example of an enzymatic process, which requires a very detailed fine-tuning.

Novel human bioactive peptides identified in Apolipoprotein B

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Abstract

Host defence peptides (HDPs) are short, cationic amphipathic peptides that play a key role in the response to infection and inflammation in all complex life forms[1]. It is increasingly emerging that HDPs generally have a modest direct activity against a broad range of microorganisms, and that their anti-infective properties are mainly due to their ability to modulate the immune response[1]. Here, we report the recombinant production and characterization of two novel HDPs identified in human Apolipoprotein B (residues 887–922)[2] by using a bioinformatics method recently developed by our research group[3].

We focused our attention on two variants of the identified HDP, here named r(P)ApoB$_L$ and r(P)ApoB$_S$, 38- and 26-residue long, respectively. Both HDPs were found to be endowed with a broad-spectrum antimicrobial activity while they show neither toxic nor haemolytic effects towards eukaryotic cells[2]. Interestingly, both HDPs were found to display a significant anti-biofilm activity, and to act in synergy with either commonly used antibiotics or EDTA[2]. The latter was selected for its ability to affect bacterial outer membrane permeability, and to sensitize bacteria to several antibiotics[4]. Interestingly, both ApoB derived peptides were found to elicit anti-inflammatory effects, being able to mitigate the production of pro-inflammatory interleukin-6 and nitric oxide in LPS induced murine macrophages[2]. Furthermore, zeta potential and electron microscopy analyses of bacterial cells were recently performed upon treatment with peptides, in order to get insights into ApoB derived peptides mechanism of action. Altogether, these findings open interesting perspectives on the therapeutic use of the herein identified HDPs.

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New insights on the functional role of URG7 in the cellular response to ER stress

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Abstract

Hepatitis B virus (HBV) induces stress in the host cell endoplasmic reticulum and through HBx, a *trans*-activating viral protein, activates the Unfolded Protein Response (UPR) pathways, favouring virus replication and cell survival[1]. Among others, HBx protein activates the expression of the Up- regulated Gene clone 7 (URG7), a protein localized into the ER[2], with an anti-apoptotic activity, due to the PI3K/AKT signalling activation[3]. In order to shed light on the molecular mechanisms underlying URG7 activity, we studied its putative role in the ER stress response. Our main results demonstrate that URG7 is able to modulate the expression of UPR markers toward survival outcomes. The analysis of its interactome suggests that URG7 is able to interact with proteins involved in the synthesis, folding and protein degradation, in concert with an increase of total protein ubiquitination. All together, these data suggest that URG7 plays a pivotal role as a reliever of ER stress-induced apoptosis.

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SUCCESSFUL ANTIBODY HUMANIZATION REQUIRES GRAFTING OF KEY STRUCTURAL RESIDUES

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Abstract

Antibodies are multimeric, multi-domain proteins able to recognize a theoretically infinite number of antigens and protect the human body against them[1]. Because of their unique specificity and affinity towards the antigen, antibodies are invaluable for both research and therapeutic applications.

Before administration to patients, antibodies of animal origin have to undergo a "humanization" procedure to reduce their immunogenic potential. This consists in the replacement of antibody regions not required for antigen binding with those of a human antibody. The resulting "humanized" antibody usually has no more than 5% of residues of animal origin.

Although antibody humanization has been extensively performed for nearly 30 years, humanized variants reported in the literature usually show absent or significantly reduced antigen binding. As a consequence, several rounds of mutagenesis have to be performed to regain suitable antigen binding properties. Our analysis of these variants revealed that one or more of the residues known to play an essential role for the maintenance of antigen binding site (ABS) structure were only present in the final variants, able to bind the antigen, but not in the previously produced, inactive sequences.

This observation prompted us to introduce a structure-based ABS grafting procedure, which takes advantage of the extensively described sequence-structure relationships in antibody molecules. We have applied this procedure to the design of two previously published antibody variants[2-3] and, more recently, of a humanized variant of an anti-cancer murine antibody, aimed at therapeutic applications. All the designed variants were able to recognize the target antigens with the same affinity and specificity as the ABS donor antibodies, without requiring additional mutagenesis rounds.

Based on these results, we propose structure-based ABS grafting to be a more efficient humanization procedure than classic complementarity determining regions (CDRs)-grafting.

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Characterization of novel inhibitors of CDC25 phosphatases: biochemical and biological analysis

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Abstract

The dual phosphatases cell division cycle 25 (CDC25) are crucial regulators of cell cycle progression, also involved in cell response to DNA damage. Their overexpression was detected in many tumors, including melanoma [1].

The characterization of molecules with an inhibitory potency on CDC25 activity, such as compound **19** (NSC 28620) [2], contributes to the research of novel anti-cancer drugs. The structure of this lead compound was engineered to produce more potent inhibitors. The most active compounds synthesized had *K*i values in the 0.79 – 10.0 µM interval. Kinetic studies identified two different mechanisms of inhibition, related to specific functional groups possessed by the inhibitors. Information on positioning/interaction of the inhibitors was also obtained by intrinsic fluorescence measurements of CDC25B.

The possible cytotoxicity of the most active compounds on melanoma cell lines was analysed. Data from MTT assay indicated that at a low concentration, i.e. 10 µM, essentially one compound (cpd **21**) significantly reduced the cell growth of melanoma cells in a time dependent manner. The effect of cpd **21** on cell viability was further confirmed by the colony-forming assay, that showed a similar trend in the ability to inhibit the clonogenicity of melanoma cells. Moreover, cpd **21** provoked an early increase of pCdk1 protein level and an arrest of melanoma cells in G2/M of cell cycle, followed by the activation of a caspase-mediated apoptotic program. Conversely, cpd **21** didn't show cytotoxic effect on BJ-5ta, a nonmalignant fibroblast cell line.

Finally, a reduction of pAkt and an increase of p53 protein level could mediate the cytotoxic effect of cpd **21** on melanoma cells.

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STRUCTURAL INSIGHTS INTO THE MOLECULAR FUNCTION OF HUMAN [2FE-2S] BOLA3-GRX5 AND [2FE-2S] BOLA1-GRX5 COMPLEXES

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Abstract

Assembly of human mitochondrial iron-sulfur (Fe/S) proteins is a key process of cells and it requires a dynamic interplay of protein:protein interactions. Defects in this process are associated with severe diseases, as the multiple mitochondrial dysfunction syndrome 2 (MMDS2)1. It results in decreased functions of specific Fe/S enzymes, respiratory complexes I and II as well as lipoic acid-dependent enzymes and it is associated with mutation in the human BOLA3 protein. The members of the BolA-like protein families and the monothiol glutaredoxin (Grxs) have recently emerged as specific interacting partners in the Fe/S cluster assembly machinery2, 3.

We solved the 3D structures of human BOLA3 (hBOLA3) and human BOLA1 (hBOLA1) proteins using triple resonance experiments. This structural knowledge has been the starting point to investigate by NMR the interaction between apo hBOLA3 (or apo hBOLA1) and both apo and [2Fe-2S] GRX5 form, performing 1H-15N HSQC experiments at different protein-protein ratios4.

Exploiting site directed mutagenesis experiments, we characterized the [2Fe-2S] cluster coordination motif in the human BOLA3-GRX5 and BOLA1-GRX5 complexes and we investigated redox properties of the [2Fe-2S] bound cluster by NMR, EPR, UV/vis and CD spectroscopies. Collectively, these data allow us to obtain experimentally driven docking models of the two heterocomplexes. The BOLA1-GRX5 complex coordinates a reduced, Rieske-type [2Fe-2S] cluster, while an oxidized, ferredoxin-like [2Fe-2S] cluster is present in the BOLA3-GRX5 complex5. The differences in the structural rearrangement and in the cluster redox properties provide the first indications for discriminating the functional roles of the two heterocomplexes.

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Insight into the mechanism of thrombin inhibition by the antihemostatic salivary protein cE5 from *Anopheles gambiae*

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Abstract

Saliva of blood-feeding arthropods carries several antihemostatic compounds whose physiological role is to facilitate successful acquisition of blood. The identification of novel natural anticoagulants and the understanding of their mechanism of action may offer opportunities for designing new antithrombotics disrupting blood clotting[1-2].

We here report an in depth structural and functional analysis of the anophelin family member cE5, a salivary protein from the major African malaria vector *Anopheles gambiae* that specifically tightly and quickly binds and inhibits thrombin[3-4]. Using calorimetry, functional assays and complementary structural techniques we show that the central region of the protein, encompassing amino acids Asp31-Arg62, is the region mainly responsible for α-thrombin binding and inhibition. As previously reported for the *Anopheles albimanus* orthologue anophelin, cE5 binds both thrombin exosite I with segment Glu35-Asp47 and the catalytic site with the region Pro49-Arg56, which includes the highly conserved DPGR tetrapeptide. Moreover, the N-terminal region of cE5 (which includes an RGD tripeptide) and the additional C-terminal serine-rich region (absent in orthologues from anophelines of the New World species *A. albimanus* and *A. darlingi*) also played some functionally relevant role. Indeed, we observed decreased thrombin binding and inhibitory properties even when using the central cE5 fragment alone. In summary, these results shed additional light on the mechanism of thrombin binding and inhibition by this family of salivary anticoagulants from anopheline mosquitoes.

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New clues into self-assembly of the fungal hydrophobin Vmh2

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Abstract

Protein self-assembly is a complex phenomenon with strong implications in the living systems [1]. This spontaneous process is gaining the interest of researchers, since understanding the mechanism of self-assembly is crucial both to shed light on amyloid diseases and their treatments, and to exploit protein layers in the design and production of novel advanced materials [2].

Hydrophobins (HFB) are fungal proteins able to self-assemble into amphiphilic films at a hydrophobic−hydrophilic interface playing a key role in the growth and morphogenesis of the majority of filamentous fungi [1]. HFBs share eight conserved cysteine residues that form four disulphide bridges. Based on the spacing of the cysteine residues and their biophysical properties, HFBs can be divided in two classes. Class I HFBs assembles are extremely stable polymeric layers composed of fibrillar structures resembling the amyloid fibrils associated with diseases states [1]. While it is widely accepted that the formation of class I HFB fibrils is accompanied by conformational change, and, in particular, the formation of β-sheet structure, speculation continues about the triggers for the conformational change and the sequence segments critical for it [2].

Vmh2 from *Pleurotus ostreatus* is one of the most hydrophobic class I HFB, which conversion into the β-sheet rich forms occurs in conditions different from those of other class I HFBs. A possible mechanism of formation of Vmh2 amyloids was proposed on the basis of a built homology model [2], and five site directed mutants were designed on the basis of the above described model to validate the proposed mechanism. Not all the obtained results were justifiable; however the behavior of all the analyzed Vmh2 mutants was clarified when a new and very reliable model was built. This model was based on a newly determined structure [3] of an HFB sharing more than 50% identity sequence with Vmh2.

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Methionine γ-lyase as Anti-Cancer Biologics

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Abstract

Methionine is an amino acid strongly required in fast replicating cells, such as cancer cells. Methionine deprivation in the cell environment by the catalytic activity of methionine γ-lyase (MGL) has been envisioned as a therapeutic strategy to reduce the viability of cancer cells that are specifically deficient in methionine synthesis pathway [1,2]. MGL is a pyridoxal 5'-phosphate dependent enzyme that catalyzes the α,γ-elimination of methionine in ammonia, methanethiol and α-ketobutyrate [3,4]. In order to increase MGL catalytic efficiency, we compared the crystal structures of MGLs from various microorganisms endowed with different efficiency, identifying five residues predicted to regulate active site accessibility. Site-saturation mutagenesis was independently performed at these five positions to generate libraries screened for variants with improved activity. We identified one mutant with a 100% increase in catalytic efficiency. In parallel, MGL was encapsulated in different matrices, such as PLGA, silica gels, alginate and chitosan [5-7]. The immobilized MGLs exhibited catalytic activities ranging from 100 to 15% with respect to the soluble enzyme [8]. Soluble and encapsulated MGLs assessed for cytotoxic activity towards a panel of cancer and nonmalignant cell lines were found to be similarly effective.

Overall, these findings indicate that encapsulated MGL is a potentially powerful strategy for cancer cell therapy. Activities are ongoing towards the selective delivery to target cells.

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Biological activities of the ribotoxin Ageritin from *Agrocybe aegerita* (V. Brig.) Singer

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Abstract

Ribotoxins are a group of extracellular rRNA ribonucleases produced by ascomycetes that display cytotoxic activity towards animal cells. They are rRNA endonucleases (EC 3.1.27.10) that catalyse the hydrolysis of the phosphodiester bond between G4325 and A4326 from the rat 28S rRNA. These nucleotides are located in the Sarcin Ricin Loop (SRL) that is crucial for anchoring the EF-G or EF-2 elongation factors on the ribosome in prokaryotes and eukaryotes, respectively. No precise biological role has yet been assigned to ribotoxins, although several studies have shown the insecticidal properties of some of them, supporting their involvement in defence and parasitism. Extensive research has been conducted to investigate their use as antiviral and antitumor agents. Moreover, they could also be used as specific tools for the study of human ribosomopathies, and they have been used in agriculture to increase resistance against virus and as bioinsecticides to control insect pests.

Recently, a ribotoxin (Ageritin) has been isolated from the basidiomycetes *Agrocybe aegerita* (Pioppino in Italy) suggesting that ribotoxins are more widely distributed among fungi than previously believed. In order to gain insights into the protective properties of Ageritin against pathogens and its putative biotechnological applications, we have tested different activities of this ribotoxin and found that Ageritin (i) displayed rRNA endonuclease activity against microbial ribosomes; (ii) was active against the Tobacco mosaic virus RNA; (iii) displayed endonuclease activity against a supercoiled plasmid; (iv) was cytotoxic to COLO 320, HeLa and Raji cells by promoting apoptosis; and (v) displayed antifungal activity against the green mold *Penicilium digitatum*, being able to enter into the cytosol inactivating the fungal ribosomes. The combined effect of these biological activities could result in a broad action against several types of pathogens.

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HUMAN HISTIDINE DECARBOXYLASE: A POTENTIAL REDOX-MODULATED PROTEIN?

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Abstract

Histidine decarboxylase (HDC) is the enzyme responsible for histamine biosynthesis from L-histidine through decarboxylation. Impairment of histamine metabolism is related to immunological and neurological pathologies, and more recently to the growth of different carcinoma types and neuroendocrine tumours (1-3). However, HDC still remains to be fully characterized, mainly due to its instability and aggregation tendency. Thus, the goal of this work is to characterize human HDC to drive the rational discovery of some promising HDC inhibitors. We produced the recombinant human active wildtype HDC and identified a reversible inter-subunit disulfide bridge, absent in the HDC structure solved in 2010 that was in the C180S and C418S double mutant form for stability reasons (4). This disulfide linkage could be reduced by increasing concentrations of different reductants, thus HDC exists both in a reduced and oxidized form whose equilibrium depends on the reducing conditions, arguing for a possible implication of HDC in redox control regulation mechanisms. Here, we demonstrate through a combination of biochemical techniques (site-directed mutagenesis, kinetic assays, chromatography, electrophoresis, absorbance, fluorescence and dichroic measurements, dynamic light scattering) together with bioinformatics analyses that Cys180 is responsible for the disulfide intermolecular bridge. Interestingly, this residue is typical of the human isoform and is not conserved in the homologues decarboxylases and even in the HDCs from different origin. Results show that the presence of the disulfide bridge causes slight structural rearrangements and increased thermal stability and hydrodynamic radius of the protein, suggesting a structure-related regulatory role for this residue, whose role is presently under investigation.

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Protein Production Platform @ Elettra: *Overcoming the bottleneck*

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Abstract

A significant majority of biomedical and basic life science research is wholly reliant on the availability of good quality biological reagents such as proteins, peptides and antibodies. The requirement of high quality proteins is even more evident in structural biology research where the difficulty in obtaining a good protein sample is often the main bottleneck for the achievement of successful results.

At the Elettra synchrotron in Trieste, taking advantage of the existing expertise of the in-house structural biology group we have implemented a protein production platform that works as a facility to support the internal and external research of academic and industrial users to express and purify recombinant proteins for functional and structural studies. In particular the lab intent is to facilitate the process to obtain recombinant proteins suitable for analysis with synchrotron radiation techniques (x-ray diffraction, SAXS, FTIR). The laboratory is also opened to less experienced researchers such as young postdocs and PhD students offering tutoring and knowledge transfer. The platform is optimized for high-throughput small scale and scale-up protein expression using prokaryotic and eukaryotic systems, with a strong expertise in insect cell, baculovirus-mediated, systems. Protein purification and characterizations are also performed in the lab to offer a tailor-sized service. A number of different scientific projects are running in parallel and the principal interest is on protein targets for cancer therapy, with the major focus on the human kinases and deubiquitinases families.

The present poster is intended to introduce the protein production platform of Elettra Sincrotrone Trieste to the SIB community, showing the major achievements and future developments of the facility in the field drawing from a panel of the most relevant results.

Session 2: Proteins session 2 and Computational and Systems Biology (P2, CSB)

Ml proteins from *Mesorhizobium loti* and MucR from *Brucella abortus*: an AT rich DNA core target site and oligomerization ability

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Abstract

The Ros/MucR transcription factor family is composed of several members that share high amino acid sequence identity. Five of the ten Ros/MucR family members from *Mesorhizobium loti*, Ml1, Ml2, Ml3, Ml4, Ml5, are well characterized structurally and functionally defined as DNA binding proteins. The Ml proteins-DNA interaction was studied using the Ros Box as DNA target site. Studies of the DNA target site naturally recognized by Ml proteins are not available to date and no evidence of the Ml proteins expression in *Mesorhizobium loti* has ever been reported. In this study, we report for the first time an expression profile of the *ml genes* in *Mesorhizobium loti* and we show the Ml proteins capability to bind a region in the promoter of the *exoY* gene from *Mesorhizobium loti* containing an AT rich DNA core target site. Moreover, we demonstrate that the Ml proteins are able to form high order oligomers through their N-terminal region. Finally, we demonstrate that the same DNA core site is specifically recognized by MucR from *Brucella abortus* and that MucR from *Brucella abortus* shares with the Ml proteins the same oligomerization capability.

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Pyrene-labelled ferritin as an excimer fluorescence based probe to study protein oligomerization

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Abstract

Pyrene is a fluorescent spatially sensitive probe, with the unique ability to form excited-state dimers called excimers by stacking interaction of two proximal molecules. Excimers manifest a characteristic emission spectrum, clearly distinct from the pyrene-monomer spectrum, and ideal for protein oligomerization studies. A new engineered ferritin from archaea bacteria (HumAfFt) has been recently demonstrated to maintain the unique divalent-cation-triggered oligomerization properties of *Archaeoglobus fulgidus* ferritin (AfFt) at neutral pH, while exhibiting the typical human H-homopolymer recognition by the transferrin receptor TfR1.[1] The HumAfFt transition from dimeric to a cage-like 24-meric state has been investigated by exploiting pyrene fluorescence properties. A single cysteine residue per subunit has been specifically introduced by mutagenesis and has been selectively labelled with N-(1-Pyrenyl)maleimide in high yield. Dramatic changes in the pyrene fluorescence emission occur as ferritin assembles into its 24-meric state, allowing for thermodynamic and kinetic investigations to be carried out. The magnesium-triggered association mechanism turned out to be highly cooperative, incredibly fast (μseconds time scale) and complete even at low $MgCl_2$ concentrations, between 1-2 mM. In addition, a chelating agent such as EDTA has been able to dissociate the ferritin into its dimeric state thus demonstrating the reversibility of the association/dissociation mechanism.

Finally, pyrene-labelled ferritin has also proved to be an ideal tool for *in vitro* two-photon fluorescence microscopy. HumAfFt internalization by HeLa cells has been followed, for the first time, by exciting the pyrene moiety in the near-IR region, with a low energy radiation thus reducing cells photodamage and photobleaching, and measuring the two-photon excimer emission florescence. Images confirmed the high extent of protein internalization and highlighted a cellular distribution in the cytoplasm and in the perinuclear space that suggest a typical clathrin-coated endocytosis pathway, mediated by TfR1.

In conclusion, the present data highlight the remarkable fluorescence properties of a pyrene-HumAfFt conjugate as a versatile probe suitable for both oligomerization and imaging studies.

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New insights into the polyubiquitin gene *UBC* transcriptional regulation under oxidative stress

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Abstract

UBC has been labelled as the most responsive gene among the Ubiquitin gene family, enabling a prompt increase in the extra-ubiquitin needed upon proteotoxic, genotoxic or oxidative stress [1]. We recently mapped and characterized the Heat Shock Elements (HSEs) responsible for the HSF1-driven induction of the polyubiquitin gene *UBC* upon treatment with the proteasome inhibitor MG132 [2]. Here we investigate the molecular players of the *UBC* transcriptional response to oxidative stress induced by Sodium Arsenite, in comparison with the proteasome inhibitor treatment, mainly focussing on the role of the Nrf2 antioxidant pathway. Incubation of HeLa cells with Arsenite up to 8 h resulted in a time-dependent increase of *UBC* mRNA, while cell viability, as well as proteasome activity were unchanged respect to control cells. Both Arsenite and MG132 treatment resulted in an increase of HSF1, HSF2 and Nrf2 protein levels in the nuclear compartment. Their contribution to the Arsenite triggered *UBC* induction was investigated by transient siRNA transfection. Depletion of HSF1, but not HSF2, significantly compromises the stress-induced *UBC* expression, as occurs for the well-stated HSP70 target gene.

Instead, Nrf2 silencing did not affect *UBC* transcription in both basal an stressful conditions, although its knockdown significantly dampens transcription of the well-known ARE genes *HO-1* and *GCLC*, at both baseline and after stress treatment. Using ChIP Real-time PCR, we verified Nrf2 binding at known Nrf2-target motifs in the promoter of *NQO1*, with a consistent signal enrichment after both stresses applied. No Nrf2 binding activity was detected in the ~1 Kb promoter sequence upstream the transcription start site of *UBC* gene. On the whole, these evidences seem to exclude the participation of Nrf2, pointing to a prominent role of HSF1 in driving the *UBC* upregulation under oxidative stress.

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Cancer-associated fibroblast metabolism may control epigenetic regulation via protein acetylation

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Abstract

The tumour microenvironment (TME) is defined by complex heterotypic interactions between cancer cells and host cells and it plays key roles in controlling tumour growth and invasion. Cancer-associated fibroblasts (CAFs), which are abundant in the stroma of carcinomas, can generate from resident normal fibroblasts which become activated and capable of remodelling the extracellular matrix to drive invasion, engaging tumour angiogenesis and affecting tumour immunity. Thus CAFs are becoming an appealing therapeutic target in cancer. Extensive comparative proteomic analysis of human mammary normal fibroblasts (NFs) and CAFs have revealed that the levels of some metabolic proteins and enzymes involved in the generation of acetyl-coA are altered in CAFs. Acetyl-CoA is the building block for protein acetylation reactions as well as for the endogenous synthesis of fatty acids. To investigate whether protein acetylation is altered in CAFs upon activation, we measured the acetylome of NFs and CAFs. We performed an antibody-based strategy to enrich for acetylated peptides from a total cell proteome digest and combined it with stable isotope labelling by amino acids in cell culture (SILAC) for accurate quantification. The acetylated peptides were fractionated using high pH fractionation and run on a Q-Exactive HF instrument. Preliminary category enrichment analysis of the regulated acetylation sites showed that proteins belonging to specific functional categories were regulated by acetylation in CAFs. In particular, histone proteins had higher acetylation levels in CAFs compared with NFs. These preliminary data indicate that CAFs may utilise acetyl-CoA to control histone acetylation and we are investigating this further. There is increasing evidence that epigenetic regulation, such as histone modifications, underpins the phenotypic alterations that occur in CAFs upon activation. Our study suggests that CAF metabolism can support such changes. Hence, targeting CAF metabolism should be explored as strategy to block tumour growth and invasion.

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PROTEOMIC SIGNATURE IN GENETIC DISORDERS OF PROPIONATE METABOLISM

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Abstract

Methylmalonic Acidemias (MMAs) are severe autosomal recessive inborn errors of metabolism caused by the deficienc activity of the enzyme methylmalonyl−CoA mutase (MUT). MUT converts L−methylmalonyl−CoA into succinyl−CoA, a Krebs cycle intermediate. A block at this enzymatic step results in elevated plasma levels of methylmalonic acid. The disorders are caused by: i) mutations in the MUT apoenzyme or ii)defective metabolism of the enzymatic cofactor, 5'-deoxyadenosylcobalamin (1). Proteomic analysis allowed us to define the altered pathways both in the pathological form due to the enzyme (MMA_MUT) and its cofactor (MMA_CHC)

In particular charactherization of in-vivo proteome of MMA_CHC lymphocytes reveals disturbance of glutathione metabolism, a deregulation in proteins involved in cellular detoxification, especially in glutathione metabolism. Moreover the cofactor defective metabolism affect also expression levels of proteins involved in intracellular trafficking, protein folding, and energy metabolism (2). Conversly proteomic and metabolomic investigation reveals changes in enzymes involved in energy metabolism, gluconeogenesis and Krebs cycle anaplerosis also on hepatic tissue from MMA_MUT patients (3).

To overcome the detection limits of previous investigations and to investigate the neuronal complication of MMA disorder a MMA in vitro model was create by using the RNA-interference strategy in neuronal SH-SY5Y cell line. The cellular model proteome was characterized by new the spectral counting proteomic approach. The new analytical approach allowed us to identify and quantify the less-expressed proteins of the analyzed proteome. Differentially expressed proteins were involved in metabolic processes, cell cycle, apoptosis processes and cellular response to stress. The proteomic dataset suggests that the high levels of

methylmalonic acid could determine cell death and, consequently, clinic manifestations affecting the nervous system in MMA patients. They represent targets to better understand its molecular basis and for the design of therapies to alleviate clinical manifestations of MMA.

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Provirus integration leads to CRISPR-mediated autoimmunity in *Sulfolobus solfataricus*

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Abstract

The CRISPR-Cas system is an adaptive resistance mechanism that several prokaryotes have evolved to specifically recognise and destroy foreign DNA elements (viruses and plasmids). It consists of a multistep mechanism by which small fragments of the invading DNA are recognized as being non-self and integrated into the host genome at CRIPSR arrays. Subsequently, these DNA sequences (referred as spacers) are transcribed, processed to small CRISPR RNAs (crRNAs) and assembled into surveillance immune machineries, together with Cas proteins. These ribonucleoprotein complexes are then able to recognized, by base pairing, the invading DNA molecules and trigger their degradation[1].

The *Sulfolobus* spindle-shaped virus 2 (SSV2) is a model system to investigate virus-host interactions in stably infected cells (lysogens), thanks to its temperate nature[2]. In this study, we have analysed the global gene expression of an SSV2-lysogenic *S. solfataricus* strain and found that the virus elicited a strong host defence response. This included the activation of CRISPR arrays as well as of several *cas* (CRISPR-associated) genes; therefore, leading to a significant decrease of the virus copy number[3].

However, given the presence of an integrated copy of SSV2 (provirus) in the *S. solfataricus* genome, this latter was forced to develop a strategy to circumvent self-attacking by the CRISPR-Cas system (autoimmunity). Indeed, since the provirus is identical in sequence to the episomal copies of SSV2, it can be targeted by the CRISPR-Cas system, thus casting a fitness cost on cells that are actively challenging the infection.

Our data show that *S. solfataricus* evolved a specific strategy to safeguard its genome integrity, i.e. throughout deletion of SSV2/self-targeting spacers, which are essential for viral DNA targeting and infection challenging[3]. The implications of such a discovery on the applicability of the CRISPR-Cas system for the production of virus-insensitive strains of *S. solfataricus* will be investigated.

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Proteotoxicity in cardiac amyloidosis through a proteome profiling of human heart cells exposed to amyloidogenic light chains

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Abstract

In light chain (AL) amyloidosis, deposition of amyloidogenic immunoglobulin light chains (LCs) as amyloid fibrils translates into life-threatening cardiomyopathy (1). In addition to organ dysfunction due to fibrils, clinical evidence indicates that proteotoxicity of soluble, pre-fibrillar amyloidogenic LCs also contributes to cardiac damage (2,3). In the experimental systems reproducing LC extrinsic cardiotoxicity, the exposure to exogenous cardiotropic LCs at concentrations commonly observed in patients' sera leads to functional and cellular dysfunction (4,5). Specifically, human cells, such as cardiac fibroblasts, internalize LCs, which can localize into mitochondria and interact with specific mitochondrial proteins (6).

In this study, using a differential proteomic approach, we identified the molecular changes that occur in primary human cardiac fibroblasts (hCF) exposed to soluble amyloidogenic and cardiotoxic LCs, purified from AL cardiomyopathy patients. To increase confidence of findings and extend the proteome coverage, we used a combination of gel-based (two-dimensional differential in-gel electrophoresis, 2D DIGE) and label-free

shotgun approaches, followed by bioinformatic analysis and an independent assessment of selected differentially expressed proteins.

Our data indicate that the proteome of hCF exposed to the cardiotoxic LCs undergoes a significant remodeling, with alterations in proteins involved in the cytoskeletal organization, mitochondrial function and protein quality control. These results demonstrate that the employed system is a powerful experimental tool to define the molecular bases of damage due to soluble, pre-fibrillar amyloidogenic LCs.

Acknowledgements. This work was supported by the Italian Ministry of Health (GR-2010- 2317596).

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Adiponectin expression is specifically related to Common variable immunodeficiency

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Abstract

Adiponectin is an adipokine widely studied for its beneficial metabolic properties. It circulates as low molecular weight (LMW), medium molecular weight (MMW) and high molecular weight (HMW) oligomers. The latter exert the most potent biological effects. Adiponectin attracted renewed interest with the finding that it was associated with the development and progression of immune disorders. The mechanisms underlying this association and the role of Adiponectin in the pathophysiology of immune-mediated conditions remain unknown. Common variable immunodeficiency (CVID) is a primary immunodeficiency characterized by chronic activation of the immune system, impaired antibody production and imbalanced cytokine production. Recently, we demonstrated that serum total levels as well as HMW Adiponectin oligomers are strongly decreased in maintenance Ig replacement therapy CVID patients compared to healthy controls. The aim of this study was to verify whether the expression of total Adiponectin and HMW oligomers is altered in treatment-naïve CVID patients. To characterized total Adiponectin and its oligomerization state, we performed ELISA test, FPLC and Western blotting analysis on serum samples from 8 CVID patients before and after the first Ig replacement therapy. As controls, we analyzed age- and sex-matched healthy subjects and age- and sex-matched non immune-deficiency Chronic Inflammatory Demyelinating Polyneuropathy (CIDP) patients before and after Ig infusion. We found that total Adiponectin and HMW oligomers were decreased in CVID but not in CIDP patients versus controls. Adiponectin and HMW levels quickly and dramatically increased after Ig infusion only in CVID patients. This finding indicates that Ig administration *per*

se is not able to induce an increase of Adiponectin , but the specific cellular and/or molecular background proper of CVID seems to be essential. In conclusion, our data indicate that Adiponectin is specifically related to CVID activity. Further studies are required to understand the biological role of Adiponectin and its possible use as disease biomarker in CVID.

DECIPHERING MOLECULAR ASPECTS OF *MYCOBACTERIUM TUBERCULOSIS* DNA REPAIR BY BIOCHEMICAL AND STRUCTURAL STUDIES

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Abstract

Mycobacterium tuberculosis (MTB) is a human pathogen that during its complex life cycle is continuously exposed to a variety of DNA-damaging stresses. The mutagenic effects of O6-alkylated guanine in DNA is counteracted by the action of the suicidal O6-methylguanine methyltransferase protein (OGT). We present here the crystal structure of wild-type MtOGT in complex with a modified dsDNA, gaining structural insights into the MtOGT cooperative DNA-binding mechanism. The MTB DNA repair toolbox also includes multi-enzymatic systems such as the Nucleotide Excision Repair (NER). The first steps in NER are carried out by the coordinated action of the UvrA, UvrB and UvrC proteins. We analysed the hydrodynamic properties and the oligomeric state of the MTB UvrB (MtUvrB) showing that the protein forms dimers in solution, which are characterized by an elongated shape, as determined by SAXS analysis. Moreover, we analysed UvrA/UvrB lesion sensing/tracking complex by adopting a SEC-based approach, revealing that the two proteins interact in solution, in the absence of ligands, with an A2B2 stoichiometry. Surface plasmon resonance analysis showed that the dissociation constant of the complex falls in the low micromolar range that could represent the basis for a fine modulation of the complex architecture.

UPLC/MRM MS Quantification of Trastuzumab in Human Serum by Selective Monitoring of a Specific Peptide Marker from the Antibody Complementarity-Determining Regions

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Abstract

Because of the large molecular weight, of the structural complexity and the similarity with endogenous immunoglobulins present in high concentrations, *in vivo* quantitative studies with therapeutic monoclonal antibodies are particularly challenging. In this work, an UPLC/MRM MS-based methodology is described for the quantification of Trastuzumab in human serum by monitoring a novel specific peptide marker located within its heavy chain Complementarity-Determining Region (CDR).

For maximum sensitivity and selectivity, specific transitions of this diagnostic proteotypic peptide were optimized and monitored at *m/z* 364.1>437.3 (quantitation ion) and *m/z* 364.1>358.0 (confirmation ion). As a proof-of-concept, the methodology was applied to the determination of Trastuzumab in human serum over a clinically relevant range from 0.02 to 200 µg/mL. According to international bioanalytical guidelines, the methodology has been validated in terms of specificity, linearity, accuracy, precision, detection and quantitation limits.

An excellent linear response has been obtained in the range from 0.005 to 0.5 µg/mL with a typical R^2 of 0.99. The limit of detection (LOD) and limit of quantification (LOQ) are 0.75 ng/mL and 7.5 ng/mL, respectively. The validation results meet the recommended acceptance criteria of ±20% accuracy and precision for quality control samples with mean bias and RSD values of 18% and 1%, respectively.

Conclusions: The strategy used for setting up the UPLC/MRM MS methodology based on monitoring specific peptide markers within CDRs can be potentially applied to the detection and quantification of other humanized or human mAbs in biological fluids.

Discovery and validation of pharmacological chaperones

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Abstract

The discovery of small molecules that act as pharmacological chaperones is a new challenge for drug discovery. The majority of mutations causing diseases affects protein stability and not functional sites. The mutant protein can be rescued by pharmacological chaperones that bind the native conformation and push back the equilibrium thus reverting the pathological phenotype. This approach was successfully employed for Fabry disease.

Fabry disease is caused by more than 400 missense mutations, mostly private, of lysosomial alpha-galactosidase (AGAL) that catalyzes the removal of α-galactosyl residues from a glycosphingolipid, globotriaosylceramide. 1-deoxygalactoojirimycin (DGJ), an iminosugar which closely resembles the natural product of AGAL galactose, has been recommended by European Medicines Agency (EMA) for the treatment of Fabry disease. DGJ is not an ideal drug because it binds the active site and couple a stabilizing effect, which is required, to an inhibitory effect, which is deleterious. A more systematic search was conducted to characterize novel and more potent pharmacological chaperones that have a better ratio between the stabilizing and the inhibitory effect. An in-silico screening was carried out to identify novel AGAL-stabilizing ligands that dock into an allosteric hot-spot. Allosteric ligands do not bind the active site, but one of the many pockets occurring on the surface of a protein so they play their stabilising action without competing with the natural substrate. A drug-like compound, 2,6-dithiopurine (DTP), was able to interact successfully with the enzyme showing a stabilizing effect against temperature and urea induced denaturation. Furthermore, DTP stabilizes lysosomal alpha-galactosidase, rescues a mutant that is not responsive to a mono-therapy with DGJ in a cell based assay.

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Antiproliferative effects of novel synthetic nucleobase analogues on U937 Histiocytic lymphoma cell line

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Abstract

Background and aims: Nucleoside and nucleobase analogues have been used for decades as cornerstone therapies for tumors treatment. Although they can act as antimetabolites, they can also inhibit essential enzymes [1] as well as interact with receptors (P1 and P2 receptors). At this time, there is a compelling need of new nucleoside analogues due to the occurrence of drug resistance. Therefore, in this study we aimed to evaluate the antiproliferative effects of novel synthetic variously-substituted nitrogenous aromatic heterocycles on a cell line (U937) of histiocytic lymphoma.

Methods: U937 were cultured in RPMI1640 medium supplemented with 10% Fetal Bovine Serum and penicillin-streptomycin. The compounds (PP1, PP2, PP3, PP4, PP5 and PP6) were dissolved in acetone and tested within the range of 238-0.93 µM for the IC50 calculation. Cytotoxicity was evaluated by MTT assay and LDH release. Apoptosis and cell cycle analyses were carried out by cytofluorimetry through Annexin-V binding assay and propidium iodide staining. Variations in intracellular calcium concentration were evaluated by FURA-2.

Results: Of the tested compounds, only PP1, PP5 and PP6 showed a cytotoxic effect (IC50 of 8.9, 0.86 and 8.1 µM) not accompanied by a significant release of LDH, but triggered both early and late apoptosis ($p<0.01$ compared to vehicle) without affecting the cell cycle. Finally, the compounds did not increase the intracellular calcium, suggesting that they do not act through P2X receptors or Gq-coupled receptors.

Conclusion: Our data suggest that the selected compounds specifically trigger apoptosis without acting on P2X receptors. However, the exact mechanism of action is still unknown and further studies are needed to shed light on the possible use of these novel compounds as possible therapies.

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Session 3: Differentiation and Neoplastic Transformation (DNT)

NANDROLONE AFFECTS CELL GROWTH AND DIFFERENTIATION IN HEPATOMA CELLS

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Abstract

Background: Sexual hormones, estrogens and androgens, determine biological response in a tissue- and gender-specific manner and have a pivotal role in endocrine-mediated tumorigenesis. Androgen signalling, mediated by the androgen receptor (AR), is critical factor influencing growth of normal and malignant cancer cells. Hepatocellular carcinoma (HCC) may be modulated by both estrogens and androgens hormones during its initiation, progression and metastasis [1]. The purpose of this study was to investigate the role of Nandrolone, an androgenic anabolic steroid, in regulating proliferation and differentiation of HCC.

Material and Methods: Human HCC cell line HepG2 was treated with Nandrolone Vetranal, a synthetic androgen ligand, for 48 hs and its viability and proliferation was assessed by MTS and cell cycle analysis, respectively. The expression of protein involved in cell cycle regulation and differentiation markers were analysed by western blot and real time PCR. Measurement of oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) were performed using Seahorse XF96 extracellular flux analyser. Respiratory chain complex activities were assayed spectrophotometrically. Stemness surface markers expression was detected by flow cytometry.

Results: Nandrolone treatment caused cell growth inhibition associated to a downregulation of cyclin D1 and an upregulation of the cyclin-dependent kinase inhibitors p21Waf1/Cip1 leading to cell cycle arrest in the G2 phase. Moreover, the activation of AKT signalling with a consequent phosphorylation of GSK-3β and a significant overall impairment of mitochondrial functions were also observed, thus suggesting a role in the control of the metabolic reprogramming. Finally, a significant increase of the stemness markers like Nanog, Myc, Lin28, KLF4 and SOX2 was detected following Nandrolone treatment, also confirmed in a human pulp mesenchymal stem cells and in an in vivo mouse model.

Conclusions: Nandrolone shows a strong anti-proliferative effect in hepatocellular carcinoma cell line promoting cancer cells stemness through cellular metabolic reprogramming.

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Interleukin-6 induced astrocytic differentiation modulates Mannose Binding Lectin (MBL)-Associated Serine Protease (MASP)-1 and MASP-3 expression in C6 glioma cells

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Abstract

Among inflammatory cytokines, Interleukin-6 (IL-6) shows a pleiotropic nature acting as a mediator in differentiation, immune response and diseases within the Central Nervous System (CNS). Exposure to exogenous or autocrine IL-6, induced by cAMP, promotes astrocytic differentiation of glioma C6 cells which assume an astrocytic phenotype correlated to the expression of glial fibrillary acidic protein (GFAP) [1].

In addition, in several mouse models of brain injury, the up-regulation of IL-6 expression was correlated to the innate and acquired immunity [2]. Glioma cells also express Mannose-Binding Lectin (MBL)-Associated Serine Proteases (MASP)-1 and 3, involved in the activation of the lectin complement pathway (LCP) in innate immunity [3].

We investigated MASP-1 and MASP-3 expression during IL-6 and dibutyryl cAMP (dbcAMP) induced astrocytic differentiation in C6 cells. As previously reported, IL-6 or dbcAMP promoted a change in C6 cell morphology towards an astrocytic phenotype, as confirmed by the increase in GFAP expression levels. During this differentiation process, we observed a highly increase in MASP-1 and MASP-3 mRNA and protein expression levels compared to untreated cells. To investigate the involvement of protein kinase A (PKA) signalling pathway in IL-6 induced effects, C6 cells were pre-treated with the H89 (PKA) inhibitor. This exposure caused an inhibition in the astrocytic differentiation and a decrease both in STAT3 phosphorylation levels and MASP-1/MASP-3 expression levels.

Taken together, these results strongly suggest that IL-6 might act as a regulatory cytokine in innate immunity enhancing MASP-1 and MASP-3 expression level through PKA signalling in C6 cells.

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CDKN1B gene alterations and human cancers: mechanicistic investigations on G9R missense mutation

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Abstract

p27Kip1 (p27) was first discovered as a key regulator of cell proliferation, modulating Cyclin-dependent Kinases activity. Nearly two decades have elapsed since the discovery of p27 roles in the regulation of cytoskeletal dynamics and cellular plasticity, stem-cell proliferation and differentiation. This versatility has been attributed to its intrinsically unstructured nature that allows p27 to bind and modulate different proteins. Depending on the cell-specific context and its cellular compartimentalization, p27 acts both as a tumor-suppressor or tumor-promoter, and several post-synthetic modifications (mainly phosphorylations) control its commitment. Recently, mutations of CDKN1B (p27 encoding gene) have been found with a statistical significance in human cancers: along with frameshifts, some missense changes were associated to Multiple Endocrine Neoplasia, neuroendocrine tumours and other cancers (1).

This study focuses on a germline mutation detected in sporadic parathyroid adenoma, Glycine9→Arginine (2). Glycine in position 9 is highly conserved in p27 across species and its substitution has been proposed to negatively affect the phosphorylation of adjacent Serine10 residue, the most abundant phosphorylation site of the protein. Therefore, we examined the phosphorylation pattern of this mutant protein by 2D-immunoblotting. Unexpectedly, Serine10 is still highly phosphorylated, at least as in the wild-type p27. Furthermore, a new phosphorylation site is probably generated by the missense mutation. Studies are in progress to identify the novel phosphorylation residue as well the protein kinase responsible for its modification.

Functionally, despite its nuclear localization, G9Rp27 might contribute to enhance cell growth, motility and invasion; it also protects cells from apoptosis.

Our studies indicate that G9Rp27 oncogenic activities depend on its phosphorylation pattern. In conclusion, this investigation confirms the importance of post-translational modifications in addressing the function of intrinsically unstructured proteins.

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CLU expression negatively modulates prostate cancer progression by inhibiting NF-kB activity

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Abstract

CLU is down-regulated in prostate cancer (PCa) where it acts, as a negative modulator of carcinogenesis and tumor spreading (1). By using the GEO2R NCBI software tool, we analyzed microarrays data in Gene Expression Omnibus (GEO) database and we found that CLU expression is significantly down-regulated in PCa primary tumors and in PCa metastasis in comparison to normal prostate tissue. In the same dataset Nf-kB (p65 subunit) is significantly up-regulated in metastatic samples, in comparison to normal tissue or primary tumors. Therefore CLU and Nf-kB are significantly inversely regulated in PCa, especially in metastasis. Consistently, by meta-analysis of microarrays data, available at the Oncomine database http://www.oncomine.org/, we found that CLU expression in PCa, inversely correlate with the expression of Nf-kB target genes, some of which are involved in tumor progression and extracellular matrix (ECM) degradation. Transient and stable overexpression of CLU in PC3 cells caused reduction of cell proliferation and impairment of cell cycle progression in comparison to mock controls. In the same cells, Nf-kB expression, phosphorylation and nuclear translocation is reduced. By luciferase assay we demonstrated that CLU overexpression inhibited Nf-kB transcriptional activity and resulted in a significantly reduced expression of extracellular matrix degrading enzymes such as urokinase-type plasminogen activator and Cathepsin B. Conversely, CLU loss of function in PC3 cells, by siRNA-CLU transduction, promoted Nf-kB phosphorylation, activation and matrix metalloproteinase transcriptional up-regulation in comparison to controls. By protein co-immunoprecipitation we demonstrated that CLU do not inhibit Nf-kB by a direct protein-protein binding mechanism, suggesting a role of CLU in the complex upstream signaling pathway of Nf-kB activation. Taken together our data are consistent with the hypothesis that progressive down-regulation of CLU during PCa development yields the release of a physiological brake on Nf-kB activity, that in turn orchestrates the execution of a transcriptional program, which contributes to the acquisition of a more aggressive/invasive phenotype.

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FPR1 modulates endothelial cell functions by VEGFR2 transactivation

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Abstract

Receptor Tyrosine Kinases (RTKs) activation can be also triggered by G-protein coupled receptors (GPCRs), which represent the largest group of cell surface receptors exerting a variety of biological functions. Transactivation of RTKs by GPCRs serves as a paradigm for inter-receptor cross-talk, combining the broad diversity of GPCR with the potent signalling capacities of RTKs.

In the present study we investigate the ability of the Formyl Peptide Receptor 1 (FPR1) to transactivate the RTK VEGFR2 in immortalized endothelial cells (ECV). Formyl-peptide receptors 1, 2 and 3 (FPR1, FPR2, FPR3) belong to the GPCR super-family and are coupled to pertussis toxin (PTX)-sensitive Gi proteins and are widely expressed in several cell lines. The results show that ECV cells express FPR1 and that the stimulation with the FPR1 agonist NfMLP induces the phosphorylation of cytosolic tyrosines of VEGFR2. This is prevented by PTX, suggesting that FPR1 is crucially involved in VEGFR2 trans-phosphorylation. Moreover, FPR1-induced RTK tyrosine phosphorylation is prevented by selective inhibition of NADPH oxidase, and by silencing of NADPH oxidase subunits. These data strongly indicate a key role of Reactive Oxygen Species (ROS) in the transactivation process. NfMLP-induced phosphorylation of tyrosine residues of VEGFR2 provide docking sites for signaling molecules which, in turn, activate intracellular cascades such as PI3K/Akt and STAT3 pathways. Our results show that the preincubation with specific RTKs inhibitors, as well as PTX, prevents both PI3K and STAT3 activation.

These results suggest that stimulation of FPR1 induces NADPH oxidase activation and VEGFR2 transactivation. FPR1-dependent ROS generation inhibit cellular phosphatase activities and is crucially involved in the VEGFR2 phosphorylation. We also observed that ECV cells upon NfMLP stimulation ameliorates cellular migration and capillary network like formation.

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The anti-apoptotic BAG3 protein is involved in BRAF inhibitor resistance in melanoma cells

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Abstract

BAG3 protein, a member of BAG family of co-chaperones, has a pro-survival role in several tumour types. BAG3 anti-apoptotic properties rely on its characteristic to bind several intracellular partners, thereby modulating crucial events such as apoptosis, differentiation, cell motility, and autophagy. In human melanomas, BAG3 positivity is correlated with the aggressiveness of the tumour cells and can sustain IKK-γ levels, allowing a sustained activation of NF-κB. Furthermore, BAG3 is able to modulate BRAFV600E levels and activity in thyroid carcinomas. BRAFV600E is the most frequent mutation detected in malignant melanomas and is targeted by Vemurafenib, a specific inhibitor found to be effective in the treatment of advanced melanoma. However, patients with BRAF-mutated melanoma may result insensitive *ab initio* or, mostly, develop acquired resistance to the treatment with this molecule.

Here we show that BAG3 down-modulation interferes with BRAF levels in melanoma cells and sensitizes them to Vemurafenib treatment. Furthermore, the down-modulation of BAG3 protein in an *in vitro* model of acquired resistance to Vemurafenib can induce sensitization to the BRAFV600E specific inhibition by interfering with BRAF pathway through reduction of ERK phosphorylation. The molecular interactions between BAG3 and mutated BRAF may represent a target for novel multi-drugs treatment design.

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Heparan sulfate proteoglycans as novel target in cancer precise therapy

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Abstract

Heparan sulfate proteoglycans (HSPGs) have crucial regulatory roles in tumor onset and progression. HSPGs are composed of a core protein and glycan chains characterized by repeated disaccharide units which can be sulfated at different amount and position. HSPG have enormous structural diversity due to the different possible modifıcations of the single saccharide units within the polysaccharide, such as position, sulphation and acetylation. As a result HSPG can bind and modulate their binding to signaling molecules such as growth factors, morphogens and chemokines [1]. HSPG proved to be important in mediating cancer development and progression by enhancing the binding of growth factors, morphogens and cytokines to their cognate receptors, thus activating signaling pathways that give rise to angiogensis, cell growth and proliferation, together with invasion and metastasis [2-3]. NT4 is a branched peptides that targets HSPGs [4-5]. NT4 specifıcally binds to sulfated glycosaminoglycans on cancer cells and tissues. NT4 can be conjugated to many different cytotoxic units and tracers. NT4 conjugated to paclitaxel produced tumor regression in a breast cancer orthotopic mouse model [6]. NT4 conjugated to tracers can discriminate between tumor and healthy tissue in different human cancer specimen [7-8]. We will show the ability of NT4 to drive tracers onto tumor lesions by means of Qdots and in vivo imaging, proving their promising features as theranostics. We will also show NT4 ability to interfere with HSPG-modulated activities such as: tumor cell proliferation, migration and invasion of matrix; as well as endothelial cells proliferation, migration and tube formation. NT4-HSPG interactions and consequent modulation of signaling pathways will prove the importance of this versatile tool, NT4, in addressing tumor cells and interfering in their cell-cell and cell-matrix communications.

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MITOCHONDRIAL "POWER" DRIVES TAMOXIFEN RESISTANCE: NQO1 AND GCLC ARE NEW THERAPEUTIC TARGETS IN BREAST CANCER

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Abstract

Drug-resistance, and the resulting treatment failure, are still significant clinical barriers, preventing more effective cancer therapy and better clinical outcomes [1].

Here, we identified two new molecular targets, which are functionally sufficient to metabolically confer the tamoxifen-resistance phenotype in human breast cancer cells [2]. Briefly, ~20 proteins were first selected as potential candidates, based on unbiased proteomics analysis, using tamoxifen-resistant cell lines. Then, the cDNAs of the most promising candidates were systematically transduced into MCF-7 cells. Remarkably, NQO1 and GCLC were both functionally sufficient to autonomously confer a tamoxifen-resistant metabolic phenotype, characterized by i) increased mitochondrial biogenesis, ii) increased ATP production and iii) reduced glutathione levels [3]. Thus, we speculate that pharmacological inhibition of NQO1 and GCLC may be new therapeutic strategies for overcoming tamoxifen-resistance in breast cancer patients. In direct support of this notion, we demonstrate that treatment with a known NQO1 inhibitor (dicoumarol) is indeed sufficient to revert the tamoxifen-resistance phenotype. As such, these findings could have important translational significance for the prevention of tumor recurrence in ER(+) breast cancers, which is due to an endocrine resistance phenotype [4]. Importantly, we also show here that NQO1 has significant prognostic value as a biomarker for the prediction of tumor recurrence. More specifically, higher levels of NQO1 mRNA strongly predict patient relapse in high-risk ER(+) breast cancer patients receiving endocrine therapy (mostly tamoxifen; H.R. > 2.15; p = 0.007).

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Protein moonlighting in cancer cells: TRAP1 and Syndesmos regulate translation and mitochondrial function

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Abstract

TRAP1, the mitochondrial HSP90, is dysregulated in several human cancers and responsible for the sensitivity to apoptosis and metabolic remodelling of cancer cells[1]. However, TRAP1 also localizes on the endoplasmic reticulum, where it binds ribosomes and translation factors, controlling expression of mitochondria-destined proteins, through a coupled control of protein synthesis and degradation[2]. Presently, the mechanisms of substrate recognition are unknown. Several lines of evidence suggest that TRAP1 might recognize its substrates as mRNAs, being potentially involved in their transport to mitochondria and localized translation. Indeed, several TRAP1 partners are putative RNA-binding proteins and the TRAP1 protein bears a ribosomal protein S5 domain 2-like. In keeping with our hypothesis, we show by PNK and eGFP-based RNA-binding assays that TRAP1 and Syndesmos are two novel, non-canonical RNA-binding proteins and that they cooperate in the regulation of protein synthesis. Notably, proximity ligation assays between phospho-rpS6, a ribosomal protein that is phosphorylated during active protein synthesis, and TOM20, a component of the mitochondrial protein import machinery, show that TRAP1 overexpression increases the number of active ribosomes near mitochondria; moreover, TRAP1 modulates translation of UQCRC2, a nuclear-encoded component of the mitochondrial respiratory chain, as shown by puromycilation coupled to proximity ligation. TRAP1 also participates to mitochondrial translation, as suggested by FRET analysis with mtEF-Tu and by translation assays in *E.coli* extracts. Stopped-flow fluorescence shows that TRAP1 slows down prokaryotic EF-Tu release from the ribosome and this modulation of kinetic of elongation factors binding to ribosomes accounts for the decreased cotranslational degradation previously associated to TRAP1 expression. As a results, activity of respiratory complexes and mitochondrial calcium increased in cancer cells upon TRAP1 silencing. Altogether, these results suggest that TRAP1 exerts an indirect control on key mitochondrial

functions in cancer cells through direct and partner-mediated translational regulation of mitochondrial proteins at multiple levels.

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Epidermal Growth Factor Receptor Signaling in a Cellular Model of Mucopolysaccharidosis IIIB

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Abstract

Mucopolysaccharidoses (MPSs) are rare genetic metabolic diseases characterized by deficiencies of lysosomal enzymes leading to the accumulation of glycosaminoglycans (GAGs) in many tissues resulting in multiple organ dysfunctions (1). To date, there are no specific treatments for MPSs and the prognosis is poor with the death of the patient during the second decade of life. We recently demonstrated that the mouse model of the MPS IIIB develops cardiac disease, valvular abnormalities and heart failure over time (2). In MPS IIIB, the enzyme α-N-acetylglucosaminidase (NAGLU) required for heparan sulfate degradation is missing, and in order to explore the molecular mechanisms underlying cardiac disorders in MPS IIIB, we generated a cellular model of the disease by silencing NAGLU gene expression in H9C2 rat cardiomyoblasts.

The results obtained demonstrated that NAGLU depleted H9C2 reproduce the lysosomal abnormalities of MPS IIIB and recapitulate the MPS IIIB cardiac hypertrophic phenotype. We identified the specific epidermal growth factor receptor (EGFR) activation through a phospho-receptor tyrosyne kinase array, and we demonstrated that EGFR triggers the hypertrophic response through ERK phosphorylation. Furthermore, we identified two pathways through which NAGLU depletion causes EGFR activation and subsequent hypertrophy in NAGLU depleted cardiomyoblasts. Our findings provide new insights into the pathogenesis of the MPS IIIB and open the way to novel therapeutic approaches for the treatment of the MPS IIIB disease by targeting components of the EGFR pathway.

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$p57^{Kip2}$ in cell biology: protein kinase network in the modulation of its phosphorylation pattern

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Abstract

$p57^{Kip2}$ is the least studied cyclin-dependent kinase inhibitor (CKI) belonging to the CIP/Kip family, also including $p21^{CIP1/Waf1}$ and $p27^{Kip1}$. Besides the canonical function of negative regulator of the cell cycle, $p57^{Kip2}$ is the only CIP/Kip protein that plays essential roles during embryogenesis and morphogenesis. Structurally, like its siblings, $p57^{Kip2}$ presents a high disorder degree, enough to be considered an intrinsically unstructured protein (IUP) [1]. Thus, it is crucial to identify and characterize its post-translational modifications (PTMs), since they, by influencing $p57^{Kip2}$ folding, might modulate its cellular localization, interactors and functions. However, few data are available, so far, on these key features of the protein. In a previous study, Borriello *et al.* [2], have underlined that $p57^{Kip2}$ might present PTMs, mainly phosphorylations. Accordingly, after a preliminary characterization of the $p57^{Kip2}$ isoforms occurring in different cell models, we focused our attention on the identification of $p57^{Kip2}$ phosphorylatable residues, and of the kinases responsible for these PTMs. By means of a bioinformatic analysis, the most probable modified residues and putative kinases have been identified. Subsequently, in order to confirm the *in silico* data, an extensive series of *site-directed mutagenesis* experiments has been performed to substitute specific serine/threonine with non-phosphorylatable residues. Then, the effect of single amino acid substitution on the $p57^{Kip2}$ bidimensional pattern of the transfected cells have been evaluated. To pinpoint the kinases that might phosphorylate $p57^{Kip2}$, we treated several cell lines with a library of selective kinase inhibitors and we evaluated their effects on $p57^{Kip2}$ phosphorylation pattern. So far, our experimental approaches allowed us to identify four novel phosphorylation sites and to recognize and select for further investigations at least ten kinases and kinases' families able to affect $p57^{Kip2}$ phosphorylation.

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Heme Oxygenase 1 Protects Myeloma Cells Against Bortezomib Through Nuclear Translocation And Regulation of Er Stress and Autophagy Proteins

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Abstract

Despite recent advances in proteasome inhibitor and immunomodulatory drug-based therapies, multiple myeloma (MM) remains largely incurable, primarily owing to acquired resistance. Heme Oxygenase-1(HO-1) is a cytoprotective microsomal enzyme that catalyzes the degradation of heme. We investigated the significance of Bortezomib (BTZ)-induced HO-1 in MM cell lines. We observed that BTZ induced apoptosis after 24h and ROS raised after 1h of treatment. BTZ was able to induce HO-1 mRNA levels after 3h of treatment. Furthermore, BTZ was able to induce the expression of ER stress proteins (Bip, IRE1α, Ero1, PERK and CHOP) in MM cells after 6h. Finally, we observed that BTZ was able to induce autophagy-related genes such as ATFG5 and BENC1 in U266 cell lines. Silencing HO-1 by shRNA, reversed the above-described effects of BTZ. Interestingly, HO-1 localized both in the cytoplasm and in the nucleus of MM cells following BTZ treatment. Blockage of HO-1 nuclear translocation by E64 sensitized MM cells to BTZ. Since nuclear HO-1 has been reported to be a regulator of DNA repair activities, we also explored its role in genomic instability of MM cells. Using the cytochinesis-block micronucleus (CBMN) assay, we observed that pre-treatment of U266 with E64 for 24h led to a significant reduction of the percentage of micronuclei and nucleolasmic bridges observed in binucleated cells. We also evaluated U266 ability to activate G2/M checkpoint after UV damage using CBPI (cytochinesis block proliferation index) assay. The percentage of monucleated cells (G2/M checkpoint activated) was higher in cells pre-treated with E64 compared to control. Our data suggest that BTZ-induction of HO-1 is linked to the activation of ER stress and autophagy pathway. Furthermore, HO-1 nuclear translocation may be involved in MM BTZ resistance and in genomic instability in MM.

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Session 4: Aging and Degenerative Diseases – Nutrition and Environment (ADD, NE)

Analysis of protease levels in brain samples from Alzheimer's patients

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Abstract

Proteolytic processing of membrane proteins is a tightly regulated activity needed for cell signaling and receptor recycling. At the same time it is a fragile process aberrantly deregulated in neurodegenerative diseases [1]. In Alzheimer's disease (AD) ADAMs metalloproteases, β- and γ-secretases, cathepsins and meprins are the main proteases involved in the complex processing of the amyloid β precursor protein (AβPP), which is strictly linked to the genesis of the disease [2,3].

In this study we analyze mRNA and protein levels, using qPCR and western blotting (WB), of pivotal metalloproteases, in frontal cortices of AD patients in comparison to non-AD control subjects, to verify whether in pathological conditions there is a imbalance in the expression of *ADAM10* and *ADAM17, ADAMTS1, CTSL, MEP1A, MEP1B and MMP9*.

The analysis show that, between the two α-secretases, *ADAM10* mRNA expression is significantly higher ($p<0.05$) in AD samples in comparison to the control group; while ADAM17 mRNA levels do not significantly differ between AD and controls. The mRNAs expression analysis on other metalloproteases shows a statistically significant different expression ($p<0.05$) only for *ADAMTS1* and *MEP1B*: 2-fold and 1,62-fold higher in AD samples than in the control group. The mRNA expression of *CTSL*, *MEP1A* and *MMP9* is not different between the groups.

Looking at protein levels, WB experiments shows that ADAMTS1 and MEP1B protein expression is significantly higher in AD samples than in control subjects: 4-fold ($p<0,0001$) and 1.9-fold higher ($p<0,01$), respectively.

These data indicate that in AD there is a significant overexpression of metalloproteases involved in AβPP processing, and this increment is controlled at trascriptional level. Whether these variations are cause or consequence of the disease is still to be determined.

NLRP3 inflammasome activation in ALS mouse model

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Abstract

Amyotrophic lateral sclerosis (ALS) is a fast-progressing neurodegenerative disease characterised by motor neuron death. Most ALS cases are classified as sporadic; the 5–10% of cases are classified as familial and are typically inherited in an autosomal dominant fashion. Approximately 20% of familial cases are associated with mutations in superoxide dismutase 1 (SOD1) gene, such as the substitution of glycine to alanine in position 93 (G93A) [1]. hSOD1(G93A) transgenic mice, which develop a progressive disease, are used as the gold standard in ALS research.

Non-neuronal cells, in particular microglial cells, contribute to motor neuron death via non-cell autonomous mechanisms [2]. The capacity of microglial cells to initiate and perpetuate chronic inflammatory responses in the absence of infection has led to speculation that these cells may play a pivotal role in ALS pathogenesis [3]. Indeed, IL-1β levels are elevated in the central nervous system of both ALS patients and mutant *SOD1* transgenic mice [4], suggesting a potentially important role in disease pathogenesis. However, the cellular source of IL-1β or the initiating stimulus driving its expression remains unclear. IL-1β is expressed as an inactive precursor that must be activated by proteolytic processing by caspase 1 that, in turn, is engaged and activated by a multiprotein complex, to form the inflammasome. We demonstrarted that inflammasome is activated in the spinal cord of hSOD1(G93A) mice and in murine hSOD1(G93A) microglial cells and showed that, oxidative/nitrosative stress, by inducing peroxynitrite formation, is one of the crucial trigger of inflammasome activation.

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$A\beta_{42}$ oligomers interact with GM1 ganglioside inducing neuronal calcium overload

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Abstract

The ganglioside GM1 is a major component of lipid raft microdomains, particularly abundant in the central nervous system. An increase in GM1 content has recently been demonstrated in the frontal and temporal cerebral cortex of elderly people and Alzheimer's disease (AD) patients. Moreover, amyloid-positive synaptosomes obtained from AD brains were found to contain high-density GM1 clusters, suggesting a pathological significance of GM1 increase at presynaptic neuritic terminals in AD. Recently, we have demonstrated that membrane GM1 specifically recruits small soluble oligomers of amyloid-β peptide 42 ($A\beta_{42}$). Among the toxic effects of these oligomeric species, our study is focused on the abnormal intracellular flux of Ca^{2+} ions, observed in primary rat hippocampal neurons and in human neuroblastoma cells. Specific membrane proteins appear to be involved in the early and transient influx of Ca^{2+} ions induced by $A\beta_{42}$ oligomers with high solvent-exposed hydrophobicity (A+), but not in the sustained late influx caused by the same oligomers and in that induced by $A\beta_{42}$ oligomers with low solvent-exposed hydrophobicity (A-) in GM1-enriched cells. In addition, A+ oligomers bind in close proximity to membrane glutamate receptors (NMDA and AMPA), inducing their activation and so the early and transient Ca^{2+} influx. However, FRET data show that the interaction is not direct. In contrast, we found a low degree of co-localization between glutamate receptors and A- oligomers, suggesting that different $A\beta_{42}$ oligomers induce the same effect with distinct mechanisms, both implicating GM1.

Our results indicate that age-dependent clustering of GM1 at the neuronal membrane could induce neurodegeneration in elderly people as a consequence of an increased ability of the lipid bilayers to recruit membrane-permeabilizing oligomers. We also show that both lipid and protein components of the plasma membrane can contribute to neuronal dysfunction, thus expanding the molecular targets for therapeutic intervention in AD.

Role of peptide from Mozzarella di Bufala Campana DOP (MBCP) on intestinal barrier breakdown associated to the inflammation process

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Abstract

The small intestine represents a barrier to noxious antigens and pathogens and disruption of the intestinal barrier has been considered a major factor in several inflammatory intestinal diseases. Maintenance of the small intestine architecture and function requires close coordination between enterocyte proliferation, apoptosis, cell-cell (AJ) and cell-matrix interactions. The main molecular component of the AJ is E-cadherin, a transmembrane protein that exhibits functional adhesion activity, when is connected to the cytoskeleton actin by a cytoplasmic complex that includes α-, β- or γ-catenin. The extracellular signals such as epidermal growth factor, transforming growth factors, interferon (IFN)-γ, and tumor necrosis factor (TNF)-α are able to regulate intercellular permeability. In inflamed gut, these junctional complexes undergo a disruption leading to a breaking of the intestinal barrier. The inflammatory gut disease are treated with anti-inflammatory drugs such as 5-aminosalicylic acid or dexamethasone which target the cyclooxygenase enzymes. However, drug-induced severe side effects occur, and most of these treatments are inadequate. Milk is a source of many bioactive peptides, that play a relevant role in preventing various disorders such as cardiovascular diseases, metabolic disorders, intestinal health. A good source of bioactive peptides after gastrointestinal digestion is the "Mozzarella di Bufala Campana DOP" (MBC) and specifically, a novel peptide (MBCP) was detected in the intestinal digest with a good stability to brush border exopeptidases and an higher bioavailability. In this study, we have focused on the therapeutic potential of MBCP in preventing or limiting intestinal barrier breakdown associated to the inflammation process. We evaluated the MBCP effects on apoptosis, differentiation and maintenance of cell-cell contacts in inflamed polarised human intestinal epithelial Caco2 cells. The data obtained have important repercussions on the therapeutic potential of MBCP in helping to

restore the intestinal epithelium integrity damaged by chronic inflammation, thereby reducing the risk of colorectal cancer.

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Mitochondrial citrate carrier deficiency causes neuromuscular transmission impairment

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Abstract

The mitochondrial citrate carrier (CIC), encoded by the SLC25A1 gene, catalyzes the export of citrate from mitochondria to the cytosol where it is broken into into acetyl-CoA and oxaloacetate. We identified for the first time two pathogenic SLC25A1 variants in a patient that suffered from a severe neurodevelopmental syndrome [1]. Recessive mutations in SLC25A1 have been since identified in more than a dozen patients and CIC deficiency has been classified as an inborn disorder of metabolism (OMIM: 615182) whose biochemical hallmark is combined D-2- and L-2-hydroxyglutaric aciduria [2]. More recently we reported a novel homozygous mutation in the SLC25A1 gene in an affected sib pair presenting with myasthenia and impaired neuromuscular junction (NMJ) transmission [3]. Upon functional reconstitution of recombinant proteins into liposomes, we showed that the newly identified mutation caused a milder activity impairment than the previously reported mutations suggesting a fundamental role of CIC in neuromuscular transmission whose defect was previously masked by the harsher phenotypes. Using the CRISPR/CAS9 approach we obtained stable lines of *Caenorhabditis elegans* knocked-out in the SLC25A1 ortholog that showed resistance to levamisole, a nicotinic acetylcholine receptor agonist, that causes continued stimulation of the worm muscles, leading to paralysis. This phenotype was, at least in part, rescued by the expression of wild-type human SLC25A1 under the control of a neuron-specific promoter, pointing towards an underlying pre-synaptic defect. Altogether these data demonstrate a conserved role of CIC in neuromuscular transmission and validate the worm *C. elegans* as a suitable animal model for further investigation of the molecular and cellular underpinnings of the NMJ transmission defect associated to CIC deficiency.

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Hypoxia induces ROS decrease in primary human dermal fibroblasts

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Abstract

Hypoxia induces severe changes in cells bioenergetics, mainly impairing the oxidative phosphorylation process, but cells adapt to hypoxia also adopting different metabolic reprogramming mechanisms depending on available substrates [1]. When the oxygen tension decreases, the hypoxia-inducible transcription factor HIF-1α is stabilized, inducing the activation of hundreds of genes involved in the hypoxia adaptation process. Several paper showed that ROS contribute to the stabilization of HIF-1α [2], although it is still a matter of debate whether ROS level increases or decreases in hypoxia.

To address this issue we investigated how moderate hypoxia affects ROS content in primary human dermal fibroblasts. For the purpose, we first validated the fluorescent responsiveness of two different ROS probes (CM-DCFDA and CellROX) to either tert-butylhydroperoxide (Luperox), as a positive control, and to N-acetyl-L-cysteine, as a negative control. After validation, we used both probes to measure ROS level in fibroblasts grown in glucose or glucose-free medium under hypoxic conditions (1% O2). Notably, we showed that ROS level markedly decreased when fibroblast were exposed to short-term hypoxia, whatever substrate was available. Moreover, cellular ROS level showed a further progressive decline under prolonged hypoxic conditions and the phenomenon was supported by two different mechanism, depending on the substrate cells experienced. In particular, in glucose-enriched medium we observed a reduced mitochondrial mass and greater fragmentation, both specific indicators of mitophagy; on the contrary in glucose free-medium we measured minor mass reduction, but a significant enhancement of antioxidant enzymes expression. In conclusion, these findings revealed that in primary human dermal fibroblasts hypoxia induces a strong decline in ROS during the first phase of exposure, mainly due to the oxygen tension decrease, and the decrease is further sustained by different metabolism-dependent mechanisms, if hypoxic conditions are extended.

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ALTERATION OF ENDOTHELIAL EXTRACELLULAR MATRIX IN INFLAMMATION

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Abstract

Atherosclerosis is an inflammatory disease of the wall of large- and medium-sized arteries. In the onset of the pathology, the artery wall thickens as a result of invasion and accumulation of white blood cells and proliferation of intimal-smooth-muscle cells (which become foam cells) and the modification of the extracellular matrix architecture, e.g. hyaluronan [HA] accumulation, changes of proteoglycans [PGs] expression and collagen deposition around the lipid-rich necrotic core. The main causes of this modifications are high levels of LDL and/or the inflammatory condition due to endothelial dysfunction. The accumulation of oxidized-LDL (oxLDL) in the tunica intima and the endothelium damage contribute to the accumulation of HA [1-3]. The treatment of endothelial cells (ECs) with the cytokine TNFα leads to the upregulation of HEPG Synecan-4 followed in 48 hours by the increase of Syndecan-1. The expression of those PGs are accompanied by the upregulation of the GAG synthetic enzymes EXT1, EXT2, and NDST1. Accordingly, HS/HE disaccharide composition shows a higher amount of N-sulfated modification. As reported in several papers, Syndecan-4 expression is related to NO production and release from ECs. NO synthases (NOSs) have a central role in modulating vascular tone and can be altered by various injuries. In our model, we observed a decrease of endothelial NOS (eNOS) expression in TNFα stimulated ECs, in according with changes in permeability and adhesiveness in vivo due to endothelial lesion. The HA synthases HAS2, HAS3 and the regulatory short mRNA HAS2-AS1 increase their expressions, too.

ECs loaded with nLDL show an increased HAS2, while oxLDL decrease both HA synthases, suggesting for HA in endothelium a protective role lost in atherosclerotic conditions. Since the cholesterol levels are dependent upon the entrance of nLDL within the ECs, the use of such molecules in association with the cholesterol levels regulator PCSK9, we evaluated the expression of LDLR, that decreases, as well as the transcytosis receptor ALK1 [2,4], increased. Alone, the regulator PCSK9 does not modify the PGs Syndecan 1 and 4 expressions, but lower HAS3. Concluding, our data indicate Syndecan-4 as an early inflammatory marker, but also suggest that HAS3 have a protective role.

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EVOLUTION OF SULFHUR-CONTAINING COMPOUNDS: FROM LIFE IN THE OCEAN TO PHARMACOLOGICAL APPLICATIONS

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Abstract

The extraordinary beauty and the richness in biodiversity of marine landscapes reflect millions of years of evolution and adaptation of life to changing environments. Sulphur-containing compounds play a key role in maintaining cellular redox homeostasis and in the survival of organisms constantly exposed to environmental constraints. Among them, 5-thiohistidines (ovothiols), isolated in large amounts in sea urchin eggs[1], display unique antioxidant properties, thanks to the peculiar position of the thiol group in the imidazole ring of histidine. In this study we: 1. integrate comparative genomic and transcriptomic analysis to reconstruct the evolutionary history of ovothiol biosynthesis and shed light on its ecological role in the ocean; 2. undertake pioneering biological studies to identify potential pharmaceutical applications of the molecule.

By *in silico* analysis we have identified homologous of the gene involved in the first step of 5-thiohistidines biosynthesis. This gene is conserved in several marine proteobacteria, microalgae, and in most invertebrates, whereas it is lost in bony fish and other vertebrates. By biochemical and molecular approaches, we have demonstrated that in marine invertebrates the biosynthesis of such molecules increases before fertilization and larval settlement, and is regulated by environmental stressors, which eggs and larvae encounter in sea water[2]. On the other hand, by pharmacological approaches, we have found that in human cancer cells, ovothiol induces cell proliferation arrest through an autophagic mechanism[3]. We have discovered an early molecular target of the molecule, a cell-surface enzyme involved in metabolic and detoxification processes. The expression of this enzyme is significantly high in several tumors, and represents an attractive pharmaceutical target against cancer. Analogous of ovothiols and pharmacological inhibitors of such an enzyme induces autophagic mechanisms, as well.

Overall, our findings shed new light on the evolution of this molecule in specific environmental niches and on its key potentiality as a marine drug.

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Platform chemicals from *Basfia succiniciproducens* BPP7 growing on lignocellulosic biomasses up to the pre-pilot scale

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Abstract

Due to its wide range of applications in the food, pharmaceutical and chemical fields microbial synthesis of succinic acid is receiving growing attention, with few relevant industrial results and constant research for improvements. In order to develop a sustainable process, a special focus is now set on the exploitation and conversion of lignocellulosic biomasses into platform chemicals. In the present work we used the recently isolated *Basfia succiniciproducens* BPP7 strain in SHF experiments on *Arundo donax* hydrolysates, exploiting batch and FB strategies to improve the titer and productivity of succinic acid in the process. A maximal production of about 30 g/l of succinic acid was demonstrated after about 40h of growth and results were confirmed on the 150L pre-pilot scale indicating process robustness. Strain potential was also explored in small scale experiments performed in the presence of high concentrations of acetic acid and succinic acid and other known inhibitors released during the hydrolysis process, to further improve strain performance and investigate strain metabolism in view of an industrial application.

Effects of a fasting mimicking diet (FMD) on peripheral blood lipid profile in cancer patients

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Abstarct

Background. Metabolic reprogramming is considered a hallmark of cancer. Recent studies suggest that exacerbated lipid metabolism is essential to sustain unrestrained growth and proliferation of cancer cells. By reducing nutrient and growth factor availability to cancer cells, cyclic fasting or fasting-mimicking diets (FMDs) inhibit the *in vivo* growth of many tumor models, and synergize with cytotoxic agents to prolong animal survival [1]. While the effects of calorie restriction on blood glucose and growth factor levels are well established, the effects on lipid metabolism have been poorly investigated.

Methods. At Istituto Nazionale dei Tumori, a prospective study is ongoing to assess the tolerability of the FMD in patients with different cancer types. We assessed the peripheral blood lipid profile in 4 patients enrolled in this study. Venous blood was taken the morning of FMD initiation and at its completion, so to assess metabolic changes occurring in the short-term (5 days) period. We measured arachidonic acid (AA) and eicopentaenoic acid (EPA), which are precursors of eicosanoids involved in the cross-talk between cancer cells and immune cells, as well as on sphingosine 1-phosphate (S1P), which is overexpressed in many human cancers to regulate tumor angiogenesis and immune system functions.

Results. We found meaningful changes in peripheral blood lipid profiles. In particular, we observed an increase of the arachidonic acid (AA)/eicopentaenoic acid (EPA) ratio in whole blood and in plasma, as well as in plasma phospholipids. We also observed a significant reduction of plasmatic sphingosine and S1P levels. Similar changes occurred *in vitro*-grown triple negative breast cancer MDA-MB-231 cells, thus suggesting that starvation may affect the lipid composition of cancer cells.

Conclusion. Nutrient starvation affects systemic lipid metabolism with possible meaningful consequences on cancer cell proliferation and anticancer treatment activity.

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Effect of selected lactic acid bacteria on antioxidant and anti-inflammatory properties of sourdough

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Abtract

Sourdough is one of the oldest biotechnological processes in leavening of cereal food production. To obtain a sourdough bread a mixture of flour and water is fermented with lactic acid bacteria (LAB), mainly heterofermentative strains, elaborating lactic and acetic acid the use of sourdough in bread making influences many aspects of bread quality thanks to the metabolic potential of LAB of organisms that enable the endogenous enzymes to metabolize flour component at the dough stage. The metabolism of sourdough microbiota and the activity of cereal enzymes are interdependent thus leading to the final product characterized by the accumulation of low-molecular weight thiol compounds, by the degradation of anti-nutritive factors, by the production of various kind of peptides, to cite some. The interest for antioxidant peptides produced during fermentation of cereal flours has increased, and evidences in preventing oxidative stresses associated with numerous degenerative aging diseases are accumulating (1)

The aim of this study was to assess the antioxidant and anti-inflammatory potential of sourdough fermentations carried out by selected lactic acid bacteria possessing different proteolytic activity. A Low Molecular Fraction from 23 different sourdoughs was obtained by RP-HPLC. The fractionated peptides have been assayed on cultured cells either for antioxidant (by spectrofluorimetric ROS detection) and anti-inflammatory properties (by western blot analysis of NFkb and IkB transcription factors and by ELISA assay of Il-1β). Results clearly show a significant reduction of antioxidant and anti-inflammatory parameters. Moreover, some LAB strains are most effective than other, enabling us the identification of some strains potentially useful in obtaining healthy sourdoughs to use in bread-making process.

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Expression, chromatin accessibility and DNA methylation of sirtuin genes in nutrient-restricted murine hepatoma cell line

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Abstract

Calorie restriction (CR), a reduction of calorie intake without malnutrition, is the key factor for extending lifespan in animal models and nutrient availability affects both metabolism and epigenetics. During CR the increased NAD+/NADH ratio, modulates sirtuin (SIRT1-7) activity. These enzymes regulate nuclear, cytosolic and mitochondrial processes. To investigate if the expression of nuclear/cytosolic sirtuins (SIRT1, 2, 6) and mitochondrial sirtuins (SIRT 3, 4, 5) is epigenetically regulated during nutrient restriction, murine Hepa-1 cells were completely or partially deprived of glucose or aminoacids for 24, 48 and 72 h. Genomic DNA was either digested with an endonuclease (cyanase) or underwent methylcytosine affinity purification. Then, chromatin accessibility at sirtuin TSSs, DNA methylation at sirtuin promoter CpG islands, and sirtuin expression were analyzed by qPCR. Our results show that total deprivation of glucose and 24-48 h of treatment are the most influential factors and sirtuin expression is the mostly affected variable. The analysis of different sirtuins highlights that, in many cases, changes in chromatin accessibility are concordant with RNA levels. On the contrary, DNA methylation at the specific analyzed CpG islands, and RNA expression, in most cases, are not concordant. Nevertheless, DNA methylation at specific CpGs islands is only one of many different epigenetic components regulating chromatin structure and, consequently, gene expression. Although other studies are necessary to understand the intricate networks of epigenetic mechanisms regulating sirtuin genes, this is the first evidence that nutrient availability affects sirtuin expression by epigenetic mechanisms. In particular, chromatin accessibility at the TSS of sirtuin genes is very sensitive to changes in glucose and aminoacid supply and its variations are associated with changes of sirtuin gene expression.

Session 5: Membranes and Other Contributions (M, O)

NOVEL DIACYLGLYCEROL KINASE ALPHA INHIBITORS FOR X-LINKED LYMPHOPROLIFERATIVE DISEASE 1 THERAPY

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Abstract

X-linked lymphoproliferative disease 1 (XLP1) is a primary immunodeficiency due to mutations in the SH2D1a gene, encoding the SAP adaptor protein[1]. SAP deficiency perturbs TCR signalling and results in a constitutive diacylglycerol kinase alpha (DGKα) activity that impairs CD8+ T cell restimulation induced cell death [2]. Indeed, pharmacological inhibition of DGKα in XLP1 animal models limits CD8+ T cell expansion and interferon-γ production, suggesting the development of DGKα inhibitors for XLP1 therapy[2].

To find new inhibitors of DGKα suitable for clinical trials in XLP1, we selected a library of 139 compounds based on chemical homology with the two commercially available DGKα inhibitors. This library comprised uncharacterized molecules, several compounds already in clinical use or development and some specifically synthetized molecules. The library was screened for inhibitory activity at 100μM concentration on DGKα overexpressing homogenates, using the two commercial inhibitors as positive controls. Active compounds (≥50% inhibition at 100μM) were tested at concentration from 0.1 to 100 μM in order to estimate the IC50.

In the primary screen 20 compounds inhibit DGKα at least by 25%. Of those, Ritanserin and CP01 showed a potency equal or superior to the two commercial inhibitors. Based on the results of the first screen we created a pharmacological model by which we synthetized a second wave of 22 compounds, among which CP02 resulted active.

In order to verify the potential usefulness of those three compounds for XLP1, we used them in a restimulation induced cell death assay. Ritanserin and CP01 restored apoptosis in SAP deficient lymphocytes at concentrations lower than the two previously available inhibitors supporting its potential for XLP1 therapy.

Concluding our work allow us to propose a pharmacological model for the rational design or DGKα inhibitors and to select three active compounds. Of those, Ritanserin is highly attractive for drug repurposing as it was previously tested in clinical trials, as it is safe in humans at doses sufficient to inhibit DGKα[3].

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New Insight Into The A_{2B} Adenosine Receptor Effects On Epithelial-Mesenchymal Transition In Human Epithelial Lung Cells

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Abstract

The epithelial-mesenchymal transition (EMT) is a complex process occurring during tissue remodelling, in which the epithelial cells change to mesenchymal-appearing cells. The dysregulation of this process has been related with the occurrence and progression of several diseases, such as tumours and fibrosis (e.g. kidney or lung)[1,2]. In the last decade, great efforts have been devoted in understanding the molecular markers that trigger and sustain the EMT. Adenosine is a purinergic signalling molecule implicated in the pathogenesis and exacerbation of chronic diseases. Among its receptors, the A_{2B} adenosine receptor (A_{2B}AR) subtypes regulate many of the adenosine-driven remodelling responses in chronic lung disease, and an up-regulation of these receptors have been found in patients with lung fibrosis[3]. However, the relationship between A_{2B}ARs and the EMT has not been investigated, yet.

Herein, the effects of the inflammatory extracellular microenvironment on the A_{2B}AR expression and functionality were investigated in the human epithelial lung cells. Moreover, the link between the A_{2B}AR stimulation and the EMT progression was explored, too. Challenging human epithelial lung cells with the cytokine TGF-β, to induce the EMT process, caused an increase in A_{2B}AR expression and functionality. The activation of the A_{2B}AR, per se, was not enough to modulate the examined transition markers (E-cad, N-Cad, Vimentin); nevertheless, the receptor activation by selective agonists potentiated TGF-β-induced EMT. In parallel, the inhibition of A_{2B}AR signalling with the inverse agonist MRS1706 partially counteracted EMT induction. These results open the way to further investigations on the A_{2B}AR role in the EMT process, and on its relationship with the EMT-related pathologies, such as lung fibrosis.

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Involvement of sAC-dependent cyclic-AMP in mitochondrial dysfunction of nephropathic cystinosis

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Abstract

The autosomal recessive disorder cystinosis, is a disease caused by mutationsin the CTNS gene encoding for cystinosin, a lysosomal transmembrane cystine/H^+ symporter which promotes the efflux of cystine from lysosomes to cytosol. In the cystinosis, the absence of cystinosin transporter results in cystine accumulation in the lysosomes leading to multi-organ dysfunctions, in particular, cystinosis represents the most cause of Fanconi Syndrome (FS) characterized by early kidney failure. The molecular basis and mechanisms underlying FS are not yet completely understood but the occurrence of FS in patients with mitochondrial disorders rises up a possible mitochondrial involvement. Although, until now, data on mitochondrial activity in cystinosis has been conflicting, the involvement of mitochondrial dysfunctions (decreased ATP, increased reactive oxygen species, abnormal mitophagy) in the renal FS has been suggested. It has been shown that cAMP can exert a regulatory effect on mitochondrial function, in term of activity of OxPhos system, biogenesis, apoptosis and mitophagy. Here, analysis of mitochondrial and cytosolic cAMP level and their mitochondrial targets in $CTNS^{-/-}$and $CTNS^{-/+}$ cPTEC cell lines with respect to a $CTNS^{+/+}$cPTEC cell line is presented showing a decreased complex I and V activities of OxPhos system, decreased mitochondrial membrane potential and decreased Sirt3 protein level associated with mitochondrial fragmentation. Reduction of complex I and V activities is accompanied by lower levels of their subunits. Based on FRET probe features, a significant decrease of the mitochondrial and cytosolic cAMP has been found. Treatment with cysteamine, which reduces the intra-lysosomal cystine, recovered the mitochondrial parameters as well as mitochondrial cAMP level in $CTNS^{-/-}$ cells. The evaluation was extended in fibroblast cell cultures from a health subject and a cystinotic patient, and validate in CTNS-silenced HK2 cells. These results show a pivotal role of mitochondrial cAMP in nephropathic cystinosis.

Ablation of type VI collagen leads to megakaryopoiesis defects

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Abstarct

In the last decades the study of the role of Bone Marrow (BM) Extracellular Matrix (ECM) components in the regulation of megakaryopoiesis has gained relevant attention. Type VI collagen is an ECM protein broadly distributed in different tissues, where it tunes fundamental aspects of cell behavior. Previous works from our group and others have localized type VI collagen within the BM and demonstrated its adhesive properties for hematopoietic cells. Recently, we also demonstrated that human and mouse megakaryocytes possess the whole collagen synthesis machinery and that are able to produce and release various types of collagen. Here we extend the list of collagens produced by human and mouse megakaryocytes to type VI collagen and we demonstrate the role of this protein in regulating megakaryopoiesis *in vitro* and *in vivo* by using a mouse model null for type VI collagen (Col6a1-/-). We found a significant increase in megakaryocyte number within BM of Col6a1-/- mice with respect to wild type (WT) mice. However, despite a higher number of megakaryocytes, the peripheral blood platelet count was comparable between the two genotypes. Defects in platelet formation were excluded as demonstrated by performing platelet formation assays both *in vitro* and *in vivo*. While, platelets from Col6a1-/- mice displayed a reduced half-life and increased tendency to activate and aggregate, with respect to WT platelets, in response to principal agonists. Searching for the mechanism, we found that Col6a1-/- platelets presented an increased expression and function of Store Operated Calcium Entry channels, Stim1 and Orai1, which was derived from alterations in megakaryocytes mTOR-signaling pathway. Consistently, *in vitro* and *in vivo* treatment with the mTOR inhibitor rapamycin rescued Stim1 and Orai1 expression and function in megakaryocytes and platelets resulting in an increase of

peripheral platelet count only in Col6a1-/- mice. These findings demonstrate the important role of self-produced type VI collagen in the regulation of megakaryopoiesis.

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Purification and characterization of extracellular nanostructures from *N.sp*. PP1Y: a novel example of Outer Membrane Vesicles

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Abstract

Outer membrane vesicles (OMVs) are nanoscale proteoliposomes of 20-200 nm diameter, derived from the surface of some gram-negative and gram-positive bacteria as part of their natural growth cycle [1] and involved in cellular communication, biofilm formation and nutrient acquisition [2].

Despite their natural function, OMVs have attracted the attention of biotechnological industries for their potential use as immobilization tools and drug delivery systems. The biotechnological use of OMVs is currently limited, among others, by the presence in of the immunogenic LPS typically produced by Gram-negative bacteria. As a consequence, much interest is currently devoted to vesiculating non-pathogen strains that do not produce LPS. An appealing source of OMVs for biotechnological applications can be found among Sphingomonadales, an order of gram-negative bacteria lacking LPS. A marine microorganism belonging to this order, Novosphingobium sp. PP1Y, was isolated in the harbor of Pozzuoli and characterized [3-5]. This bacterium is endowed with the ability to grow in a polluted environment using polycyclic aromatic hydrocarbons (PAHs) as only source of carbon and energy. Moreover, N. sp. PP1Y genome has been completely sequenced and annotated [4].

We have successfully isolated OMVs from *N. sp*. PP1Y. Different growth conditions were tested in order to identify the best experimental setting for vesiculation. Atomic Force Microscopy (AFM) and Scanning Electron Microscopy (SEM) were used to confirm OMVs production, which resulted to occur only when PP1Y was grown in minimal medium supplemented with 0.4% glutamate. Vesicles were purified from exhausted growth media and AFM, DLS and Nanosize analysis showed that *N. sp.* PP1Y OMVs have a circular morphology with a diameter of ≈ 200 nm and are uniform in size and shape. Moreover, proteomic and fatty acid analysis

of these extracellular nanostructures were performed and all data obtained suggested a specific protein and fatty acid fingerprint of these structures.

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Advancements in Hyaluronan Chemical Modification: Relevance to Tissue Engineering and Other Biomedical Applications

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Abstract

Hyaluronan (HA) is widely exploited in aesthetic medicine, in the treatment of osteoarthritis, in cosmetics, ophthalmology, surgery, wound healing, topical drug delivery, tissue engineering (TE). The biopolymer may be chemically modified to improve performances in many of the aforementioned uses. Low degrees of modification are highly desirable to obtain products better resembling the natural macromolecule, thus eventually eliciting an appropriate biological response. Our research group has set up novel conditions for HA chemical modification aiming at slightly modifying the biopolymer in view of clinical applications.

HA was modified by reaction with 1,4-butandiol-diglycidyl-ether (BDDE) to produce gels intended for facial rejuvenation or with Lysine-methyl-ester (Lys) aiming at scaffolds for TE purposes[1,2]. Crosslinking conditions were varied in respect to conventional protocols to maximize reaction efficiency. The products (XHA) were evaluated for the HA modification extent, swelling, mechanical properties, morphology, stability in physiological conditions and to hydrolysis catalyzed by hyaluronidases. Hydrogels outperforming conventional similar products but with far less extensive HA modification were obtained, an attracting attainment in view of clinical uses. The biological response of the products was studied *in vitro*. The XHA-Lys materials were characterized in combination with primary human chondrocytes considering applications in cartilage engineering. The scaffold-cells constructs were observed with optical microscopy, TEM, and TPM, and evaluated with immunohistochemical and molecular analyses. Cells were viable, maintained the chondrocytic phenotype, and secreted cartilage-specific matrix. The XHA-BDDE gels are being evaluated for their capacity to promote skin restoration, when injected in an *in vitro* 3D-skin model, in comparison to commercial products. Finally, the novel protocols for HA modification have interesting implications in the composition and performance of HA-based dermal fillers and scaffolds for TE.

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Nucleolipidic-based liposomes effectively deliver a Ru(III) complex across cell membranes to nuclei promoting in concert apoptosis and autophagy in human models of breast cancer

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Abstract

In search of novel and effective metallotherapeutics for cancer treatment, in the last few years several ruthenium-based drugs have been proposed as safe alternative to cisplatin. In this context, we have recently developed a novel approach for the *in vivo* delivery of Ru(III) complexes, preparing stable ruthenium-based formulations endowed with significant antiproliferative activity. In particular, aiming at improving the suitability of Ru(III) complexes for biomedical applications - specifically of AziRu, a NAMI-A analog - we have designed innovative nanoaggregates by means of high-functionalized nucleolipidic Ru(III) complexes, *ad hoc* mixed with zwitterionic or cationic lipids to provide stable and biocompatible liposome formulations for cancer therapy.

Behind an in-depth microstructural characterization, *in vitro* bioactivity profiles in the context of preclinical studies have revealed high antiproliferative effects for our liposomes on different cancer cells from human solid tumours, as breast cancer (MCF-7, MDA MB-231, MDA-MB 436, CG5). Using especially designed fluorescent formulations and confocal microscopy approaches for targeted studies of intracellular localization, in addition to subcellular fractionation and inductively coupled plasma-**mass** spectrometry (**ICP**-MS) to assess cellular accumulation, we have detected, unlike the naked AziRu, a wide both cytosolic and nuclear distribution of the active Ru(III) complex. This would allow the ruthenium to interact with both mitochondrial and nuclear molecular targets, accounting for its ability to inhibit breast cancer cell proliferation by the activation of multiple cell death pathways, such as intrinsic apoptosis and autophagy, possibly *via* mitochondrial perturbations involving Bcl-2 family members, as well as Ru(III) ions incorporation

into double-stranded DNA. To limit chemoresistance and counteract uncontrolled proliferation, multiple cell death pathways activation is a promising strategy for targeted therapy development. These outcomes provide original knowledge on ruthenium-based candidate drugs and new insights for future optimized cancer treatment protocols.

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A combined treatment approach of p53 null colon cancer cells based on 5-FU plus the proapoptotic rpL3 gene

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Abstract

Colon cancer is one of the leading causes of cancer-related death worldwide and the therapy with 5-fluorouracil (5-FU) is mainly limited due to resistance. Recently, we have demonstrated that nucleolar stress upon 5-FU treatment leads to the activation of ribosome-free rpL3 as proapoptotic factor. In this study, we analyzed rpL3 expression profile in colon cancer tissues and demonstrated that rpL3 mRNA amount decreased with malignant progression and the intensity of its expression was inversely related to tumor grade and Bcl-2/Bax ratio. In addition, rpL3 overexpression in 5-FU treated colon cancer cells decreased clonogenic potency, cell migration and cell viability, and stimulated late apoptosis. The potential of a strategy based on 5-FU plus the proapoptotic protein rpL3 for the treatment of colon cancer was investigated by using novel polymeric nanoparticles (NPs) with a core of poly(lactic-co-glycolic) acid (PLGA). To this aim, 10 μM 5-FU and 2 μg of rpL3 were encapsulated in biocompatible NPs chemically conjugated with HA to achieve active tumor-targeting ability in CD44 overexpressing cancer cells. We showed the specific intracellular accumulation of NPs in cells and a sustained release for 5-FU and rpL3. Analysis of cytotoxicity and apoptotic induction potential of combined NPs clearly showed that the 5-FU plus rpL3 were more effective in inducing apoptosis than 5-FU or rpL3 alone. Furthermore, we showed that the cancer-specific chemosensitizer effect of combined NPs was dependent on rpL3 ability to affect 5-FU efflux by controlling P-gp (P-glycoprotein) expression. These results led us to propose a novel combined therapy with the use of 5-FU plus rpL3 in order to establish individualized therapy by examining rpL3 profiles in tumors to yield a better clinical outcomes.

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Posters

Aging and Degenerative Diseases (ADD)

Oxidative modification of proteins in rotenone-induced neurotoxicity in PC12 cells

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Rotenone has been suggested as the primary environmental risk factor for Parkinson's disease (PD). The exposure to rotenone causes a systemic defect in mitochondrial complex I and consequently an impairment of respiratory chain with high production of reactive oxygen species (ROS). The alteration of mitochondrial function lead to apoptosis and oxidative stress, which are the main contributors to the etiology of PD[1].

In the present study, we explored the effects of rotenone in pheochromocytoma PC12 cell line to clarify molecular mechanisms underlying its neurotoxicity. The well-known effects of rotenone in increasing intracellular ROS and apoptosis were first confirmed and then, oxidative modifications of proteins were assayed. Protein oxidation was evaluated by labeling protein carbonyls (PCO) with 2,4-dinitrophenyl hydrazine (DNPH) followed by immunoblotting of mono- or two-dimensional SDS-Page electrophoresis[2]. Lipid peroxidation was evaluated by measuring TBARS levels.

PC12 treatment for 24h with rotenone caused apoptotic cell death and elevated intracellular ROS which induced protein and lipid oxidation. Rotenone induced an increase of PCO and TBARS levels in dose depend manner. Interestingly, differential carbonylated protein pattern resulted from image analysis of 2D western blots obtained from treated and controls cell-cultures. Mass spectrometry analysis of differentially expressed carbonylated protein spots is in progress. Identification of protein carbonylated, could provide information regarding cellular pathway affected during respiratory chain impairment.

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In vitro evaluation of novel hybrid cooperative complexes (HCC) based on high and low molecular weight hyaluronic acid in wound healing: a step towards improvement in bioreparation

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The usefulness of hyaluronic acid and its formulations in tissue regeneration and engineering, epidermal diseases (wound healing), ophthalmic surgery, and plastic surgery is well documented.[1]. It is know that HA stimulates fibroblast(HDF, cells assigned to the collagen deposition [2, 3] . In addition, hyaluronic acid also favors tissue regeneration by increasing keratinocyte (HaCat) proliferation and mobility. [4,5].

Hydrodynamic and rheological characterizations were accomplished for the HCC samples, using a SEC-TDA system and a oscillatory rheometer respectively.

The HCC developed through the patented NahyCo technology, were tested at diverse concentrations (0.2-0.4%), using different *in vitro* cell models: standardized scratch test on HaCat and on HaCat/HDF monolayer or modified by adding interleukin 1β (IL-1β).

The latter was applied as in vitro inflammation model, better resembling *in vivo* wound repair.

The reparation was followed by time lapse video microscopy (TLVM) and by evaluating molecular pathways strictly involved. In particular the gene expression of representative cytokines (TGF-β, TNF-α, IL-6, IL-8) was investigated and, furthermore we evaluated MMPs and elastin that are crucial towards remodeling and exclude scar formation.

The results showed that novel formulations fastened the natural wound closure either in single than in co-culture model. Also in the presence of IL1β the repair rate was superior with HCC. These data were supported by gene expression in which there was a general reduction of inflammatory mediators while MMP based remodeling was prompted, when only scratched HaCat and HaCat/HDF monolayers were treated with novel formulations. Treated cells were definitely expressing more elastin confirming that the remodeled tissue has a sound consistence and supporting the idea of a reduced "danger" of scar formation.

The data induced positive perspectives for novel hybrid complex also as medical device used in the specific field and a superior concentrations than previously reported in literature (6,7,8).

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Funding for this project was received by MIUR PON03PE_00060_3 entitled "Development and testing of molecules with nutraceutical and cosmeceutical properties".

Ergothioneine oxidation protects against high-glucose induced endothelial senescence via SIRT1 and SIRT6

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Ergothioneine (ESH), 2-mercapto-L-histidine betaine (ESH), is a diet-derived compound with antioxidant properties acting through a redox mechanism different from alkylthiols. In vitro studies demonstrated that ESH, a powerful scavenger of hydroxyl radicals, superoxide anion, hypochlorous acid and peroxynitrite, protects against endothelial oxidative damages. Here, experiments were designed to evaluate the molecular mechanisms underlying the beneficial effect of ESH against hyperglycaemia-induced senescence in endothelial cells (ECs). Results indicated that cell viability was not affected by mM concentrations of ESH and that the high-glucose (25 mM) cytotoxicity was prevented by ESH with the highest efficacy at 0.5 mM. ESH was also effective in reducing the cytotoxicity of H_2O_2 and paraquat (PQT), an inducer of superoxide anion production. The cytoprotective effect of ESH was paralleled by reduced ROS production, cell senescence, and, interestingly, by the formation of hercynine (EH), a betaine produced during the ESH oxidation pathway. Importantly, HPLC-ESI-MS/MS analyses revealed that ESH oxidation generated also the sulfonic acid derivative (ESO_3H) whose amounts was dependent on the oxidative stress employed. Notably, ESH exerted a beneficial effect against high-glucose induced EC senescence through the upregulation of sirtuin 1 (SIRT1) and sirtuin 6 (SIRT6) expression, and the downregulation of p66Shc and NF-κB, as confirmed by inhibition of SIRT1 activity and SIRT6 gene silencing. In summary, results highlight the cellular antioxidant properties and mechanism of ESH, whose peculiar redox behavior makes it an attractive candidate for the prevention of oxidative stress-associated endothelial dysfunction during hyperglycemia.

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A specific nanobody prevents amyloidogenesis of D76N β2-microglobulin in vitro and modifies its tissue distribution in vivo

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Systemic amyloidosis is caused by misfolding and aggregation of globular proteins in vivo for which effective treatments are urgently needed. Inhibition of protein self-aggregation represents an attractive therapeutic strategy. Studies on the amyloidogenic variant of β2-microglobulin, D76N, causing hereditary systemic amyloidosis, have become particularly relevant since fibrils are formed in vitro in physiologically relevant conditions(1,2). Here we compare the potency of two previously described inhibitors of wild type β2-microglobulin fibrillogenesis, doxycycline and single domain antibodies (nanobodies). The β2-microglobulin -binding nanobody, Nb24, more potently inhibits D76N β2-microglobulin fibrillogenesis than doxycycline with complete abrogation of fibril formation. In β2-microglobulin knock out mice, the D76N β2-microglobulin/ Nb24 pre-formed complex, is cleared from the circulation at the same rate as the uncomplexed protein; however, the analysis of tissue distribution reveals that the interaction with the antibody reduces the concentration of the variant protein in the heart but does not modify the tissue distribution of wild type β2-microglobulin. These findings strongly support the potential therapeutic use of this antibody in the treatment of systemic amyloidosis.

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Cyclo (His-Pro) protects SOD1G93A microglial cells from oxidative stress

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Amyotrophic lateral sclerosis is a fatal disease which affects motor neurons and causes death of the patient from respiratory failure within a few years following diagnosis. Most patients suffer from sporadic amyotrophic lateral sclerosis, but about 5–10% of all amyotrophic lateral sclerosis cases can be attributed to familial forms, which are caused by mutations in the gene encoding for superoxide dismutase1(SOD 1)[1]. The mutant SOD1 proteins can catalyze oxidative reactions that damage substrates critical for viability of the affected cells (oxidative damage hypothesis) [2]. High levels of oxidative damage within the brain is a prominent feature in patients with Amyotrophic Lateral Sclerosis (ALS) [3]. Based on non-cell autonomous mechanism, glial cells play a crucial role in the pathogenesis of ALS. Transgenic mice overexpressing the human gene encoding for SOD1 mutated in Gly-93-Ala (SOD1G93A) recapitulate several aspects of the disease, besides providing a powerful model system to identify pathophysiological mechanisms associated with ALS, and they represent a golden standard to screen potential therapeutics [4].

Cyclo (His-Pro), an endogenous cyclic dipeptide produced by the cleavage of the hypothalamic thyrotropin releasing hormone, crosses the blood brain barrier and improves recovery in models of traumatic injury to the brain and shows antioxidant and anitinflammatory properties(5). The protective effects are sustained by the ability of the cyclic dipeptide to interfere with the Nrf2–NF-κB signalling systems, the former governing the antioxidant and the latter the proinflammatory cellular response. By exposing microglial cells overexpressing the mutated human gene superoxide dismutase1 to a prooxidant insult, we showed that, by activating Nrf2 transcription factor, cyclo (His-Pro) is able to alleviate the oxidative stress in an ALS pathological environment.

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The role of clusterin on alpha-synuclein aggregation in an "*in vitro*" model of Parkinson disease

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Clusterin (CLU) is a glycosylated intra and extracellular protein, exhibiting an activity similar to that of the small heat shock proteins (HSPs)[1]. CLU expression is up-regulated both in plasma and in cerebrospinal fluid of Parkinson disease (PD) patients[2-4]. Aggregation of alpha-synuclein (αSyn) is linked to the pathogenesis of PD, although very little is known about its role in PD onset and progression. Recent studies suggest that αSyn oligomers have the capacity to spread out of the neuronal cells, expanding the cellular damage to proximal regions[5]. HSPs, such as HSP70, can prevent αSyn misfolding, oligomerization and aggregation[6], representing potential targets for innovative therapeutic strategies[7]. The focus of our research is to investigate if CLU plays a role in the formation and clearance of αSyn aggregates.

We first established SH-SY5Y cell clones stably overexpressing αSyn or a mock construct. CLU is up-regulated in αSyn_SH-SY5Y in comparison to mock, possibly as a cell strategy to cope with the formation non-native αSyn inclusions. We then blocked the proteasome by MG132, to favour αSyn misfolding and accumulation. Under these experimental condition CLU mRNA is up-regulated similarly and concomitantly with HSP70. WB analysis showed the simultaneous presence of CLU and αSyn in the insoluble intracellular protein fraction and in the cell medium. We demonstrated the co-localization of CLU with phospho-αSyn, the main neuropathological hallmark of PD[9], by fluorescence confocal microscopy. Interestingly following MG132 treatment, phospho-αSyn staining was detected in cytoplasm, nucleus and in vacuoles resembling auto-phago-lysosomes. The colocalization with CLU is limited to the cytoplasm/nucleus. We suggest that CLU binds to and assists αSyn to lysosome delivery promoting clearing of αSyn aggregates by chaperone-mediated autophagy.

Co-immunoprecipitation assays and gain- and loss-of-function studies of CLU, are currently ongoing to better characterize the role of CLU in the formation/degradation of toxic αSyn inclusion bodies associated with PD.

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Novel multi-targeted anti-Alzheimer's ligands from marine sources

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Alzheimer's disease (AD), one of the most common cause of dementia in elderly population, is a complex neuro-degenerative disorder of the central nervous system. It is characterized by a progressive and irreversible degeneration of cholinergic neurons with the concomitant decrease of the hippocampal and cortical neurotransmitter acetylcholine (Ach) levels, and by the presence of amyloid plaques and neurofibrillary tangles. The current pharmacological therapy for AD is mainly based on acetylcholinesterase inhibitors (AChEI), aimed at restoring the Ach tone in the brain. However, due to the multifactorial aetiology of this pathology, compounds able to act simultaneously on two or more relevant biological targets are high desirable. In this study, the combination of AChE inhibition with the impairment of β-amyloid peptide's aggregation and deposition by inhibition of β-secretase enzyme (BACE- 1), is approached.

The previous identification of a pseudozoanthoxanthin variant from the zoanthid crust coral *Parazoanthus axinellae*, as an acetylcholinesterase inhibitor prompted us to search other molecules sharing a similar scaffold in our collection of natural marine compounds, and to explore the potentiality of the newly-selected molecules as multiligand agents, by including as potential target BACE-1 and investigating the ability of this scaffold to inhibit β-amyloid aggregation. The most similar compounds among those available in suitable quantities for subsequent experimental validation within our collection were a pseudo-zoanthoxanthin from an unidentified caribbean zoanthid (CUNC2), differing from the analog reported in the literature for the number and position of methyl substituents on the azulene ring, and the bromo-pyrrole alkaloid stevensine (STEV). Docking and molecular dynamics studies were carried out on both AChE and BACE-1 enzymes as well as on β-amiloid fibrils. The positive results obtained in silico were then confirmed by biochemical assays that showed an inhibition for both enzymes in the low/sub-micromolar range.

POTENTIAL INVOLVEMENT OF D-ASPARTATE METABOLISM IN SCHIZOPHRENIA

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It is long acknowledged that the N-methyl D-aspartate receptor (NMDAR) co-agonist, D-serine, plays a crucial role in several NMDAR-mediated physiological and pathological processes, including schizophrenia[1]. Besides D-serine, another free D-amino acid, D-aspartate, is involved in the activation of NMDARs acting as an agonist of this receptor subclass, and is abundantly detected in the developing human brain. Based on the hypothesis of NMDAR hypofunction in the pathophysiology of schizophrenia[2] and considering the ability of D-aspartate and D-serine to stimulate NMDAR-dependent transmission[1,3], we assessed the concentration of these two D-amino acids in the post-mortem dorsolateral prefrontal cortex and hippocampus of patients with schizophrenia and healthy subjects. Consistent with previous work[4], we found that D-aspartate content was selectively decreased by around 30% in the dorsolateral prefrontal cortex, but not in the hippocampus, of schizophrenia-affected patients, compared to healthy subjects. Interestingly, such selective reduction was associated to greater (around 25%) cortical activity of the enzyme responsible for D-aspartate catabolism, D-aspartate oxidase. These results reveal the potential involvement of altered D-aspartate metabolism in the dorsolateral prefrontal cortex as a factor contributing to dysfunctional NMDAR-mediated transmission in schizophrenia.

We also evaluated the potential therapeutic relevance of D-aspartate metabolism in schizophrenia, by assaying DDO enzymatic activity in vitro in presence of different first- and second-generation antipsychotics. This analysis revealed that the second-generation antipsychotic olanzapine, but not clozapine, chlorpromazine, haloperidol, bupropion, fluoxetine and amitriptyline, inhibits the human DDO activity. In line with in vitro evidence, chronic systemic administration of olanzapine induces a significant

extracellular release of D-aspartate and L-glutamate in the prefrontal cortex of freely moving mice, which is suppressed in Ddo knockout animals. These results suggest that the second-generation antipsychotic olanzapine, through the inhibition of DDO activity, increases L-glutamate release in the prefrontal cortex of treated mice.

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EPIGENETICS IN HYALURONAN SYNTHESIS CONTROL

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Epigenetics has emerged as a key aspect in the synthesis of hyaluronan (HA) influencing gene expression of hyaluronan synthases 1, 2 and 3 on cell membranes. Beside covalent modulation throughout phosphorylation and O-GlcNAcylation, HAS2 shows an intriguing regulation with epigenetic relevance. Smooth muscle cells (SMC) in the presence of different stimuli, as inflammation, oxLDL, aging, diabetes, arteriosclerosis and cancer, produced an altered ECM where hyaluronan (HA) is the major component [1]. UDP-sugar availability as well as the cellular energy are critical for the synthesis of HA and for HAS2 activity. The AMP activated protein kinase, a sensor of the energy status of the cell, leads to HAS2 T110 phosphorylation inhibiting HA secretion [1]. The most general sensor of cellular nutritional status is the UDPGlcNAc, which can lead to intracellular protein glycosylation (O-GlcNAcylation). O-GlcNAcylation of serine 221 residue of HAS2 induces a stabilization of the enzyme on the membranes increasing HA production [2]. Eventually we found a long non-coding RNA (NAT) (HAS2-AS1) positively controls in cis the HAS2 expression involving p65 and NFkB pathway. More recently, by using an array approach based on the stable knock down of HAS2-AS1 in cancer cell lines, we have evidences that NAT HAS-AS1 is able to regulate miRNA 186 availability altering the translation of several key mRNAs as those for c-MET, LC3-B, p21 and P2RXT, which correlate with cancer aggressiveness. The histone acetylation by P300 and histone de-acetylation by HADC and sirtuin 1 have a critical effect as transfection of P300 increased the HAS2 expression and HA synthesis whereas transfection of HDAC1 and sirtuin activation has opposite effects, as resveratrol and other sirtuin activators confirmed.

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THE OCCURRENCE OF A KV7.4 POTASSIUM CHANNEL IN NEURONAL MITOCHONDRIA

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The mitochondrial potassium permeability remains still a matter of investigation; in addition to mitoK$_{ATP}$, mitoBK and others only recently reported/proposed to play a role (for refs see[1]), here we show that mitochondria of F11 cells, a neuronal cell model, express a member of the Kv7 voltage dependent potassium channel family[2]. Functional evidence of this was obtained by monitoring membrane potential (ΔΨ) of isolated mitochondria by means of safranine o[3]. Externally added K^+ to succinate energized mitochondria resulted in ΔΨ decrease whose rate showed a dependence on K^+ concentration of hyperbolic nature; K-dependent ΔΨ decrease was not affected by iberiotoxin (mitoBK blocker) and only partially inhibited by either glybenclamide or ATP (mitoK$_{ATP}$ blockers); furthermore, kinetic analysis of glybenclamide inhibition confirmed the occurrence of a glybenclamide-insentitive K^+ transport in these mitochondria. Interestingly, K-dependent ΔΨ decrease was reduced by XE-991 (Kv7 blocker) and enhanced by retigabine (Kv7 activator); more importantly, the stimulatory effect of retigabine was abolished in the presence of XE-991, but not glybenclamide. Accordingly: *i)* RT-PCR experiments revealed that the mRNAs encoding for Kv7.4 subunits were expressed in F11 cells; *ii)* Western-blotting (WB) experiments using subunit-specific antibodies detected Kv7.4 expression in mitochondrial fraction (lacking in BK); *iii)* in immunocytochemistry experiments, a strong overlap between the distribution of anti-Kv7.4 antibodies and mitochondrial marker mitotracker signals was found.

Finally, in WB experiments we detected Kv7.4 subunits also in mitochondria from mouse brain, this showing that mitoKv7.4 occurrence is not limited to F11 cell model. Therefore, the tangled puzzle of mitochondrial potassium permeability in neurons is added of another piece, mitoKv7.4, whose role in neuroprotection must be checked in the future.

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The methylation state of *D-aspartate oxidase* gene predicts D-aspartate concentration in the mammalian brain.

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The flavoenzyme D-aspartate oxidase (DDO) catalyzes the degradation of the NMDA receptor agonist, D-aspartate [1]. In the mammalian brain, DDO is expressed selectively during postnatal life, thus reducing the embryonic storage of D-aspartate and keeping this D-amino acid at low levels during adulthood[2]. In the present work, we show that D-aspartate content in the mouse brain drastically decreases after birth, whereas *Ddo* mRNA levels concomitantly increase. Interestingly, postnatal *Ddo* gene expression is paralleled by progressive demethylation within its putative promoter region. Consistent with an epigenetic control on *Ddo* expression, treatment with the DNA-demethylating agent, azacitidine, causes increased mRNA levels in embryonic cortical neurons. These results suggest that during crucial phases of brain development the levels of D-aspartate are critically regulated by changes in the methylation state of *Ddo* promoter[3].

We then evaluated whether a similar molecular mechanism might be also predictive of the regional variations of D-aspartate levels occurring in the mammalian brain. To answer this question we used *post-mortem* human samples deriving from two different brain regions: dorsolateral prefrontal cortex (DLPFC) and hippocampus. First of all, we detected the levels of D-aspartate in these two areas by HPLC and we found that the content of this D-amino acid is higher in the hippocampus compared to the DLPFC. After we measured the *DDO* mRNA levels and the methylation state of the putative *DDO* promoter[4]. These analyses revealed a higher *DDO* gene expression in the DLPFC compared to the hippocampus while the methylation

percent of the CpG analyzed is lower in the former brain region compared to the latter. This evidence suggests that the methylation state of the DDO gene may also control the regional variations of D-aspartate in the mammalian brain.

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Unraveling the TZAP interactome by BioID proximity labeling: a step toward the understanding of the telomeres trimming pathway

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The proximity-dependent Biotin Identification (BioID) approach coupled to high resolution tandem mass spectrometry represents an elegant strategy for mapping local protein interactomes. This methodology provides a useful tool to explore the interaction network of a protein of interest[1], by expressing the bait as a fusion protein with a mutant form of the biotin ligase enzyme BirA (BirA). Following biotin incubation, proximal endogenous proteins are biotinylated by BirA and streptavidin-purified biotinylated proteins are then identified by MS/MS.

In the present study, the proximal interactome of the Zinc finger Telomere-Associated Protein (TZAP), involved in telomere length regulation[2], has been performed by using a BioID-based approach coupled to nano LC- high resolution tandem MS. Over 63 potential novel interactors have been identified by applying very stringent criteria for data filtering including the number of matching peptides in replicate injections and high confidence scores at both peptide and protein level. Among the identified interactors, several nuclear proteins involved in nucleosome assembly, chromosome maintenance and cell cycle regulation were identified.

Our results provide an integral view of the TZAP local interactome, thus opening novel perspectives for elucidating key players involved in the shortening telomere process potentially relevant in aging, cancer and other related diseases.

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Alzheimer's disease (AD) and Frontotemporal dementia (FTD): investigating the overlap of genetic mutations focusing on *CD33* and *TREM2*.

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Frontotemporal dementia (FTD) and Alzheimer's disease (AD) are two complex neurodegenerative disorders with associated several genes thus suggesting that a common molecular pathway could exist and is yet unknown. Despite massive research and drug development, there are still no therapies that slow down or stop their progression. Mutations in genes related to neuroinflammation, including *CD33* and *TREM2*, may be risk factors and could be entry points for therapeutic intervention.

Two *CD33* SNPs and one in *TREM2* were described to be predisposing factors to Late Onset Alzheimer disease (LOAD). In particular, *CD33* SNPs rs3865444 and rs12459419 in minor alleles were found to confer strong protection while conferring elevated risk of LOAD in major alleles. These SNPs directly modulate *CD33* exon 2 splicing efficiency. Moreover, the rare heterozygous missense variant rs75932628-T in *TREM2* exon 2 was strongly associated with the capacity of TREM2 to activate microglial cells.

In order to assess the presence of these polymorphisms in our cohort, we analyzed 216 Caucasians diagnosed with LOAD and 50 healthy controls. We performed High Resolution Melting analysis (HRM) on genomic DNA from whole blood of the patients and we sequenced by Sanger method individuals showing different melting curves and we used these results as reference in our analysis.

Our patients exhibited the coinheritance of SNPs rs12459419 and rs3865444. In addition, we identified a third SNP in *CD33* exon 2, rs2455069, which belongs to a previously identified LD SNP block associated with an increased rate of cognitive decline. We found that all patients analyzed for SNP rs75932628 in *TREM2* gene are homozygous for the wild-type allele. However, some individuals are heterozygous for the nearby SNP rs143332484, which could be potentially associated with AD in our population. Further investigations are in progress to understand the mechanism of action of these two genes.

IMPACT OF UBIQUINOL SUPPLEMENTATION ON ENDOTHELIAL FUNCTION IN SUBJECTS WITH MODERATE CARDIOVASCULAR RISK: A DOUBLE BLIND, RANDOMISED, PLACEBO-CONTROLLED CLINICAL STUDY

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Most of the major cardiovascular risk factors, including hypercholesterolemia, are characterized by an increased production of reactive oxygen species (ROS), leading to endothelial dysfunction through breakdown of the vasodilating compound nitric oxide (NO)[1]. Supplementation with Coenzyme Q10 (CoQ10) positively affects heart performance in congestive heart failure and ischemic heart disease, among with a significant blood pressure lowering effect[2].

To understand the effects of a supplementation of ubiquinol on endothelial function, measured through non-invasive ultrasonographic assessment of flow-mediated dilation (FMD)[3], a double-blind, randomized, parallel-groups clinical trial was carried out on 60 subjects with moderate cardiovascular risk. Subjects were males (aged 35-65 years) and post-menopausal females (<65 years), with untreated dyslipidaemia (LDL-Cholesterol 130-200 mg/dl) and endothelial dysfunction (baseline FMD <6%). Subjects were randomized to receive ubiquinol, 100 or 200 mg daily, or placebo. During each visit (T0, 4-weeks, 8-weeks) blood pressure and FMD were measured; laboratory parameters (including lipid profile), reduced/oxidized CoQ10 levels and oxidative stress markers (NO, peroxynitrite, conjugate dienes) were also evaluated.

Although at time of submission the study code has not been broken, red/ox CoQ10 plasma levels from 48 subjects who completed the treatment were determined. Subjects with increased CoQ10 plasma bioavailability after treatment showed an increased FMD (FMD difference = 1.32±1.20%, n=32) compared with subjects with unchanged CoQ10 plasma levels (FMD difference = -0.41±1.51%, n=16, $p<0.001$). FMD increase is positively related to improvement in plasma CoQ10 oxidative status. No significant change in lipid profile was observed after treatment. Oxidative stress biomarkers will be determined after study completion.

FMD and plasma oxidative status are significantly improved following administration of Ubiquinol in subjects with confirmed endothelial dysfunction. Moreover, bioavailability of ubiquinol is positively correlated with FMD improvements. Positive influence of CoQ10 supplementation seems to be independent from cholesterol and triglycerides levels, suggesting a direct effect of ubiquinol on endothelial cells.

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Beneficial Effects of Late-life Enalapril Administration on Heart Mitochondria of Old Rats

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Mitochondrial heart functionality decreases during the aging process. It is known that the inhibition of the renin-angiotensin system ameliorates age-related mitochondrial alterations and increases rodent lifespan. In our study, we investigated the beneficial effects of late-life enalapril administration on mitochondrial biogenesis and oxidative stress modulated via NO-dependent and -independent pathways. Four groups of Fischer 344×Brown Norway rats were randomly assigned to receive enalapril (n=4), the NO synthase (NOS) inhibitor NG-nitro-L-arginine methylester (L-NAME; n=4), enalapril + L-NAME (n=4) and placebo (n=4) from 24 to 27 months of age. Enalapril was able to prevent the age-related decrease of relevant mediators of mitochondrial biogenesis, namely peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α) and mitochondrial transcription factor A (TFAM). Such an effect was independent from NO signalling. In line with the higher TFAM content in old rats treated with enalapril, an increase in mitochondrial mass was found as measured by citrate synthase activity and mtDNA relative content. We also evaluated the level of mitochondrial ROS scavenger proteins, namely peroxiredoxin III (PRXIII) and manganese superoxide dismutase (MnSOD) showing an increase of both factors in sample treated with enalapril and L-NAME, suggesting an increase in ROS defences. Conversely, no change was found in the overoxidated PRX isoforms in the same groups.

Our results indicate a beneficial effect of enalapril on the heart by preventing the increase of ROS during aging that ensures a functional mitochondrial pool.

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Hyaluronan Hybrid Cooperative Complexes and Linear and Crosslinked Ha Based Injectable Medical Devices Differently Affect *in Vitro* Human Adipose Stem Cell Differentiation and Proliferation

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In the last decade, Human Adipose-derived Stem Cells (hASCs) hold a key position in regenerative medicine. Now different clinical applications were found: fat grafting, overcoming wound healing difficulties and scar remodeling. We evaluated Hyaluronan based gels on adipose stem cell in *in vitro* differentiation and proliferation: comparative analyses of treatments with high and low Molecular Weight (MW) hyaluronans (HA), Hyaluronan Hybrid Cooperative Complexes (HCCs) obtained through the NAHYCO Technology and commercially available, and cross-linked hyaluronan based dermal fillers available in the market were performed. hASCs were characterized by flow cytometry. Cells were treated for 7-14 and 21 days with HCCs, linear high and low MW HA and cross-linked commercially available HA based products. ASCs differentiation towards adipocytes was evaluated at first using Oil red-O staining to evidence the intracellular fat accumulation, and through the expression analyses of specific biomarkers. In particular, adiponectin, leptin, PPAR-γ and LPL expression were evaluated using qRT-PCR. Also, western blotting, ELISA assay and immunofluorescence for adiponectin and leptin were accomplished to evaluate in the time course the modulation of differentiation markers. Adiponectin and PPAR-γ proved a remarkable increase depending on the incubation time and the specific treatments. HCCs were able to improve differentiation and keep the viability of the ASCs up to 21 days. High and low MW HA modulate the biomarkers only in the short time. Cross-linked HA seemed damage the ASCs causing a loose of viability in the long term treatments. In the framework of this research, *in vitro* experiments based on human ASCs proved that HCCs-based formulations enhance adipogenic differentiation and viability more than linear HA and cross-linked hyaluronan based gels. These findings may be considered very valuable also from a translational perspective: it may be suggested

that injection of HCCs in the subdermal fat compartment may prompt remodeling improving fat tissue renewal.

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Computational and Systems Biology (CSB)

Structure-Based Virtual Screening for the search of novel SMO antagonists: a ligand repurposing approach

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Dysregulation of the Hedgehog Signaling Pathway is involved in the development of a wealth of solid tumors, as it has been well established1. In particular, the aberrant activation of this pathway is linked to metastasis growth and acquisition of resistance to traditional chemotherapeutic agents. The GPCR-like receptor Smoothened (SMO) is part of this pathway and represents an attractive target to antagonize in cancer treatment. Thus, we devised an in silico protocol which is fine-tuned to identify new potential ligands for this receptor and coupled this method with the intrinsic advantages of a drug repurposing approach2. Such a protocol employs the docking software AutoDock Vina, which allows for fast and comprehensive virtual screening (VS) campaigns. To probe the predictive power of our method, we screened a database of inhibitors active against the tyrosine kinase MET, whose overexpression is also heavily implied in cancer progression3. The most promising hits resulting from this campaign proved to be active in the nanomolar range in biological assays against SMO, representing the first dually-active ligands against this two structurally different targets.

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The role of irbesartan in inflammatory processes and oxidative stress triggered by cardiac ischemia

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The hypoxic conditions that predispose to the occurrence of myocardial infarction, leading to activation as well as neuronal systems (SNS) also hormonal systems such as the renin-angiotensin system. It plays a very important role in determining the inflammation and stress oxidative processes. In this experimental procedure, we investigated the effect of irbesartan cardioprotective as an antagonist of the last stage of the renin-angiotensin system because Angiotensin II signals through receptor subtypes, i.e., type 1 (AT-1), increased after myocardial ischemia. The activation of inflammation appears to play an important role in patients with cardiovascular disease. Clinical studies have shown that this drug is able to make an improvement of the cardiac and hemodynamic parameters in myocardial infarction patients. In addiction, has shown an anti-inflammatory and antioxidant efficacy *in vitro* that is independent from its receptor antagonism. In particular, it has shown to reduce the expression of pro-inflammatory mediators such as, TNF alpha, iNOS in atrial cardiomyocytes HL-1 as well as a reduction in NO production in stressed cells.

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BAR 3.0: GOING BEYOND PROTEIN FUNCTION ANNOTATION

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BAR 3.0 [1] updates our server BAR (Bologna Annotation Resource) for predicting protein structural and functional features from sequence. This new version is built on a bigger database and features new query capabilities and information presented to the user. The core of BAR 3.0 is a graph-based clustering procedure of UniProtKB sequences, following strict pairwise similarity criteria (sequence identity ≥40% with alignment coverage ≥90%). Each cluster contains the available annotation downloaded from UniProtKB, GO, PFAM and PDB. After statistical validation, GO terms and PFAM domains become cluster-specific and annotate new sequences entering the cluster according to the similarity criteria.

BAR 3.0 includes 28,869,663 sequences in 1,361,773 clusters, of which 22.2% (22,241,661 sequences) and 47.4% (24,555,055 sequences) have at least one validated GO term and one PFAM domain, respectively. 1.4% of the clusters (36% of all sequences) include PDB structures and the cluster is associated to a Hidden Markov Model that allows building template-target alignment suitable for structural modelling.

When evaluated on the CAFA2 (Critical Assessment of Function Annotation) targets, BAR 3.0 largely outperforms our previous version. We benchmarked BAR 3.0, simulating an in-house CAFA2 experiments. BAR 3.0 outperforms the previous version BAR++ in all the sub-ontologies, reaching F1-scores as high as 0.54, 0.35 and 0.42 for Molecular Function, Biological Process and Cellular Component, respectively.

Besides that, BAR 3.0 features also cross-links clusters thanks to connections built on the basis of known protein-protein interactions and protein complexes. This approach may be useful to gain further insights that go beyond the assignment of protein functions.

BAR 3.0 is available at https://bar.biocomp.unibo.it

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N6L interacts with NPM-1 and sensitizes leukemic cells to chemotherapy

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NPM1 is a multifunctional nucleolar protein implicated in several processes such as ribosome maturation and export, DNA damage response and apoptotic response to stress stimuli. The *NPM1* gene is involved in human tumorigenesis and is found mutated in one third of acute myeloid leukemia patients, leading to the aberrant cytoplasmic localization of NPM1. Recent studies indicated that the N6L multivalent pseudopeptide, a synthetic ligand of cell–surface nucleolin, is also able to bind NPM1 with high affinity. N6L inhibits cell growth with different mechanisms and represents a good candidate as a novel anticancer drug for a number of malignancies of different histological origin. In this study we investigated whether N6L treatment could drive antitumor effect in acute myeloid leukemia cell lines. We found that N6L binds NPM1 at the N-terminal domain, co-localizes with cytoplasmic, mutated NPM1, and interferes with its protein-protein associations. N6L toxicity appears to be p53 dependent but interestingly, the leukemic cell line harboring the mutated form of NPM1 is more resistant to treatment, suggesting that NPM1 cytoplasmic delocalization confers protection from p53 activation. Moreover, we show that N6L sensitizes AML cells to doxorubicin and cytarabine treatment. These studies suggest that N6L may be a promising option in combination therapies for acute myeloid leukemia treatment.

System biology analysis for dissecting the epithelial mesenchymal transition metabolic program

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Metabolic reprogramming is a hallmark of several physiopathological conditions including cancer [1]. The activation of the epithelial mesenchymal transition (EMT) program is accompanied by proteomic modifications including alterations in the expression of metabolic enzymes. Deciphering the functional implications of this dysregulated metabolism requires the analysis of multiple molecules including mRNAs, proteins, and lipids at omics level.

Here, we applied a LC-MS/MS approach to define proteomic alterations in breast cancer models with epithelial and mesenchymal features. Significant changes in metabolic pathways promoting cell growth were identified. Overall, the metabolic protein expression program characterised mesenchymal models for a limited metabolic flexibility with a functional consequent dependence on specific metabolic substrates such as lipids. This metabolic phenotype reflects changes in lipid-signaling pathways and enzymes that satisfy the increasing uptake of lipids from the microenvironment. This is in difference with epithelial cells that metabolizes glucose in the mitochondria to produce fatty acids. We also demonstrated how these proteomic changes impact on lipid content profile by *in situ* lipidomic analysis of our models. Moreover, metabolic alterations identified from MS profiling were integrated with genomic and transcriptomic data revealing a comprehensive and complex picture of regulation.

In the contest of EMT metabolism, this omics approach improved our understanding of the genetic and molecular events associated with the metabolic phenotype of epithelial and mesenchymal cells.

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NMR-based metabolomic investigation of metastatic melano

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Malignant melanoma is the most serious type of skin cancer because of its high metastatic ability (1). While an extensive genomic analysis of metastasis has already been described (2), there is limited information regarding the metabolic pathways that are altered during the melanoma progression. In this work the endo-metabolome of a panel of melanoma cell lines was analysed using an NMR-based metabolomic approach. The intracellular metabolites were extracted through a dual phase extraction procedure introduced by Tyagi et al. in 1996 (3) with slight modifications. A combination of untargeted and targeted metabolomic analysis was performed to understand the metabolic differences between the primary and the metastatic tumour. The targeted metabolomics profiling allowed the identification of 32 metabolites, thus quantifying a subset of them that significantly change across the cell lines under consideration. First findings from these experiments suggest that glycolysis/TCA intermediates as well as amino acid and phospholipid derivatives could have a relevant role in the metastatic process. Future works will focus on understanding the metabolic pathways that are altered during the melanoma progression.

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Analysing the relations among genes and polygenic diseases with eDGAR

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Modern sequencing technologies allow dissecting the genetic component of phenotypic traits, with a focus on diseases. While determining the gene-disease associations, it emerges that the number of diseases associated with multiple genes is increasing. The molecular mechanisms at the basis of the pathogenesis are often uncharacterized; investigating the functional relations among genes involved in the same disease may give fundamental indications about the disease development. We develop eDGAR[1], a database collecting and organizing data on gene-disease associations as derived from OMIM, Humsavar and ClinVar. eDGAR lists 2672 diseases related to 3658 different genes, for a total of 5729 gene-disease associations. We used eDGAR as a resource to investigate the features of polygenic diseases, corresponding to 23% of the dataset, and we found that the greatest majority of polygenic diseases have a couple of associated genes sharing the biological pathway (96% considering GO terms for Biological Process, while considering other resources like REACTOME and KEGG this percentage remains greater than 50%). In most cases, proteins are also linked in interactomes: about 14% of polygenic diseases are associated with a pair of proteins forming a stable complex, about 46% are associated to proteins in direct interactions in STRING or BIOGRID and another 25% involve proteins in indirect interactions. Regulation relationships are also present: 6% of polygenic diseases are associated with a transcription factor/target pair from TRRUST and another 44% is linked to genes controlled by the same transcription factor. In conclusion, eDGAR offers a resource to address the question why different genes are related to the same disease by investigating the molecular mechanisms and the functional features that are related to a specific set of genes. eDGAR is available at: edgar.biocomp.unibo.it

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Microalgae in Circular Economy: from Waste to High-Value Products

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Microalgae have gained considerable importance in recent decades due to a broad range of market applications, from simple biomass production for food and feed to high-value products [1,2]. However, for most of these applications, the market is still developing and, in order to compensate the high cost of cultivation, the biotechnological use of microalgae is extending into new areas [3]. In particular, there is an increasing interest in the valorization of starch fraction for food and other applications. In this context, different microalgae extracts are used as skin care products or as sun protection and hair care products [1].

A recent protocol for starch purification discards a fraction very rich in antioxidants [4]. Therefore, in the idea of circular bioeconomy, we analyzed two extracts obtained from *Chlorella sorokiniana* Shihiraet Krauss on an *in vivo* system (*C. elegans*). We found that both extracts can reduce accumulation of ROS and hsp-16 and increase sod-3 levels. This mechanism is mediated by DAF-16/FOXO transcription factor. Moreover, when the two extracts were combined, a synergistic effect was observed. Overall, our results clearly indicate a protective effect of microalgae extracts against oxidative stress. Accordingly, we can conclude that a "waste" product could be used for cosmetic applications.

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Metabolomic approach in the study of inborn errors of metabolism

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Inborn errors of metabolism are genetic disorders due to impaired activity of enzymes, transporters, or cofactors resulting in accumulation of abnormal metabolites proximal to the metabolic block, lack of necessary products or accumulation of byproducts. Many of these disorders cause serious clinical consequences to the affected neonate, which sometimes can be avoided by an early and accurate diagnosis to establish the appropriate therapy. In other cases, treatment is not completely curative but early diagnoses relieve families from long and difficult diagnostic work-ups and allow suitable genetic counselling. Newborn screening by LC-MS/MS analysis on dried blood spot samples (DBS) can detect several disorders by a single injection. In May 2007 we initiated an expanded newborn screening for inborn errors of metabolism in Campania region by analyzing amino acids and acylcarnitines in DBS samples using tandem mass spectrometry (1). Here we report the most recent results of our experience from this program.

Analysis of amino acids and acylcarnitines in DBS samples performed on 31519 infants between 48 and 72 h of life allowed us to identify, through the period January 2015-june 2017, 11 new affected newborns. Diagnoses on newborns with elevated metabolites were confirmed by gas chromatography-mass spectrometry for an overall incidence of about 1: 2865. Three infants were identified with isolated methylmalonic acidemia (MMA), 1 with a vitamin B12 metabolism/absorption defect, 3 with 3-methlycrotonyl-CoA carboxylase (3-MCC) deficiency. Three infants were found with alterations indicative of urea cycle defects: 1 showed citrullinemia, 1 evidenced hyperornitinemia, 1 was affected by deficit of ornitine transcarbamylase. The last affected patient identified by the newborn screening exhibited a significant hypermethioninemia.

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PEPTIDOMIC PROFILE OF IN VITRO DIGESTED BOVINE, CAMEL, GOAT AND SHEEP MILK

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Milk and dairy products have long traditions in human nutrition since they are a rich source of nutrients such lipids, proteins, amino acids, vitamins and minerals. Nowadays, milk may also be considered as a source of health-promoting compounds. Bioactive peptides deriving from milk proteins may play an important role in the prevention and treatment of metabolic syndrome and its complications [1].

This study aimed to characterize the peptidomic profile of *in vitro* digested skimmed bovine, camel, goat and sheep milk. Milk samples were *in vitro* digested following the harmonized INFOGEST protocol [2]. The peptide fractions were extracted from fully digested milk by ultrafiltration (cut-off 3 kDa) and the permeate were characterized by nanoflow-LC-ESI-QTOF MS/MS analysis. Digestibility and biological activities of the peptide fractions were also determined at the end of the digestion.

The results showed a faster and more efficient gastric and duodenal degradation of goat milk proteins than camel, bovine and sheep milk. More than 100 peptides were identified in the permeate of digested milk, most of them arising from beta-casein. Goat, sheep and bovine milk showed the highest similarity in peptide sequences than camel milk. Peptides with previously demonstrated biological activities were found in the permeate of all the milk samples after *in vitro* digestion. Biological activities analysis showed that bovine milk peptides were the most effective in scavenging hydroxyl radical and in the inhibition lipid peroxidation. Bovine milk was also the best source of DPPIV-inhibitory peptides (IC_{50}=6.87 mg of peptides/mL) whereas sheep milk was the best source of ACE-inhibitory peptides (IC_{50}=625.4 µg of peptides/mL). The quantitative diversity in the identified bioactive peptides explained the observed differences in the biological activities.

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A COVALENT DOCKING APPROACH TO SIMULATE THE INTERACTIONS OF A NOVEL CEPHALOSPORIN DERIVATIVE WITH THEIR BIOLOGICAL TARGETS

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One of the most important and growing problems in public health is the development of antibiotic resistance, which allows bacteria to escape to the activity of antimicrobial compounds. Moreover, no new chemical classes of antibiotics have been discovered during the last 30 years; therefore, researchers worldwide are actively involved not only in searching for innovative antibiotics, but also in developing new molecules belonging to traditional classes that could be more resistant towards bacteria's defence mechanisms.

We have developed a new class of cephalosporin derivatives in which an additional isolated beta-lactam ring is bound to the classic 7-aminocephalosporanic ring. The prototype compound has proven to be effective against Gram-positive bacteria, especially *S. aureus*, with no cytotoxic effects. Starting from this compound, we have introduced substituents with the aim of modulating its activity, and we have simulated the interactions of the new derivatives with their biological targets (penicillin binding proteins and beta lactamases) by using a covalent docking approach to take into account the irreversible bond formed between the antibiotics and the enzymes.

The new derivatives are able to bind to their biological targets with a predicted negative binding energy compatible with a binding affinity in the low nanomolar range. The isolated 2-azetidinone ring shows an affinity lower than that of the traditional cephalosporin nucleus. We have also performed a detailed analysis of the complexes obtained in order to identify the residues involved in different kind of interactions with the chemical groups of the antibiotics.

ACKNOWLEDGEMENTS

The present project is funded by FARB (Fondo di Ateneo per la Ricerca di Base), cod. ORSA151138 and ORSA161582 (A.M).

Differentiation and Neoplastic Transformation (DNT)

Hippo signaling pathway in human astroglial tumors

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The Hippo signaling pathway is considered as a key regulator of tissue homeostasis, cell proliferation and apoptosis, and alterations of this pathway seems to contribute to tumorigenesis. Yes-associated protein (YAP1) is a downstream target of the Hippo pathway and acts as a transcription co-activator. Different studies suggested a role of YAP1 in in various types of cancer, such as colorectal cancer, gastric cancer and human hepatocellular carcinoma. YAP1 has often been described as an oncogene, but different researchers argued that YAP1 could also act as a tumor suppressor gene in some malignancies, such as breast cancer.

Astroglials tumuors /Gliomas are highly invasive and vascularized neoplasms accounting for more than 70% of all brain tumors. Despite recent advances in surgery, radiotherapy and chemotherapy, survival of high-grade glioma patients remains poor. The lack of robust treatment options has propelled a search for markers that could identify subgroups of patients likely to benefit from molecularly targeted therapies. For this reason, the study of Hippo pathway, could clarify further molecular mechanisms of aggression and the progression of these tumor types.

We have investigated the expression of: phosphorylated/Unphosphorylated form and epigenetic regulation of YAP1 in human astroglial tumors with different grade and the expression of Survivin as one of the downstream product of the Hippo pathway. Our results show an over expression of the unphosphorylated YAP1 in glioblastoma versus anaplastic and low grade glioma. Survivin is more expressed in glioblastoma tissues versus the other grade. However the miRNA involved in Hippo signaling regulation via LAST1/2 protein kinase were over expressed. This preliminary data confirming a crucial role of Hippo pathway in cancer proliferation and suggesting that this pathway could be a potential therapeutic target.

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$p57^{Kip2}$ phosphorylation modulates its interaction with cyclin-dependent kinases

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$p57^{Kip2}$ protein, identified for its homology with $p21^{Cip1}$ and $p27^{Kip1}$, modulates the activity of cyclin-dependent kinases (CKR) and represents a key regulator of cell division cycle progression. However, it remarkably differs from the other Cip/Kip family members (i.e. $p21^{Cip1}$ and $p27^{Kip1}$) for its C-terminal sequence. So far, $p57^{Kip2}$ is the less studied component of the Cip/Kip family and for a long time its importance has been associated to a role in embryonic development. Recently, $p57^{Kip2}$ decreased levels, found in several tumor types, are in agreement with potential tumor-suppressor function(s). Moreover, the restricted distribution of $p57^{Kip2}$ in adult human tissues, different from that of $p21^{Cip1}$ and $p27^{Kip1}$, suggests that this CKR plays specialized roles. In this study, $p57^{Kip2}$ functional characterization has been performed by comparing the CKR binding with cyclin/CDK2 complexes. Employing co-immunoprecipitation experiments, we established that endogenous $p57^{Kip2}$ is mainly bound to cyclin A/CDK2 rather than to cyclin E/CDK2. Unexpectedly and clearly distinct from $p21^{Cip1}$ and $p27^{Kip1}$, $p57^{Kip2}$ has been found to mainly bind Thr-160 phospho-CDK2, the activated isoform of the kinase. The selective binding suggests that major differences may exist between members of Cip/Kip family in terms of their binding affinity for specific CDKs, although the kinase inhibitory domain is, at least apparently, well conserved among CKRs. Post-translational modifications (PTMs) of an intrinsically unstructured protein IUPs, like $p57^{Kip2}$, has been suggested to direct the protein toward specific conformations and, thus, interactors. Recently, by an experimental strategy based on analysis through bidimensional electrophoresis followed by immunoblotting (2D/WB) of mutagenized $p57^{Kip2}$, we have characterized four phosphorylatable residues of the protein, not reported in literature. On the basis of these data, specific pattern of $p57^{Kip2}$ PTMs has been identified as associated with the modulation of Cyclin A(E)/CDK2, strongly indicating that PTMs control the CDK modulatory activity of $p57^{Kip2}$.

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SPHINGOSINE 1-PHOSPHATE INSIDE-OUT SIGNALING IS INVOLVED IN BRADYKININ-INDUCED MYOGENIC DIFFERENTIATION

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Skeletal muscle has a remarkable capacity to regenerate that relies on the activation of quiescent resident stem cells that in pathological conditions or after tissue damage proliferate and migrate towards the site of injury. Subsequently, myogenic differentiation of these immature progenitors is responsible for skeletal muscle repair. In the regenerative microenvironment, cytokines, kinins and growth factors, are released after injury by leukocytes and macrophages, being responsible for the inflammatory response aimed at clearing cell debris, while also affecting skeletal muscle regeneration. Sphingosine 1-phosphate (S1P) has been identified as an intracellular mediator, even though it is well accepted that mainly acts via G protein-coupled receptors (S1P1-5), after its export through specific and unspecific transporters, spinster2 (Spns2) and ATP-binding cassette (ABC) family members, respectively. Among other tissues also in skeletal muscle growth factors exploit sphingosine kinase (SK), the enzyme that synthetises S1P, and S1P receptor signalling for the accomplishment of specific biological effects (1).

The present data report that bradykinin (BK), the leading member of kinin/kallicrein system, is capable of inducing myogenic differentiation in C2C12 myoblasts. In particular, both S1P metabolism and signalling are involved in this effect, since pharmacological treatment and RNA interference that target SK1/Spns2/S1P2 axis significantly affect BK-induced myogenic differentiation. Moreover, the molecular mechanism initiated by BK has been clarified by time-lapse immunofluorescence analysis: BK exerts a rapid translocation of SK1 to plasma membrane that is not dependent on SK1 phosphorylation. The present study highlights the role of SK1/Spns2/S1P2 signalling axis in BK-induced myogenic differentiation, thus confirming the crucial involvement of this pathway in skeletal muscle cell biology.

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Exogenous fatty acids modulate ER composition and lipid metabolism in breast cancer cells

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De novo lipogenesis is required in all cancers for synthesis of new membranes, and then for cellular transformation and cancer progression. Cancer cells utilize exogenous fatty acids (FAs) for energy and membrane formation. Moreover, exogenous administration of saturated and unsaturated FAs might alter the anabolic and catabolic activity, the structure and organization of biological membranes and thus influence tumor cell survival [1]. We have investigated the alterations induced by treatment with exogenous palmitic acid (PA), and docosahexaenoic acid (DHA, omega-3), in human breast adenocarcinoma cells, both sensitive (MCF-7) and insensitive to oestrogen (MDA-MB-231). Given the importance of the endoplasmic reticulum (ER) in lipid metabolism and assembly of biological membranes, we have quantified changes of different lipid classes isolated from ER. The results obtained demonstrate that the two cell lines are influenced in different ways by PA and DHA. In MDA-MB-231, both PA and DHA are able to modify ER lipid composition supporting the hypothesis of their action at this level. On the contrary the MCF-7 cells result less sensitive to PA, while they incorporate DHA, although less efficiently than MDA-MB-231 cells.

Moreover, our data suggest that cancer cells incorporate FAs not only to synthesize structural lipids, such as PLs, but also lipids with signalling potential such as eicosanoids, phosphoinositides, that control important cellular processes, including cell proliferation, apoptosis, metabolism and migration. In addition, FAs might also influence cholesterol biosynthesis and are incorporated into intracellular triglycerides and cholesterol esters. In conclusion, our data indicate that in oestrogen insensitive cells the saturated and polyunsaturated FAs are able to modify the lipids of ER, suggesting an activity at lipid biosynthetic level. On the contrary, the oestrogen sensitive cells are less sensitive to exogenous FA incorporation. This different sensitivity to lipid environment might be related to cell malignancy: MDA-MB-231 cells, the most aggressive line, seem to be more metabolically oriented towards the biosynthesis of lipid than the relatively sensitive and less aggressive MCF-7 cells. Our data suggest a correlation between the onset of cancer and lifestyle, diet, obesity.

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Investigation of Lipid Metabolism in Mucopolysaccharidosis type IIIB mouse model

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Mucopolysaccharidosis type IIIB (MPS IIIB), also known as Sanfilippo syndrome B, is a genetic disorder caused by mutations in the NAGLU gene. The gene codifies for the alpha-N-acetyl-glucosaminidase (NAGLU) enzyme, which is responsible for the hydrolysis of large molecules of mucopolysaccharides called glycosaminoglycans (GAGs). In particular, the defect leads to the storage of a class of GAGs called heparan sulphates (HSs) in liver, kidney, spleen, heart and in the central nervous system, resulting in multiple organ dysfunctions including hepatosplenomegaly, cardiac defects and mental degeneration with behavioural abnormalities (1).

In order to explore the metabolic changes underlying the haepatic and cardiac defects in the MPS IIIB, here, the murine model of the disease ($NAGLU^{-/-}$ mice) (2) was used to investigate the lipid metabolism. Livers and hearts from $NAGLU^{-/-}$ and wild type (WT) mice were homogenized, and metabolites were extracted in methanol from samples. A complete panel of metabolites – acylcarnitines – was then identified and quantified by liquid chromatography - tandem mass spectrometry (LC-MS/MS).

Acylcarnitines are essential intermediates for the transport of fatty acids across the mitochondrial membrane for the β-oxidation. Variations in acylcarnitines profiles might reveal alterations in the lipid metabolism caused by the absence of NAGLU. The point of breakdown in the β-oxidation pathway and the consequent damage in mouse can be recognized from their chain-length profile. The altered pathways could represent new molecular targets for the therapy of human patients.

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DEVELOPMENT OF A NOVEL THERAPEUTIC APPROACH FOR MUCOPOLYSACCHARIDOSIS DISEASES

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Mucopolysaccharidoses (MPSs) are a group of inherited metabolic diseases, belonging to the class of the Lysosomal Storage Diseases (LSDs), caused by the absence or malfunctioning of lysosomal enzymes needed to metabolize glycosaminoglycans (GAGs). Four types of therapies are available at the moment for these diseases: enzyme replacement therapy (ERT), substrate reduction therapy (SRT), gene therapy, and hematopoietic stem cells transplantation (HSCT). However, all of these therapeutic approaches are still under development with variable clinical efficacy.

We have recently developed a new therapy for the MPS diseases, based on the use of a recombinant protein that is able to bind the excess of extracellular accumulated substrate. This protein shows the capability to reduce GAG content and lysosomal defects in primary fibroblasts from affected MPS patients. Furthermore, the therapy is able to reduce GAG content in the organs and urine of the murine model of MPS IIIB, thus preventing also the symptoms of the disease in mice. Finally, we demonstrated the mechanism of action of our therapeutic approach unraveling a new therapeutic target for these diseases. We believe that this therapy has the potential to transform the standard of care for patient affected by MPS by the use of this technology alone or in combination with the others already available.

Standardization of an experimental system of adherent primary mesenchymal colon cancer cells and paired tumorspheres for the study of cancer cell migration, stemness features and drug response

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Colorectal cancer (CRC) is one of the main causes of cancer deaths, with metastases representing the first cause of death. It has been recently demonstrated that epithelial to mesenchymal transition (EMT) could play a critical role in invasion and metastasis of CRCs. We have isolated primary mesenchymal colorectal cancer cells from CRC patients, which expressed epithelial (E-Cadherin and Cytokeratins), mesenchymal (N-Cadherin and Vimentin) and stemness markers (Oct4, sox2, nanog, ALDH1, LGR5, CD133, CD44 and CD44v6), together with high level of EMT-transcription factors (Snail and Twist), suggesting that these were epithelial cells undergone EMT [1]. Afterwards, we generated tumorspheres from these cells, by using hanging drop assay. Finally, since GSK-3-β is a multifunctional serine/threonine kinase over-expressed in colorectal and pancreatic cancer, we studied the effects of GSK-3-β inhibition, by incubating cells with LiCl, an its specific inhibitor. Thus, we established a system of adherent primary mesenchymal colon cancer cells and their paired tumorspheres allowing us to study cell migration, stemness features and drug response. By using this experimental system, we were able to obtain the following results: a) Epithelial-mesenchymal colon cancer cells showed stemness features that were reverted by GSK-3-β inhibition; b) GSK-3-β inhibition reduced migration of these cells; c) primary mesenchymal colon cancer cells were able to form tumorospheres, indicating the acquisition of a dedifferentiated state; d) GSK-3-β inhibition affected stem cell-like properties and altered cell plasticity. Thus, we suggest that GSK-3-β and LiCl could represent an eligible target and a potential drug for CRCs therapy. Finally, we have identified a panel of biomarkers (including E- and N-

cadherin, Snail, Oct-4, Sox2, Nanog and LGR5), all showing a nuclear localization, that could represent prognostic and/or predictive biomarkers supporting CRC follow-up and therapy.

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MSH2 3'UTR AS PUTATIVE BINDING SITE OF MIR-137

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In this work, we reported the results of the study performed to the c.* 226A> G variant detected in the 3'UTR of the MSH2 gene, in order to demonstrate the pathogenicity. First, the c. * 226A> G variant was identified in the index case who developed colon cancer with high microsatellite instability at age 38 and that was belonging to Lynch syndrome family. The RT-PCR analysis showed that this variant determined a increase of MSH2 mRNA expression; the IHC analysis and by functional luciferase in vitro assay also showed that this variant increased the MSH2 protein levels. Therefore, we performed an computational analysis of this mutation in order to clarify the pathogenetic role. In this manner, we showed that the region in which falls the mutation was identified as a putative target point of miRNAs (as -miR-137). Thus, we speculated that the overexpression effect was related to the loss of MSH2 down regulation from the hsa-miR-137.In order to confirm our hypothesis, the wilde-type (WT) and mutant (MUT) MSH2 3'-UTR were cloned downstream the Renilla luciferase reporter gene. The reporter gene constructs were transfected into SW480 cells and 48 hrs later cells were collected for luciferase assay and quantitative mRNA analysis. The MUT MSH2 3'-UTR showed higher luciferase activity than the construct with the WT MSH2 3'UTR. Moreover, using a defined in-vitro miRNA processing system, we also showed that miR-137 regulates MSH2 expression through base pairing with the MSH2 3'-untranslated region. Therefore, the c.* 226A> G variant preventing the binding of miR-137 to specific MSH2 3'UTR region determines an increase of MSH2 expression. It is known that loss or overexpression of the key mismatch repair protein leads to genome instability and tumorigenesis, thus, it is likely that this variant is really pathogenic. Moreover, this study also indicate an involvement of miR137 in the pathogenesis of the Lynch syndrome. In the next future, the miRNAs may be considered as biomarkers or novel therapeutic targets.

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NICOTINAMIDE N-METHYLTRANSFERASE: A NEW POTENTIAL BIOMARKER FOR CUTANEOUS MALIGNANT MELANOMA

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Unlike other types of skin cancer, cutaneous malignant melanoma is highly aggressive, such as even small lesions can metastasize systemically to multiple organs, resulting in a very poor prognosis. Despite recent advances in chemo- and immunotherapy, treatment options for advanced disease are limited. Therefore, the identification of biomarkers that can be used for the detection of early stage disease as well as for monitoring the therapeutic response is urgently needed.

In the present study, we evaluated the expression level of nicotinamide N-methyltransferase (NNMT), an enzyme which catalyses the N-methylation of nicotinamide and other structural analogues[1]. NNMT was found to be upregulated in a variety of solid tumors, but no data are currently available for cutaneous malignant melanoma. Immunohistochemistry was performed to analyse NNMT expression in 34 melanomas and 34 nevi, used as controls. In addition, statistical analyses were carried out to explore the correlation between enzyme levels and tumor prognostic parameters.

Results obtained demonstrated that NNMT display significantly ($p<0.05$) higher expression in melanoma samples compared with that detected in nevi. Interestingly, a significant ($p<0.05$) inverse correlation was found between enzyme levels and Breslow thickness, Clark level, the presence/number of mitoses and ulceration. Taken together, these data seem to suggest that NNMT could represent a molecular biomarker for melanoma, thus highlighting its potential for both diagnosis and prognosis of this neoplasm.

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GATA-1 isoforms differently contribute to the production and compartmentation of reactive oxygen species in myeloid cells

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A correlation between aberrant expression of GATA-1, a master regulator of several hematopoietic genes, and a poor prognosis has been well established in myeloid leukemias, suggesting that GATA-1 may be part of the malignant phenotype. GATA-1 exists at least as two naturally occurring isoforms that play opposite roles in the differentiation and proliferation processes: the full-length protein (GATA-1_{FL}) which comprises an N-terminal transactivation domain (TD), two conserved zinc finger domains and a C-terminal domain, and a shorter isoform named GATA-1_S which lacks the N-terminal TD. Maintenance of a normal GATA-1_{FL}/GATA-1_S ratio plays a crucial role in normal hematopoiesis whereas its disruption correlates with a variety of hematopoietic disorders.

Recently we found that GATA-1 isoforms are differently involved in the expression levels of the subunit C (SDHC) of the succinate dehydrogenase complex, a component of the complex II of the electron transport chain. According to a mechanism recently proposed for ROS production by complex II, we evaluated mitochondrial and cytoplasmatic oxidative stress levels in K562 cells over-expressing GATA-1 isoforms. Intriguingly, in GATA-1_S over-expressing cells we found elevated superoxide mitochondrial levels associated with a significant increased mitochondrial mass whereas in GATA-1_{FL} over-expressing cells we observed lower mitochondrial superoxide levels and a slight reduction in the mitochondrial mass. On the other hand, ROS cytoplasmatic levels were strongly increased in GATA-1_{FL} over-expressing cells with respect to GATA-1_S.

Our findings indicate that GATA-1 isoforms could differently contribute to modulate the proliferation and differentiation programs in hematopoietic cells via production and

compartmentation of reactive oxygen species. This study also highlights a mechanism through which aberrant expression of GATA-1 isoforms could contribute to sustain the leukemogenic process in myeloid cells. Expectedly, a better understanding of this mechanism could eventually lead to design more effective ROS-based therapies in myeloid leukemias.

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Identification of a retinoid-responsive element in the GATA-1 promoter and its role in the promyelocytic differentiation induced by *all*-trans retinoic acid (ATRA)

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Acute promyelocytic leukemia (APL) is characterized by a unique chromosomal translocation t(15;17) which results in the PML-RARα gene fusion and chimeric protein. PML-RARα behaves as an altered retinoic acid receptor with an ability of transmitting oncogenic signaling leading to accumulation of undifferentiated promyelocytes. *All*-trans retinoic acid (ATRA) acts by binding to specific retinoic acid ligand-dependent nuclear receptors targeting genes that further control cell growth and differentiation. Recent studies indicate that GATA-1, a master regulator of several hematopoietic genes, may be part of the malignant phenotype in myeloid leukemias. GATA-1 exists at least as two naturally occurring isoforms: the full-length protein, GATA-1_{FL}, and a shorter isoform, GATA-1_S. Aberrant GATA-1 isoforms ratio plays a crucial role in a variety of hematopoietic disorders since GATA-1_{FL} and GATA-1_S play opposite roles in the differentiation and proliferation processes.

On the basis of these findings, we aimed to evaluate the involvement of GATA-1 isoforms in the differentiation pathway activated by ATRA treatment in HL60 promielocytic cells. We firstly found that in these cells ATRA induces a dramatic decrease in GATA-1 expression, particularly with regard to GATA-1_S, the GATA-1 isoform with pro-survival and proliferation activity. To investigate whether retinoids are directly involved in GATA-1 expression, we performed a bioinformatic analysis of the GATA-1 proximal promoter region that revealed putative binding sites for RXRα, RARα and VDR. Interestingly, ChIP analysis performed in ATRA-treated HL60 cells showed variations in the RXRα/RARα/VDR binding dynamics on this promoter region compared to untreated cells. Taken as

a whole, these results suggest a mechanism through which ATRA could promote promyelocytic differentiation by down-modulating GATA-1 expression levels and by increasing the GATA-1_{FL}/GATA-1_S ratio.

Furthermore, these findings are also suggestive of a potential role of GATA-1 and its isoforms as novel promising therapeutic targets in ATRA-resistant promyelocytic leukemia.

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Effect of hypoxia on H_2S metabolism in colon cancer cells

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Along with nitric oxide (NO) and carbon monoxide (CO), hydrogen sulphide (H_2S) belongs to a small group of gaseous signalling molecules termed 'gasotransmitters'. H_2S has been recognized to play a key role in tumour cells where it sustains cell bioenergetics both acting as a reducing substrate of the mitochondrial respiratory chain and stimulating glycolysis (reviewed in [1]). The human enzymes involved in H_2S synthesis and catabolism are currently investigated by our group. Namely, the activity of cystathionine β-synthase (CBS), a major source of H_2S in humans, was found to be fine-tuned through an intricate interplay between the positive allosteric effector S-adenosyl-L-methionine and the heme-mediated inhibitory action of CO and NO [2-3]. Dysregulation of such control mechanism was more recently proposed to represent a pathogenic mechanism in classical homocystinuria, a rare genetic disease associated with mutations in the CBS-encoding gene [4].

H_2S catabolism is accomplished by a mitochondria-associated enzymatic sulphide-oxidizing unit that feeds the respiratory electron transport chain with the electrons derived from H_2S oxidation. Here, the H_2S catabolism was investigated in colon cancer model cells (SW480). Cells were grown under either normoxic (20% O_2) or hypoxic (1% O_2) conditions and their ability to metabolize H_2S at the level of the mitochondrion was assayed by high resolution respirometry. Intriguingly, exposure to hypoxic conditions for 24 hours, while reducing the mitochondrial mass, was found to enhance the mitochondrial H_2S metabolism in cancer model cells. The potential implications of this unexpected finding will be discussed.

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Downregulation of microRNA-125a in Hepatocellular Carcinoma and Overexpression of its Targets Sirtuin-7, Matrix Metalloproteinase-11, and c-Raf

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MicroRNA-125a is a vertebrate homolog of lin-4, the first discovered microRNA (1). It appeared early in evolution, about 550 millions of years ago, and today is present in all animals with bilateral symmetry (2). MiR-125a plays a well-conserved primary role in downregulating Lin-28 protein, thus promoting phase transitions in development and/or cell differentiation in nematodes, insects and mammals (3-5). Many other targets of miR-125a are membrane receptors or transductors of mitogenic signals and the main effect of its transfection in cultured cells is inhibition of cell proliferation. This evidence and the ubiquitous expression of miR-125 suggest that its role in differentiated tissues is fine-tuning of cellular response to mitogenic signals. In accordance with this hypothesis, miR-125a is downregulated in several types of tumors, such as breast, lung, ovarian, gastric, colon, and cervical cancers, neuroblastoma, medulloblastoma, glioblastoma, and retinoblastoma.

In this study, we focused on hepatocellular carcinoma (HCC) and showed that miR-125a inhibits proliferation of cultured HCC cells by p21/p27-dependent cell cycle arrest in G1. Then, the analysis of a number of miR-125a validated targets revealed that sirtuin-7, matrix metalloproteinase-11, Zbtb7a, and c-Raf were downregulated. Interestingly, miR-125a was found to be induced by sorafenib, the antitumor drug for treatment of advanced HCC, and to be part of its mechanism of action. The expression of miR-125a was then evaluated in 55 tumor biopsies of HCC and in matched adjacent non-tumor liver tissues, showing its down-regulation in 80% of tumors with a mean 4.7-fold decrease. Sirtuin-7, matrix metalloproteinase-11 and

c-Raf were conversely up-regulated by 2.2-, 3-, and 1.7-fold, respectively. Finally, we studied the molecular mechanisms governing miR-125a expression and identified its transcription promoter.

The availability of a luciferase-based reporter assay for testing miR-125a promoter activity will be useful to screen compounds increasing its activity, eventually leading to treatments to restore miR-125a expression in tumor cells.

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Looking for novel antitumor compounds from Fabaceae species

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Colorectal cancer (CRC) is one of the most frequent cancers in developed countries and one of the major causes of cancer-related deaths worldwide. Chemotherapeutic agents used for the treatment of metastatic CRC (mCRC) are not entirely effective to date. In particular, the possibility to cure mCRC patients is limited by the side effects of the drugs and by the development of drug resistance. Nature have been a source of medicinal products for millennia with many useful drugs developed from plants. Most of chemotherapeutic agents for cancer treatment are molecules isolated from natural sources.

In this study, we report a new screening method for a rapid identification of cytotoxic metabolites in fourteen Mediterranean Fabaceae species. This approach combines an NMR-based metabolomic profiling with antiproliferative assays toward a panel of human colon cancer cell lines. The NMR analysis of plant extracts revealed different metabolic profiles with various secondary metabolites including phenols and terpenoids. The potential antiproliferative activity of the extracts was then evaluated by MTT assays on three different colon cancer cell lines, Caco-2, HT-29 and HCT-116, selected for having different mutation profiles in key oncogenes such as *KRAS*, *NRAS*, *BRAF* and *PIK3CA*. Results from MTT analysis revealed that *Astragalus boeticus* and *Trigonella corniculata* extracts have a strong antiproliferative activity in all cell lines. Then, extensive 2D NMR analyses allowed to identify triterpenoidc and steroidic saponins as principal constituent of these two plant extracts, respectively. The spectroscopic analyses along with biological assays led to a full characterization of the main metabolites and bio-guided isolation of compounds responsible for the antiproliferative activity. More importantly, the purified compounds showed antiproliferative activity against a panel of CRC drug-resistant cell lines. These findings encourage further studies aimed at

investigating their mechanism of action for the development of new promising therapeutic approaches.

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Activation of the hypoxia-inducible factor 1α promotes myogenesis through the non-canonical WNT pathway leading to hypertrophic myotubes

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Regeneration of skeletal muscle is a complex process that requires the activation of quiescent adult stem cells, the *satellite* cells, which are resident in hypoxic niches in the tissue. This process is mainly regulated through a group of transcription factors known as the *hypoxia-inducible factors (HIFs)* (1). In particular, HIF-1α activation has been described as beneficial for the cell to overcome an hypoxic insult (2), while it has been observed that its chronic activation completely inhibits skeletal muscle differentiation (3). Therefore, oxygen deprivation and HIF-1α may play a role in activating the initial steps of the regeneration process. Herein, we investigated whether a 24h pre-conditioning under hypoxic culture conditions could alter the differentiation of C2C12 myoblasts.

In this work we report that a controlled hypoxic stimulus can trigger the hypoxia inducible factor HIF-1α, activating MyoD through the non-canonical Wnt/β-catenin pathway and resulting in muscle hypertrophy. In particular, results show that an hypoxic pre-conditioning promotes the increase of all differentiation markers and the up-regulation of WNT4, WNT7a, and WNT9, which are known to be involved in myogenesis.

Finally, HIF-1α silencing significantly reduced cell differentiation, down-regulating MyoD and MHC as well as the expression of WNT7a. Altogether these results support the notion that hypoxia plays a pivotal role in activating the regeneration process by directly inducing myogenesis through HIF-1α and the non canonical WNT pathway.

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FR051, a novel Hexosamine Biosynthetic Pathway inhibitor, induces cell death in primary and metastatic pancreatic cancer models

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Pancreatic Ductal Adenocarcinoma (PDAC) is an aggressive disease, characterized by invasiveness, rapid progression and profound resistance to treatment, due to reduced vasculature and to surrounding stroma that protects cancer cells(1). Glucose and glutamine have critical role for pancreatic cancer growth, in particular our data revealed that glucose depletion strongly affects cell proliferation, compared to glutamine reduction, and decreases O-glycosylation level and activates Unfolded Protein Response (UPR) in time dependent manner.

Hexosamine Biosynthetic Pathway (HBP) altered flux has been associated to abnormal cell behavior(2)(3)(4). In this regard, by using specific inhibitors (azaserine and tunicamycin) it has been shown that PDACs cells are strictly dependent on HBP and more specifically on N-glycosylation, in order to sustain cell proliferation, survival and anchorage-independent growth. Since the available HBP inhibitors are quite unspecific and in some cases toxic, we decided to synthetize an inhibitor targeting the Phosphoacetylglucosamine mutase 3 (PGM3) enzyme, involved in an important step of HBP. According to a computational analysis, we designed and synthetized a mini-library of putative PGM3 inhibitors, mimicking the chemical structure of its natural substrate. Among the different inhibitors, FR051 was selected as the best hit. In fact, upon treatment it strongly reduced cell proliferation and viability of both primary tumor cells (Mia Paca-2 and BxPC-3) and of metastatic ones (Capan-1 and Su.86.86) as compared to HPDE cells (immortalized pancreatic cells). FR051-dependent cell death was associated to a strongly UPR activation, as confirmed by increased expression of several markers such as DDIT3 and ATF4. Importantly, FR051 was also able to reduce PDACs cells adhesion and migration in correlation with a reduction of membrane proteins N-glycosylation. All these findings make FR051 a promising molecule for cancer cell therapy and the starting

point for the synthesis of more efficient molecules.

Supported by the Italian Association Cancer Research (grant No. IG-15364) and by University of Milano Bicocca (FAR 2016).

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Production of spheroids from human primary skin myofibroblasts: an experimental model to study myofibroblast deactivation

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Myofibroblasts are activated fibroblasts, involved in tissue repair and cancer, characterized by *de novo* expression of alpha smooth muscle actin (α-SMA), increased secretion of growth factors and immunoregulatory phenotype [1]. At the end of wound healing myofibroblasts undergo apoptotic cell death, whereas *in vitro* they are also subjected to a programmed necrosis-like cell death, termed nemosis, associated with cyclooxygenase-2 (COX-2) expression induction and inflammatory response [1,2]. Moreover, fibroblasts form clusters during wound healing, fibrotic states and tumorigenesis.

In this investigation, we produced and analysed clusters such as spheroids from human primary cutaneous myofibroblasts to evaluate apoptotic or necrotic cell death, inflammation and activation markers during myofibroblasts clustering. The spheroids formation does not induce apoptosis, necrotic cell death and COX-2 protein induction. The significant decrease of α-SMA in protein extracts of spheroids, the cytostatic effect exerted by spheroids conditioned medium on both normal and cancer cell lines and the absence of proliferation marker Ki-67 after 72 h of three-dimensional culture indicated that myofibroblasts undergo a deactivation process within spheroids. The cells of spheroids, reverted to adhesion growth, preserve their proliferation capability and are able to reacquire a myofibroblastic phenotype. Furthermore, the spontaneous formation of clusters and spheroids on plastic and glass substrates suggests that aggregates formation could be a physiological feature of cutaneous myofibroblasts.

This study represents an experimental model to analyse myofibroblasts deactivation and indicates that fibroblast clusters could be a cell reservoir regulating tissue turnover.

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uL3 is a key regulator of oxidative stress response genes in multidrug resistant lung cancer cells

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Lung cancer is one of the most common causes of cancer-related death among adults. Chemotherapy is recognized as an important component of treatment for all stages of lung cancer and is crucial in determining patient survival and quality of life. However, intrinsic or acquired multidrug resistance (MDR) is the main reason for tumor recurrence continuing to pose a significant challenge in the management of cancer. We previously reported evidence of the role of uL3 (formerly rpL3) in mediating drug resistance showing that the resistance of A549 lung cancer cells to Cisplatin correlates to the loss of uL3 expression. In this study we identified a key role of uL3 in the control of the redox status conferring multidrug resistance to lung cancer cells lacking p53. We established and characterized a multidrug resistant Calu-6 lung cell line. We found that uL3 down-regulation correlates positively with multidrug resistance. Restoration of the uL3 protein level re-sensitized the resistant cells to the drug by regulating the reactive oxygen species (ROS) levels, glutathione content, glutamate release, and cystine uptake. Chromatin immunoprecipitation experiments and luciferase assays demonstrated that uL3 coordinated the expression of stress-response genes acting as transcriptional repressors of solute carrier family 7 member 11 (*xCT*) and glutathione S-transferase α1 (*GST-α1*), independently of Nuclear factor erythroid 2-related factor 2 (Nrf2). Altogether our results describe a new function of uL3 as a regulator of oxidative stress response genes and advance our understanding of the molecular mechanisms underlying multidrug resistance in cancers.

These data suggest the possibility of targeting uL3 to modulate the redox status of cancer cells for future therapeutic purposes in order to overcome MDR.

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The Lysophosphatidic Acid Acyltransferase (LPAATs) Enzymes and their Role in Membrane Transport Alterations in Cancer

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The 1-Acyl-Glycerol-3-Phosphate AcylTransferase enzymes (AGPATs) is an emerging class of enzymes, and the AGPAT2 member is a potential prognostic/diagnostic marker for different tumors. AGPAT2 is upregulated in several tumors and its increased expression/activity correlates with tumor aggressiveness. AGPAT2, also known as LysoPhosphatidic Acid AcylTransferase-β (LPAATβ), catalyses the acylation of lysophosphatidic acid to form phosphatidic acid, a phospholipid precursor involved in membrane transport and signalling for cell survival, proliferation and tumor progression[1]. Alteration in phospholipid membrane composition and enhanced cellular secretion are associated to tumor progression and migration/invasion. Indeed, during tumorigenesis the secreted factors of cancer cells are key actors in setting the microenvironment that leads to tumor progression.

In addition to AGPAT2, also AGPAT3, 4, 8 and 11 are up-regulated in cancer cells. AGPAT4, like AGPAT2, is a LPAAT enzyme, and we have identified AGPAT4 as an important controller of secretion in cancer cells[2]. AGPAT4 expression/activity increases in prostate cancer, and this correlates with enhanced tumor aggressiveness. Conversely, its depletion impairs cells migration. However, the role of AGPATs in the exocytic pathways that drive tumor progression and migration/invasion remains unknown.

We performed a proteomic approach to identify, in a sensitive, and at high-resolution manner, factors differentially secreted in the conditioned medium of prostate cancer *versus* non-cancer cells, by LC-MS/MS. Then, we have used the same differential proteomic approach to identify among these cancer-specific secreted factors those with reduced secretion under AGPAT4 depletion. Among them, we have found the human Growth Hormone and the membrane type 1- and type 9-matrix metalloproteases[3], which are all involved in invasion/migration.

The role of the identified AGPAT4-dependent cancer-secreted factors in tumor invasion/migration, are under investigation.

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Human acetyl-CoA carboxylase 1 mRNA translation is enhanced in HepG2 cells under endoplasmic reticulum stress, serum deprivation or hypoxia

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A wide range of biochemical, physiological and pathological stimuli have been associated to dysregulation of protein folding process which causes accumulation of unfolded or misfolded proteins in lumen of endoplasmic reticulum (ER), a condition referred to as ER stress. Among the responses raised by the ER stress, an induction of lipogenic genes expression has been observed. Activation of lipid synthesis in hepatocytes upon ER-stress contributes to the development of some liver disease such as steatosis. Acetyl-CoA carboxylase 1 (ACC1), encoded by ACACA gene, catalyzes the ATP-dependent carboxylation of acetyl-CoA to form malonyl-CoA. This reaction is the rate limiting step in the *de novo* fatty acid biosynthesis. The expression of ACC1 is dysregulated in metabolic disorders associated to obesity, diabetes, insulin resistance, and metabolic syndrome. The aim of this work was to deepen the regulation of ACC1 expression at translational level, characterizing the leader region of ACC1 mRNA. By using several approaches, in HepG2 cells treated with an ER stressor, such as tunicamycin or thapsigargin, we observed an increment of ACC1 expression, at transcriptional and translational level. An internal ribosome entry site (IRES), identified in the leader region of ACC1 mRNA, allows its translation through a cap-independent mechanism. Cellular stress conditions, such as serum starvation and hypoxia, caused an increase in the level of ACC1 protein in HepG2 cells, through the cap-independent translation mediated by IRES. All together, these findings indicate that the presence of IRES in the ACC1 5'-UTR allows translation of ACC1 mRNA under conditions that inhibit the cap-dependent translation.

Cladosporols A and B from *Cladosporium Tenuissimum* act as PPARγ ligands and inhibit adipogenesis in 3T3L1 cells

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Cladosporols, secondary metabolites from *Cladosporium tenuissimum,* have previously been characterized for their ability to control cell proliferation of human colon cancer cells through PPAR-mediated mechanisms of action (1, 2). More recently, we demonstrated that cladosporol B, an oxidate form of cladosporol A, acts as a PPAR partial agonist with lower affinity and reduced transactivation potential but sustained proapoptotic activity, as compared to the full agonists cladosporol A and rosiglitazone (3). We suggested that the lower transactivation potential, higher antiproliferative and sustained proapoptotic activity of cladosporol B can be ascribed to the different binding to PPAR.

Identification of PPAR partial agonists with no adverse side-effects as those reported for full agonists has received great attention for developing new therapeutical tools to manage obesity and diabetes. To this goal, we verified whether both cladosporols display regulatory properties in 3T3L1 preadipocytes by virtue of their differential binding to the PPAR binding domain.

We show here that cladosporols A and B hamper adipocyte differentiation and lipid storage through downregulation of mRNA and protein levels of early (C/EBPα, PPAR) and terminal (LPL, FAS, GLUT-4, Adiponectin, Leptin) differentiation markers. Analysis of stored triglycerides in 3T3L1 mature adipocytes and simultaneous release of glycerol and fatty acids in the culture medium upon cladosporols treatment indicate that both isoforms A and B inhibit lipogenesis. To our knowledge, this is the first report describing that cladosporol A and B, as PPAR natural ligands, inhibit adipogenesis *in vitro* through modulation of early and late differentiation gene expression.

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Acknowledgements
This work was supported by funds from DST-University of Sannio (FRA 2014 and 2015).

Membranes (M)

Fad Synthase (Fads) Localization and Function in Neuronal Cell Models: a possible Involvement of Fads in The Pathogenesis of Neurodegenerative Diseases

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BACKGROUND AND AIMS: FAD synthase (FADS, EC 2.7.7.2), coded in humans by *FLAD1* gene, is the last enzyme in the pathway converting riboflavin into the redox cofactor FAD, essential for the activity of hundreds of flavoenzymes. In non-neuronal cells FADS performs a citosolic, mitochondrial and nuclear localization (Giancaspero TA et al., 2013). Interestingly, a case report (Lin J et al., 2009) showed that in a ALS patient with an IgA gammopathy, the neuronal surface antigen was represented by FADS. Subcellular localization of FADS in human neuronal cells is still unknown. Also, a significant reduction of FADS mRNA levels was observed in the blood of ALS patients. Thus, here we aimed at studying the subcellular localization and the effect of altered expression levels of FADS on cellular bioenergetics in experimental models, i.e. neuronal cell models.

METHODS: Confocal and sub-fractionation studies were performed on human neuroblastoma (SK-N-SH) and mouse motor neuron (NSC-34) cell lines. After overexpression and silencing, FADS levels were assessed by RT-PCR and WB, while Riboflavin, FMN and FAD content was measured by HPLC. The effects of altered FADS levels on neuronal bioenergetics were evaluated by measuring oxygen consumption rate and levels of ATP, ROS and glutathione reductase.

RESULTS: Confocal data collected in neuronal cells showed a partial co-localization of FADS with a lipid raft marker and a high degree of co-localization with a vesicle marker in both the neuronal cell lines. Also, a clear toxic effect on bioenergetics was shown in FADS overexpressing SK-N-SH cells, as indicated by the specific reduction in complex I functionality.

CONCLUSIONS: These data straightforwardly demonstrate the localization of FADS in membrane domains of two neuronal cell models, suggesting a novel role for FADS in neuronal physiology and, possibly, in neurotransmission. The relationships with results collected in *other models* will be discussed.

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Membrane transporters as target of xenobiotics.

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The maintenance of cell homeostasis results from of a network of different pathways in which membrane transporters play fundamental roles. Indeed, the number of human pathologies related to defects of membrane transporter genes is increasing. Moreover, membrane transporters can be considered targets of drugs and xenobiotics. Indeed, these proteins can mediate absorption of drugs or can be targets of "off site" interactions. In the last case, transporters become responsible of drug side effects. The human genome accounts for almost 600 genes encoding membrane transporters. Therefore, the International Transporter Consortium (ITC) highlighted the need of systematic studies for revealing transporter-xenobiotics interactions. The most used experimental models are based on cell lines in which an exogenous transporter is transiently or stably over-expressed. An up to date tool for studying membrane transporters is the proteoliposome model. In this system, transporters extracted from cells or derived from heterologous expression are inserted in artificial membrane with the same orientation they have in cells.

An important advantage of this tool consists in reducing or abolishing the interferences due to other transporters or enzymes present in entire cells. This strategy has been applied to the study of interaction of heavy metals and potential drugs with transporters either extracted from rat tissues or obtained by recombinant techniques. The effect of Hg^{2+}, methyl-Hg, Cd^{2+}, Cu^{2+} and of some dithiazole derived organic compounds has been evaluated on plasma membrane carnitine (OCTN2), acetylcholine (OCTN1) and neutral amino acids (ASCT2, B0AT1 and LAT1) transporters. The tested xenobiotics were strong inhibitors of some or all the transporters with a common mechanism based on covalent bonds with Cys residues. This structural alteration contributes to the well-known toxicity of the metals. Indeed, the transporters underneath hold variable number of Cys residues, potential targets of SH-reagents. These studies contributed in understanding the molecular bases of heavy metal toxicity. A further interesting outcome is the finding that the antioxidant scavenger N-acetyl-cysteine is able to revert the inhibition suggesting its use in detoxification. The transporters ASCT2 and LAT1 are strongly inhibited by the SH reacting organic compounds with potential pharmacological application

Is the Pseudoxanthoma Elasticum (PXE) a purinergic disease?

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Mutations in the human *ABCC6* gene, a member of ABCs protein superfamily that encodes MRP6 protein which is involved in multi-drug resistance phenomena [1], cause Pseudoxanthoma Elasticum (PXE), a multisystemic disorder characterized by progressive ectopic calcification of the mineralized elastic fibers in dermal, ocular and vascular tissues [2]. MRP6 is mainly present in liver and in kidney, especially in the basolateral plasma membrane of hepatocytes, and it was found that MRP6 is involved in the release of ATP out of the cell. ATP is immediately hydrolyzated in adenosine and in PPi (a mineralization inhibitor) by ENPP1 and CD73 proteins [3]. In order to characterize the role of *ABCC6* in hepatic cells and in the physiopathology of PXE, *ABCC6* gene expression was silenced in HepG2 cells. In HepG2 cells with silenced *ABCC6*, a variation of expression in genes involved in mineralization processes and a downregulation of *NT5E* gene have been observed [2]. In this work the effects of an inhibitor of ABC proteins activity in HepG2 cells have been evaluated. The results obtained show that, in the presence of this inhibitor, both CD73 and MRP6 protein levels are downregulated, while TNAP protein level is unchanged. In order to characterize the effects of the inhibition of purinergic pathway due to MRP6 low activity, the HepG2 cells were treated with this inhibitor in the presence and in the absence of adenosine. The results obtained show that there is a variation of genes involved in mineralization processes regulated by the purinergic pathway, such as *NT5E* and *TNAP*.

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Bax activation blocks self-renewal and induces apoptosis of human glioblastoma stem cells

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Glioblastoma (GBM) is characterized by a poor response to conventional chemotherapeutic agents, attributed to the insurgence of drug resistance mechanisms and to the presence of a subpopulation of glioma cancer stem cells (GSCs)[1]. GBM cells and GSCs present an overexpression of anti-apoptotic proteins and an inhibition of pro-apoptotic ones, which help to escape apoptosis. Among pro-apoptotic inducers, the Bcl-2 family protein, Bax, has been recently emerged as a promising new target in cancer therapy along with the first Bax activators (BAM7, Compound 106 and SMBA1)[2]. Herein, the effects of Bax activation (by the BAM7 derivative BTC-8)[3] were explored in human GBM cells and in their derived-stem cell subpopulation. Bax activation caused an inhibition of GBM cell proliferation, arrested cell-cycle arrest and induced apoptotis through the induction of mitochondrial membrane permeabilization.

Most importantly, Bax activation decreased proliferation and self-renewal of GSCs, and induced their apoptosis. These data shed light on the important role of the protein activation not only in classified tumors but also in their stem cell component.

Noteworthy, Bax activation sensitizes GBM cells and GSCs to the standard alkylating agent temozolomide. Such findings indicate that affecting two different targets (i.e. nuclear DNA and mitochondrial Bax) can enhance toxicity against tumor cells.

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Funding sources: The study was supported by PRIN2015FCHJ8E.

Mechanisms of CtBP-1/BARS-mediated mitotic Golgi fragmentation

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One of the control mechanisms that regulate the correct cell cycle progression is the so-called "Golgi mitotic checkpoint". During mitosis the Golgi complex undergoes extensive fragmentation through a multistage process that allows its correct partitioning into the daughter cells. This processes is required not only for correct cell inheritance, but also for mitotic entrance itself, since its block results in the arrest of the cell cycle in the G2 phase[1]. We have identified CtBP1-S/BARS as key controller of the Golgi ribbon unlinking during mitosis[2]. Moreover, CtBP1-S/BARS controls also the membrane fission processes required during several intracellular trafficking pathways such as: formation of basolateral post-Golgi carriers, fission of COPI-coated vesicles, macropinocytosis and fluid-phase endocytosis[3].

The CtBP1-S/BARS-complex components involved in membrane fission have been identified and characterized[2]. This complex comprises CtBP1-S/BARS bridged to PI4KIIIβ by a 14-3-3γ dimer and includes also ARF, PLD1/2 and the two stabilizing kinases PKD and PAK[3]. Once incorporate into this complex, CtBP1-S/BARS binds to and activates two Golgi localized lysophosphatidic acid (LPA) acyltransferase enzimes, namely LPAAT3 and LPAAT4[4]. In order to define the molecular mechanisms underlying the CtBP1-S/BARS-mediated Golgi fragmentation during mitosis, we are studying the role of these protein-complex components in cell-cycle synchronized HeLa cells. The specific depletion of CtBP1-S/BARS, or LPAAT3, or LPAAT4, or PAK1/2, or PLD1/PLD2 complex-components strongly inhibits the mitotic Golgi fragmentation (although to different extents) revealing the relevance of this CtBP1-S/BARS protein-complex in the mitotic Golgi partitioning.

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Mitochondrial ATP-Mg/phosphate carriers transport divalent inorganic cations in complex with ATP

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The ATP-Mg/phosphate carriers (APCs) modulate the intramitochondrial adenine nucleotide pool size. In this study the concentration-dependent effects of Mg^{2+} and other divalent cations (Me^{2+}) on the transport of [^{3}H]ATP in liposomes reconstituted with purified human and Arabidopsis APCs (hAPCs and AtAPCs, respectively, including some lacking their N-terminal domains) have been investigated. The transport of Me^{2+} mediated by these proteins was also measured. In the presence of a low external concentration of [^{3}H]ATP (12 μM) and increasing concentrations of Me^{2+}, Mg^{2+} stimulated the activity (measured as initial transport rate of [^{3}H]ATP) of hAPCs and decreased that of AtAPCs; Fe^{2+} and Zn^{2+} stimulated markedly hAPCs and moderately AtAPCs; Ca^{2+} and Mn^{2+} markedly AtAPCs and moderately hAPCs; and Cu^{2+} decreased the activity of both hAPCs and AtAPCs. All the Me^{2+}-dependent effects correlated well with the amount of ATP-Me complex present. The transport of [^{14}C]AMP, which has a much lower ability of complexation than ATP, was not affected by the presence of the Me^{2+} tested, except Cu^{2+}. Furthermore, the transport of [^{3}H]ATP catalyzed by the ATP/ADP carrier, which is known to transport only free ATP and ADP, was inhibited by all the Me^{2+} tested in an inverse relationship with the formation of the ATP-Me complex. Finally, direct measurements of Mg^{2+}, Mn^{2+}, Fe^{2+}, Zn^{2+} and Cu^{2+} showed that they are cotransported with ATP by both hAPCs and AtAPCs. It is likely that *in vivo* APCs transport free ATP and ATP-Mg complex to different degrees, and probably trace amounts of other Me^{2+} in complex with ATP.

Palmitate lipotoxicity in enteric glial cells

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The enteric nervous system is a complex neural structure devoted to the control of nutrients uptake and gut functions, which is mainly represented by two cell populations, neurons and enteric glial cells (EGCs). EGCs are components of the intestinal epithelial barrier that can be targeted by pathogens, with the opportunistic aim to affect the cell fate. Following bacterial insult by *C. difficile*, the produced enterotoxin B can cross the mucosal membrane and reach EGCs, due to breaching of the intestinal epithelial barrier. Here it exerts cytopathic and cytotoxic effects and activates a NADPH oxidase/ROS/JNK/caspase-3 axis, leading to death a restricted EGCs pool[1]. Disrupted intestinal epithelial barrier could favor access to EGCs of digestion products that possess an intrinsic toxic effect, such as saturated fatty acids.

In this work, we evaluated the lipotoxic effect of fatty acids on EGCs by incubating cells with palmitate (PA) or linoleate (LA) in complexes with albumin. PA, but not LA, decreased cell viability. Nile red staining of cells evidenced lipid droplets accumulation in both treatments, as the result of increased triacylglycerol content, determined by TLC analysis of lipids. Among polar lipids, increased phosphatidic acid, and decreased cardiolipin levels were found only in PA-treated cells. Moreover, MALDI-TOF mass spectrometry indicated that phosphatidic acid was enriched in PA-containing species, which justifies the decreased cardiolipin level, due to low specificity of cardiolipin synthase towards saturated precursor species. PA lipotoxicity is exerted through a respiratory burst and the decrease of mitochondrial membrane potential. Altogether, data support the conditions for cyt c release in the intermembrane space to be poured outside mitochondria for caspase-3 activation. The molecular mechanisms of cyt c release in the cytosol do not involve MPT, but rather the overexpression of BIM, through ROS/JNK axis.

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Mitochondrial cAMP prevents apoptosis of cardiac myoblast cells modulating Sirt3 protein level and OPA1 processing

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Mitochondria, responding to a wide variety of signals, including oxidative stress, are critical in regulating apoptosis that plays a key role in the pathogenesis of a variety of cardiovascular diseases. A number of mitochondrial proteins and pathways have been found to be involved in the mitochondrial dependent apoptosis mechanism, such as optic atrophy 1 (OPA1), sirtuin 3 (Sirt3), deacetylase enzyme and cAMP signal. cAMP can be produced inside and outside mitochondria by, respectively, a soluble adenylyl cyclase (sAC), present in the mitochondrial matrix, and transmembrane adenylyl cyclases in the cellular plasmamembrane. sAC-dependent cAMP production in mitochondria was shown to regulate cytochrome c oxidase activity (1), ATP production (2), the turnover of nuclear-encoded subunits of complex I (3) and to regulate the functional activity and structural organization of the ATP synthase (4).

In the present work we report a network among OPA1, Sirt3 and cAMP in ROS-dependent apoptosis. Rat myoblastic H9c2 cell lines, were treated with tert-butyl hydroperoxide (t-BHP) to induce oxidative stress-dependent apoptosis. FRET analysis revealed a selective decrease of mitochondrial cAMP in response to t-BHP treatment. This was associated with a decrease of Sirt3 protein level and proteolytic processing of OPA1. Pretreatment of cells with permeant analogous of cAMP (8-Br-cAMP) protected the cell from apoptosis preventing all these events. Using H89, inhibitor of the protein kinase A (PKA), and protease inhibitors, evidences have been obtained that ROS-dependent apoptosis is associated with an alteration of mitochondrial cAMP/PKA signal that causes degradation/proteolysis of Sirt3 that, in turn, promotes acetylation and proteolytic processing of OPA1.

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Nutrition and Environment (NE)

Neuroprotective effect of different virgin olive oil extracts in SH-SY5Y cells

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Neurodegenerative diseases such as Alzheimer's disease and Parkinson's disease are the largest growing area of neurological research still in search of an effective therapy. Virgin olive oil is a rich source of phenolic compounds that have been demonstrated to interfere with different mechanisms involved in neurodegenerative disorders[1]. Olive oil polyphenols can be divided into three categories: secoiridoids such as oleocanthal, simple phenols such as hydroxytyrosol and lignanes. Here, we evaluated the neuroprotective effect of four different virgin olive oil extracts against neurodegeneration by determining their effect in modulating oxidative stress and pro-survival pathways. The different olive oil extracts were characterized for their content in tyrosol, hydroxytyrosol, oleacein and oleocanthal by HPLC. SH-SY5Y cells were treated with different concentrations (1-10 µg/mL) of the extracts. Cell viability was evaluated by MTT assay and the results showed that three of the tested extracts significantly increased cell viability. Furthermore, two of these extracts decreased basal reactive oxygen species level measured by dichloro-dihydro-fluorescein diacetate (DCFH-DA) assay. Interestingly all the extracts increased GSH level measured by monochlorobimane (MCB) assay. These extracts were also able to modulate phase II antioxidant enzymes and MAPK signalling pathways as measured by RT-PCR and immunoblotting. Our findings support the idea that virgin olive oil has a beneficial health effect in counteracting neurodegeneration and these effects are strongly related to the specific pattern of olive oil phenols.

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Synthetic and Natural Avenanthramides from Oat: Antioxidant, Anti-proliferative and Anti-inflammatory Activities

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Oats (*Avena sativa* L.) are a source of unique phenolic compounds, termed avenanthramides (AVNs), that exhibit antioxidant, anti-inflammatory and anticancer activities [1]. The three most important AVN forms, out of 30, are constituted by 5-hydroxyanthranilic acid linked to *p*-coumaric (**2p**) or ferulic (**2f**) or caffeic acid (**2c**). In our recent work [2], we purified an AVN-enriched mixture (**n-MIX**), containing the three forms, from oat sprout and demonstrated that the n-MIX was able to activate the extrinsic apoptotic pathway in colon cancer (CaCo-2) and hepatocarcinoma cells (HepG2). The aim of this study was to compare the biological effects of the synthetic AVNs (s-2c, s-2p, s-2f) with those exerted by the n-MIX.

Antioxidant capacity was evaluated through the Oxygen Radical Absorbance Capacity (ORAC) method, as well as through the intracellular antioxidant activity (DCFH-DA assay). The s-2c showed the highest ORAC value, whereas the DCFH-DA assay provided similar values for all AVNs.

Anti-proliferative and pro-apoptotic effects were evaluated through the Sulforhodamine B assay and caspase 8, 3, 2 activity assays, respectively, on CaCo-2 and Hep3B cells. On CaCo-2 cells, the s-2c showed the highest cytotoxic effect, whereas on Hep3B cells, no significant difference was observed among the s-AVN and n-MIX. As regards the pro-apoptotic effect, on both CaCo-2 and Hep3B cells, n-MIX and s-AVNs were able to activate the caspases 8 and 3, whereas only n-MIX and s-2c were able to increase the caspase 2 activity level.

Anti-inflammatory effect was evaluated through the COX-2 activity assay and the RTqPCR assay. n-MIX and s-2c were able to reduce the COX-2 mRNA and activity levels in CaCo-2 cells; otherwise only n-MIX was able to downregulate COX-2 expression and activity in Hep3B cells.

In conclusion, both synthetic and natural AVNs are able to provide a pleiotropic anticancer effect, which is worthy to be investigated in *in vivo* studies.

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Glycoxidative Stress and Paraoxonase-2 in Intestinal Cells: Effect of Apple Polyphenols

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Oxidative stress is one of the primary processes underlying the initiation and progression of inflammation and tissue injury in inflammatory bowel disease (1). Under physiological conditions, the balance between reactive oxygen species (ROS) generation and ROS scavenging is tightly controlled in intestinal cells. Elevated plasma glucose levels and advanced glycation end products (AGEs) generate free radicals and can cause inflammation and cell damage.

Apple fruit antioxidant effect has been related to high polyphenol content. The aim of the study was to investigate the role of apple polyphenolic extracts (*Calville White Winter cultivar)* on glycoxidation in intestinal Caco-2 cells. In particular, we studied the expression and activity of the antioxidant enzyme paraoxonase-2 (PON2) in cells exposed to high-glucose (HG) stress in the absence and in the presence of apple extract. PON2, a member of the multigene family of paraoxonases, is expressed in all human tissues. The enzyme localizes in mitochondria and cell membrane and exerts a protective role against ROS within cells (2). Therefore, PON2 may be involved in the antioxidant and anti-inflammatory response in intestinal cells (3). Our results showed a lower cell viability and higher intracellular ROS and AGEs formation in HG-treated cells. A significant decrease in PON2 protein levels and activity was observed in cells treated with high glucose. Treatment with apple extract reduced glycoxidative stress and induced a significant increase of PON2 levels and activity.

The intestine is highly vulnerable to glycoxidative damage due to its constant exposure to high glucose, AGEs and oxidants from ingested nutrients. A diet rich in apple antioxidants might prevent or delay cell oxidative stress and inflammation, by increasing PON2 activity and reinforce cell antioxidant defence.

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The uncommon redox mechanism of ergothioneine

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Ergothioneine (ESH), the betaine of 2-mercapto-L-histidine, is a water-soluble naturally occurring amino acid with antioxidant properties. Animals and humans, which absorb ESH only via their respective food chains, accumulate this compound in certain tissues at relatively high concentrations. Some foods, including several species of mushrooms, black and red beans, red meat, liver, kidney, and grains possess particularly high levels of ESH. Numerous evidence demonstrated the antioxidant and cytoprotective effects of ESH, including protection against cardiovascular disease, chronic inflammatory conditions, ultraviolet radiation damages, and neuronal injuries. Although more than a century after its discovery has gone by, our understanding on the *in vivo* antioxidant mechanism of ESH is limited, as it does not seem to provide any advantage compared to alkylthiols, which are better reducing agents. This study was designed to investigate the products of ESH oxidation formed by neutrophils during oxidative burst, a source of hypochlorite in humans. Furthermore, we also tested other biologically relevant oxidants, such as peroxynitrite and hydrogen peroxide. Results from ESI-MS analyses show that treatment of human neutrophils with phorbol 12-myristate 13-acetate in the presence of ESH leads to a remarkable production of the sulfonated form (ESO_3H), a compound never described before, and hercynine (EH), the desulfurated form of ESH. Similar results were obtained when ESH was subjected to cell-free oxidation in the presence of hypochlorite, as well as hydrogen peroxide or peroxynitrite. Interestingly, these oxidation products were also found when the disulfide of ESH was reacted with those oxidants. These data reveal a unique ESH redox behavior, entirely different from that of alkylthiols, suggesting a novel mechanism through which ESH acts as an antioxidant in cells.

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Quercetin supplementation decreases erythrocytes oxidative damage at resting and after an acute bout of eccentric exercise in humans

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Quercetin (Q) has been shown, in vitro and in murine models, a wide range of biological actions however, its effect on redox status has been minimally examined in combination with exercise in humans. The purpose of this investigation was to evaluate if Q ingestion 1g/day for 2 weeks would result in an anti-hemolytic effect and would increase red blood cells (RBCs)/plasma antioxidant measures as well as attenuate increases in exercise-induced oxidative damage.

Fourteen volunteer males were randomly assigned, in a double-blind crossover design, to a placebo or Q groups. Blood samples were taken under resting conditions, after 2 weeks of Q/placebo supplementation, and after an acute bout of eccentric exercise (EE). Glutathione homeostasis (GSH, GSSG, GSH/GSSG), thiobarbituric acid reactive substances (TBARs), enzyme antioxidant activities (CAT, SOD GPx) as well as AAPH–induced oxidative hemolysis were evaluated.

After Q supplementation, the time to reach 50 % of hemolysis increased compared to placebo (T50, +13.7 minutes). Evaluation of TBARs levels showed a decrease in RBCs (-26.21%) compared to placebo. No differences were found in RBCs CAT, SOD and GPx activities as well as in RBCs/plasma redox status. After EE, quercetin increased T50, reduced TBARs as well as reduced the GSSG increase exercise-induced.

Q supplementation before and after a strenuous eccentric exercise improves RBCs resistance to AAPH-induced oxidative stress and decreases lipid peroxidation therefore making cells more able to cope to oxidative insult. This effect do not involve an up-regulation of endogenous antioxidant processes.

Valorization of olive mill wastewaters by membrane processes to recovery natural antioxidant compounds for cosmeceutical and nutraceutical applications

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Olive trees play a vital role in the economy, ecology and social life of Mediterranean countries. In fact it is the principal fat source of the traditional Mediterranean diet that has been associated with a low incidence of coronary heart disease [1]. Unfortunately, olive oil production also generates a huge quantity of wastewaters. Neverthless, OMWs may become an important source for the extraction of natural antioxidants such as polyphenols [2].

The objective of this work was to reach the optimization of a downstream process for the recovery of the polyphenols from the olive mill wastewaters to evaluate potentialities of polyphenols as cosmeceutical active ingredients on human dermal cells *in vitro*. The processing started with an initial flocculation step, then a downstream process was developed based on the use of ultra and nanofiltration membranes. The mills wastewater were then used for the extraction of biologically active components (polyphenols with antioxidant activity) and for the recovery of purified water to be potentially reused within the same manufacturing processes. The nanofiltration permeate showed a reduction of about 95% of the organic load with a COD values ranging from 1500-6000 mg/kg lower than the initial wastewater. The polyphenols recovery after two filtration steps was of about 65%. The nanofiltration residues were freeze dried using the spray dryer technique and tested on human epidermal cells *in vitro* to evaluate potentialities as cosmeceutical active ingredients, and improving cell reparation in scratch assays assisted through time lapse video-microscopy. In particular, MTT test was performed after oxidative stress induction using human immortalized keratinocytes (HaCaT) as epidermal cell model *in vitro*. MTT test also allow us to select the best concentration to test for evaluating the bio-revitalizing effect of these samples performing a scratch assay on HaCaT monolayers and evaluating reparation rate through time lapse video-microscopy.

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This project was financed under the measure 124 of the PSR Campania 2007/2013 (Project "*VALORE"*) and by MIUR PON03PE_00060_3 entitled "*Development and Testing of molecules with nutraceutical and cosmeceutical properties*".

Phytochemical profile, antioxidant and anti-ageing properties of *Citrus bergamia* juices[a]

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Ageing is one of the main risk factor in the developing cardiovascular diseases. The increase of oxidative stress associated with ageing can be overcome with a high intake of food antioxidants (1). In this context, a number of studies have been addressed to assess the anti-ageing potential of antioxidant natural compounds. Recently, it has been shown that bergamot juice ('Fantastico" cultivar), a fruit mostly produced in the Ionian coastal areas of Calabria (Italy), possesses a good quality and a valuable source of health promoting constituents, contributing to its antioxidant properties and cholesterol reduction capacities (2,3). In order to investigate potential anti-ageing effects of this natural antioxidant source from the Mediterranean area, fresh bergamot juices of three different cultivars ('Fantastico', 'Femminello', and 'Castagnaro') were herein investigated for the evaluation of total polyphenolic (TPC) and flavonoid (TFC) contents, the high performance liquid chromatography-photodiode array-electrospray ionization-tandem mass spectrometry (HPLC-PDA-ESI-MS/MS) profiles, in tube antioxidant activities and *in vitro* anti-ageing properties on myocardial H9c2 subclonal cell line derived from embryonic rat hearts.

Each cultivar showed different phenolic and flavonoid contents and the highest values, obtained by *Folin-Ciocalteu* (for TPC) and *aluminum complex formation* (for TFC) tests, were found in 'Fantastico' cultivar. The HPLC-PDA-ESI-MS/MS profiles confirmed that juices were rich in flavonoids, both flavone and flavanone glycosides. In addition, two limonoid glycosides were also identified in all cultivars. Moreover, the *in tube* and *in vitro* results showed that 'Fantastico' cultivar juice possesses the strongest antioxidant activity and counteracts chemical-induced senescence in myocardial H9c2 cells. The overall results support the hypothesis that the 'Fantastico' cultivar possesses beneficial health effects that could make it useful for nutraceutical purpose.

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Relevance of oleic acid and hydroxytyrosol, main compounds of extravirgin olive oil, in the inhibition of cholesterol and fatty acid biosyntheses in C6 glioma cells

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Extra virgin olive oil (EVOO), the principal source of fat in the Mediterranean diet, positively affects human health, reducing the incidence of cancer, hypertension and cardiovascular diseases. Clinical studies support the efficacy of the EVOO also against the onset and progression of a number of neurodegenerative diseases. Brain fatty acid and cholesterol syntheses are critically challenged in several of these diseases.

The finding that natural compounds are capable of modulating nervous system function has revealed new perspectives for an healthier brain. We investigated the effects of oleic acid (OA) and hydroxytyrosol (HTyr), two important EVOO compounds, on lipid synthesis in C6 glioma cells. Experimental data demonstrated that OA and HTyr inhibited within 4 h both de novo fatty acid and cholesterol syntheses; the inhibitory effect was more pronounced when OA and HTyr were administered in combination, revealing a synergic action. A remarkable reduction of polar lipids, but not of triglycerides biosynthesis was also observed in OA- and HTyr-treated C6 cells.

The effects of OA and HTyr on the activity of the key enzymes of fatty acid biosynthesis, (acetyl-CoA carboxylase-ACC and fatty acid synthase-FAS) and cholesterologenesis (3-hydroxy-3-methyl-glutaryl-CoA reductase-HMGCR) were investigated in C6 cells. ACC and HMGCR activities were reduced by OA and HTyr treatment. No change in FAS activity was observed. The inhibition of ACC and HMGCR activities, observed in OA- and HTyr-treated C6 cells, is corroborated by the decrease in the corresponding mRNA abundance and protein level. Overall, our data indicate a direct and synergic effect of OA and HTyr on lipid synthesis in C6 cells. The modulation of the activity and expression of the key enzymes of lipid synthesis suggests a putative role of the two EVOO bioactive compounds in the prevention of several neurological diseases.

Vegetable waste biomass as a renewable source of value-added polyphenols and polysaccharides

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Vegetable biomass is one of the most promising renewable sources of energy and chemicals. Indeed, its exploitation for biofuels and chemicals production has been the focus of research over the last years since the gradual shift toward the so-called bio-based economy (an economy system based on exploitation of renewable resources), has determined a global increasing demand for goods mainly derived from vegetable biomass. In line with the biorefinery approach for the re-use of waste biomass, the residues of industrial processing of lemon (Citrus limon), tomato (Lycopersicon esculentum var. ''Hybrid Rome''), fennel (Foeniculum vulgare var. dulce) and carrot (Daucus carota), were used as sources of two kinds of value added-products i.e polysaccharides and polyphenols. Here we described the main properties of the polyphenol extracts and the polysaccharides fractions that could be obtained by each selected waste biomass. Both the polyphenol and polysaccharides fractions were investigated for their chemical composition and for the biological and biotechnological properties.

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Natural antioxidants to improve cell culture conditions of amniotic fluid stem cells

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Human amniotic fluid stem cells (AFSCs) are a powerful source of cells with a plethora of trophic and immunomodulatory functions. These cells can be appropriately expanded *ex-vivo* to be differentiated in many cell types, in order to dissect developmental pathways, unravel pathogenic mechanisms and replace defective tissues. Traditionally, *in vitro* AFSCs are exposed to non-physiological concentrations of O_2 increasing the production of reactive oxygen species (ROS). The unbalance in the redox signals may impair the AFSC stemness machinery[1]. In this context the use of natural antioxidants could be proposed as a strategy to prevent the loss of self-renewal and the extensive cellular damage due to abnormal ROS production. To this aim, AFSCs were exposed to sulphorafane (SF) and epigallo-3-catechingallate (EGCG) to counteract ROS deleterious effects. AFSCs were co-treated with 1 μM SF and 10 μM EGCG. After 72 h a slight increase in their metabolic activity was observed. However, cell population doubling over a cell culture period of 25 days was not different between untreated and co-treated samples. Interestingly, the co-treatment with SF and EGCG synergistically reduced intracellular ROS level in respect to SF or EGCG treatment alone, as measured by DCFH-DA assay. In addition, co-treated AFSCs displayed higher total GSH level in respect to the untreated samples. Self-renewal capacity was investigated evaluating the expression of pluripotent gene markers such as OCT4, NANOG and SOX2 by RT-PCR. Interestingly, the co-treatment was able to up-regulate the expression of all the three genes. Moreover, the chronic treatment for 21 days with SF and EGCG counteracted the expected decrease of OCT4 and NANOG expression during cell passaging.

In conclusion, we propose the co-treatment with SF and EGCG as a strategy to counteract oxidative stress and maintain the functionality of AFSCs. Supported by Fondazione Cassa di Risparmio di Bologna (Italy)

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Nutritional values and chemical composition of Eurasian woodcock (*Scolopax rusticola* L.) meat

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Meats from birds are rich sources of proteins for human diet and represent an attractive alternative to red ones. At present, many birds are domesticated and farmed commercially for food, while others are hunted for the same purpose (1). Indeed, the consumption of meat from game birds (common quail, common pheasant, wild duck, wood pigeon and woodcock) has gained increasing favor among consumers, which appreciate its texture and flavor, as well as the low content in saturated fatty acids and cholesterol.

The Eurasian woodcock (family Scolopacidae) is a medium-small wading bird found in temperate and subarctic Eurasia, hunted as game bird in many countries and considered one of the best meats for culinary purposes (2). Despite this meat is appreciated, until today there is the lack of information about its nutritional values and chemical composition. Therefore, the present study was designed to evaluate the nutritional aspects of the meat from this bird.

Thus, nutritional values, amino acids (free and total) and fatty acid composition of Eurasian woodcock meat were carried out on fresh weight basis and compared with those of chicken meat (3).

Woodcock meat contains high levels of proteins (22.5 g/100 g) and essential amino acids (6.3 g/100 g). Furthermore, the levels of unsaturated fatty acids (1.3 g/100 g) give a great contribute to the total lipid amount. Oleic acid (0.97 g/100 g) is the most abundant among monounsaturated fatty acids, while the mayor essential PUFA is linoleic acid (0.19 g/100 g).

The information presented in this study, suggested that woodcock meat is a nutritionally dense food, representing a rich source of macronutrients with high nutritional value.

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A new antioxidant system for the human intestinal mucosa: the archaeal peroxiredoxin Bcp1 adsorbed to spore of *Bacillus megaterium*

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Peroxiredoxins (Prxs), are ubiquitous thiol peroxidases involved in reduction of peroxides. It has been showed that the prokaryotic Prxs display a greater structural robustness than their eukaryotic counterparts making them less prone to inactivation by over-oxidation (1). Bcp1 is an archaeal peroxiredoxin of *Sulfolobus solfataricus* (1) previously characterized that works both under physiological pH and temperature conditions typical of eukaryotes, and it can use heterologous recycling system Thioredoxin reductase (TrxR) / Thioredoxin (Trx) from yeast (2). These features have inspired the exploration of this enzyme as new antioxidant to be used as possible therapeutic BioDrugs in association with spore for oral admistration (3). The use of the oral route for BioDrugs is still limited, mainly because of drug susceptibility to proteases, endonucleases and to the extremes of pH of the gastrointestinal (GI) tract and because of the lack of efficient mucosal delivery systems.

With the aim to develop a suitable system to deliver antioxidant in the gut, a Bcp1-spore system using *Bacillus megaterium*, was set up. We evaluated the stability of binding of enzyme to the spore surface and the stability of spore-bound enzyme in the presence of different artificial gastric juice formulations and artificial intestinal fluid. Preliminary results show both the binding of Bcp1 with *B. megaterium* spore, and the protection of the enzyme.

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17β-estradiol mediated potentiation of sulforaphane effects on cardiac cells

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Different studies demonstrated that female gender is associated with improved heart failure survival, and estrogens seem to play a fundamental role in this protection. It has been suggested that 17 β-estradiol (E2) reduces apoptosis in animal model of myocardial infarction through the modulation of MAPKs involved in oxidative stress[1]. Moreover recent evidences suggest that gender can influence the response to cardiovascular medications[2,3]. Therefore, we hypothesized that sex hormones could also modulate the cardioprotective effects of nutraceutical compounds, such as the isothiocyanate sulforaphane (SF), present in *Brassica* vegetables. In a previous study we evidenced that low concentrations of SF, in the presence of E2, counteracted oxidative damage better than SF alone. This effect seems to be related to the up-regulation of several antioxidant enzymes (GST, HO-1, GR, Trx, NQO1) and to the activation of pro-survival pathways (PI3K/Akt and ERK1/2). Aim of this study was to better clarify the mechanisms of E2-mediated potentiation of SF effects. H9c2 cardiomyoblasts were treated with SF in the absence/presence of E2 for different times. After 6h treatment, the co-treatment with SF and E2 induced a higher activation of Nrf2 transcription factor in respect to SF treatment alone. The study the involvement of E2 in SF induced Akt activation specific agonists for estrogen receptors were used. GPR30 (G protein-coupled receptor 30) and ER-ß (estrogen receptor beta) emerged to be involved in Akt activation. Moreover the co-treatment increased free $[Ca^{++}]_I$ evaluated by Fura-2 probe suggesting its involvement in E2 mediated effects. Our results increase the knowledge on E2 mediated potentiation of SF effects in cardiac cells.

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Hydrocolloid-based coatings to prevent acrylamide formation in fried foods

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Acrylamide (Acry) is formed when certain foods are prepared at temperatures above 120°C and low moisture, especially carbohydrate-rich foods containing free asparagine and reducing sugars (such as french fries, potato chips, crisps, crispy bread, breakfast cereals, and biscuits). By comparing the data on the cancer-causing potential of Acry to dietary exposure, the EFSA's experts in 2015 concluded that Acry in food is a health concern for consumers. Even if people could escape to Acry exposition not eating such kind of foods, it is well known that fried and baked foods are preferred in many countries and cultures. Thus, it could be useful to find a way to reduce Acry food content to preserve human health. Recently, the use of hydrocolloid materials has been proposed to prevent the Acry formation in some foods, like banana and potato chips. Our group studies edible films and coatings made of hydrocolloids (proteins and/or polysaccharides) using transglutaminase (TG) to modify protein component to influence both mechanical and barrier properties. In fact, TG is able to produce inter- and/or intra-molecular ε-(γ-glutamyl)lysine isopeptide bonds covalently reticulating hydrocolloid edible films. The present study is aimed to assess whether or not edible films affect the formation of Acry in fried potatoes using coating solutions containing TG-modified proteins.

By the means of RP-HPLC, we measured the Acry content of fried samples. In particular, we demonstrate the effectiveness of coatings prepared in the presence of TG which are able to reduce the Acry content in fried potatoes up to 37% in comparison to uncoated samples, being more effective than coatings prepared in the absence of the enzyme. These results may be explained assuming that the protein reticulation produced by TG in the coated foods provides a higher barrier to water during food frying that is responsible for the Acry reduced formation.

Expression of mitochondrial pyruvate carrier in different metabolic conditions in HepG2 and HEK 293 cells

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Mitochondrial import of pyruvate is a central step which links cytosolic and mitochondrial intermediary metabolism and determines whether glycolysis is followed by mitochondrial oxidative phosphorylation, or by lactic fermentation. Genes encoding mitochondrial pyruvate carrier (MPC), a heteroligomeric complex composed of MPC1 and MPC2 subunits, were identified [1][2]. Lower level of MPC protein expression has been correlated to poor survival in different types of tumors [3] and to higher expression of the stem cell markers [4]. In order to further explore the role of MPC in metabolism of cells, we evaluated correlations between growth with different fuels and the expression of both subunits of this transporter in HEK293 and HepG2 cell lines. In particular, we investigated the relationship between MPC activity and histone acetylation levels. Our results suggest that modulation of MPC expression is involved in the metabolic reprogramming of cells.

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Bioactive peptides from buffalo milk

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Food-derived constituents represent important sources of several classes of bioactive compounds. Among them peptides have gained great attention in the last two decades thanks to the scientific evidence of their beneficial effects on health in addition to their established nutritional value. It is now assessed that milk contains physiologically bioactive peptides, which can be both endogenous and released during gastrointestinal digestion or food processing. Several physiological functions are assigned to those peptides including antioxidative, antihypertensive, anti-inflammatory, antimicrobial and immunomodulating activity. Food peptidomics is a sub-field of proteomics that has recently had a significant boost. This technique deals with the identification and quantification of nutritionally relevant peptides that can individually or in combination be bioactive. Bioactive peptides are a key category of molecules for functional food application. Most known bioactive peptides are small (less than 5 amino acids) and hence represent a challenge in terms of analysis when using current proteomics techniques.

In this study we analyzed various samples from buffalo milk in order to identify and characterize bioactive peptides. The process included screening of endogenous peptides isolated from the whole fresh milk by centrifugation and filtration steps. Peptides were analyzed by a HPLC ion trap mass spectrometer. Peptides were identified by fragments characterization of MS/MS spectra through MASCOT search engine using Swiss-Prot and biopep (http://www.uwm.edu.pl/biochemia/index.php/pl/biopep) databases.
Results indicated the presence of 60 endogenous peptides from buffalo milk of which, 6 reported to possess an angiotensin-converting-enzyme inhibitory activity, 5 antibacterial activity, 11 with immunomodulating activity, and 4 with anticancer activity. These results suggest that, similarly to cow milk, Buffalo milk is a rich source of bioactive peptides.

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Development of new nutraceutical formulations and their evaluation on *in vitro* simplified steatosis model

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Non Alcoholic Fatty Liver Disease (NAFLD) is the most common chronic liver pathology worldwide that among many concurrent factors, depends on the accumulation of intracellular fatty acids. So, fat diet is the most important external factor associated with the incidence of steatosis. Nowadays, only nutritional guidelines are indicated to reduce its progression. The aim of this study is to combine different nutraceutical compounds, mainly reported in literature as nutraceutical agents, to evaluate their synergistic effects on a hepatic steatosis *in vitro* model compared to their separate use. Human hepatocellular carcinoma cells (HepG2 line) were treated with a mixture of fatty acids (oleic and linoleic acid ratio 1:1) in order to induce *in vitro* steatosis. In particular, three different formulations based on vitamin E, silymarin, curcumin, choline, docosahexaenoic acid (DHA) and phosphatidylcholine were assayed. After the treatment, the amount of intracellular fat was evaluated by Oil Red staining. Peroxisome proliferator-activated receptor-γ (PPARγ) expression, closely correlated to lipid metabolism, was evaluated by western blot analysis. In addition, superoxide dismutase (SOD-2) and, malondialdehydes (MDA) in lipid peroxidation, were assayed as specific biomarkers. The oxidative stress damage, was efficiently faced by formulation 1 and formulation 3 that reduced SOD-2 expression, the former was also more effective on PPARγ activation. Taken together, our results proved that the formulations tested could be considered a suitable support to the dietetic regimen to face steatosis. New nutraceutical approaches may help to prevent the pathology and/or even to reduce fat deposits. This experimental design could be a promising approach supporting future clinical studies on the development of nutraceutical formulations targeting steatosis.

This work was supported by the national grant PON 01_1226 Nutrafast.

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EFFECT OF SULFURAPHANE ON AQUAPORIN-8 IN A LEUKEMIC CELL LINE

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Cancer cells are characterized by an increase in spatially localized reactive oxygen species (ROS) production and by an altered redox environment compared to normal cells. Consequently, signalling pathways that promote cell proliferation, survival, angiogenesis and metastasis are hyper-activated. In particular, hydrogen peroxide (H_2O_2) derived from NOX family is involved in various redox signal transduction pathways and the aquaporin 8 (AQP8) has been identified as a H_2O_2 transport facilitator across the plasma membrane. Recent evidence demonstrated that many tumor cell types express elevated level of aquaporin isoforms and highlighted a positive correlation between histological tumor grade and the AQP expression [1].

Sulforaphane (SFN), an isothiocyanate compound present in abundance in cruciferous vegetables, has been found to induce therapeutic effects against a wide array of malignancies in both experimental and epidemiological studies [2]. Therefore, this study aimed at the evaluation of the potential effect of SFN on the modulation of AQP8, NOX2 and p-VEGFR-2 expression in B1647 cell line, a model of acute myeloid leukemia. In fact, we previously demonstrated that AQP8 funnels NOX-derived H_2O_2, triggered by endogenously generated VEGF, which, in turn, provokes VEGFR-2 phosphorylation and the consequent modulation of many cellular activities, resulting in cell survival and proliferation [3]. As peroxiredoxin (Prx1) represents a member of the so called thiol-based antioxidant system that acts as redox switches to modulate redox signalling pathways, the effect of SFN on Prx1 expression was also evaluated.

Unravelling the role of AQP8 in cancer redox signalling, and the effect exerted by SFN on its modulation, can offer new potential target for anti-cancer therapy, suggesting the importance of dietary co-treatment.

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Spermidine, a New Cationic Plasticizer for Polysaccharide- and Protein-Based Films

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The use of bio-based polymers for manufacturing biodegradable/edible materials, both to protect pharmaceuticals and to improve the shelf life of food products, is greatly increasing. Among them, several plant polysaccharides and proteins, representan abundant, inexpensive and renewable raw source. In addition, a major component of such hydrocolloid films is the plasticizer. The presence of a plasticizer generally reduces the intermolecular forces and increases the mobility of the polymeric chains, thereby improving the flexibility and the extensibility of the derived biomaterial. The most commonly studied plasticizers are polyols, like glycerol (GLY) and some mono or oligosaccharides. In particular, GLY not only increases film extensibility, but also migrates inside the film network causing often the loss of desirable mechanical properties of the material. Therefore, replacing GLY with a different plasticizer might help to improve film characteristics allowing potential industrial applications. To improve film properties it seemed of interest to test as plasticizers hydrophilic small molecules, like spermidine (SPD), containing amino instead of hydroxyl functional groups, thereby able to trigger ionic interactions with either polysaccharides or proteins. Pectin and bitter vetch (*Vicia ervilia*) seed proteins (BVP) were used to prepare hydrocolloid films, whereas GLY and SPD were added as film plasticizers, either singly or in combination, at various concentrations. Our results indicate that SPD increased the tensile strength and reduced the elongation at break of both pectin and BVP films, whereas blending of different amounts of both plasticizers were able to give rise to hydrocolloid films with mechanical properties tailored for specific applications.

Acknowledgements

This work was supported by the Italian Ministry of Foreign Affairs and International Cooperation through the "Fourth Executive Program of Scientific and Technological Cooperation between Italy and the U.S. of Mexico ", 2016.

Endocrine distruptors: bisphenols citotoxicity and effects on cellular iron homeostasis

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Bisphenols are a class of chemicals largely used in industry, e.g., in the production of food contact materials, thermal paper, medical devices. The principal mechanism by which the bisphenols exert their effects involves the binding to the estrogen (ER) and aryl hydrocarbon (AhR) receptors, even if other mechanisms of action can be considered. Numerous studies have shown that the endocrine distruptors are able to induce an abnormal ROS production, which mediates their toxic effects. The iron, in form of labile iron pool (LIP), is a potent catalyst for ROS production, and given that the estrogens modulate the iron metabolism, it can be hypothesized that the bisphenols interfere with the maintenance of iron homeostasis. The toxicological potential of nine bisphenols (BPA, BADGE, BPS, BPF, BPB, BPE, BPM, BPAF, BFDGE) was evaluated on different cell lines by MTT assay and supported *in silico* by using the software ADMET Predictor™. Bisphenol lipophilicity was estimated by a chromatographic method allowing to measure their phospholipid affinity (IAM-HPLC). Moreover, the effects of BPA, BADGE and BPS on cellular iron metabolism were assessed on human enteric epithelial cells (CaCo-2) differentiated in enterocytes, as the intestine is the preferential adsorption site of bisphenols, on murine fibroblasts (3T3-L1) differentiated into adipocytes, as the adipose tissue is the primary site of bisphenols biological accumulation, and on MCF-7 cells, as a model of ER-responsive cells. Then, the expression of the main proteins involved in iron homeostasis (transferrin receptor, ferritin, IRP1, ferroportin), the intracellular LIP levels and ROS production were evaluated.

The results show that the toxicity of bisphenols is strictly related to their physicochemical properties, as well as to ER affinity. Moreover, BPA, BADGE and BPS differently modulate cellular iron metabolism, suggesting that the adverse biological effects, in particular of BPA and BADGE, may be mediated by perturbations in iron homeostasis.

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Synthesis of 5-S-lipoylhydroxytyrosol with enhanced ability in protecting human erythrocytes from mercury toxicity compared with its precursor, the natural phenol hydroxytyrosol

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Mercury (Hg) is a highly toxic, redox-active, heavy metal which represents one of the main agents responsible for environmental pollution. Increased formation of reactive oxygen species (ROS) is generally agreed to be one of the underlying mechanisms responsible for Hg-induced toxicity. This heavy metal, indeed, endowed with high affinity for sulfhydryl groups, is able to react with low molecular-weight cellular thiols, including glutathione (GSH). In this connection, we recently provided experimental evidence that the olive oil phenolic antioxidant hydroxytyrosol (HT) modulates the oxidative stress triggered by Hg ions in erythrocytes (RBC) (1,2).

In the search for a more potent tool to counteract Hg toxicity, in this study we synthesized and tested the protective ability of the HT derivative 5-S-lipoyl-hydroxytyrosol (compound 1), including the active sulphydryl moiety of dihydrolipoic acid in the molecular scaffold. When administered to cells (5-20 μM), compound 1 exhibited marked protective capacity against the increase in ROS generation and hemolysis induced in human RBC by exposure to 40 μM of $HgCl_2$; furthermore, GSH depletion was also significantly reduced by compound 1 treatment. Interestingly, at all tested concentrations, compound 1 exhibited overall superior cytoprotective ability compared to native HT. The effects observed can be ascribed to formation of a Hg complex involving the free secondary SH group followed by a redox reaction that would spare GSH. Taken together the reported data encourage the use of compound 1 as an innovative approach in designing nutraceutical strategies to contrast Hg toxicity in humans.

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MODULATION OF CYTOKINE RELEASE FROM HUMAN GINGIVAL MESENCHYMAL STEM CELLS: EFFECTS OF *RIBES NIGRUM* BUD EXTRACT

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The gingival mesenchymal stem cells (GMSCs) have arisen as a promising tool to promote the repair of damaged tissues[1]. The GMSCs secrete trophic mediators that play a pivotal role in the modulation of the different stages of tissue regeneration, affecting in the first stage the proliferation and migration of endothelial cells. The blackcurrant, *Ribes nigrum* has been widely used in the traditional herbal medicine for its regenerative properties[3]. However, its ability to affect the stem cell regenerative properties in soft tissue regeneration is still unknown. Herein, the effects of the *R. nigrum* bud extract on GMSCs and endothelial cell were investigated.

The HPLC-PDA/UV-ESI-MS/MS analyses revealed that R. nigrum bud preparation was rich in polyphenol constituents, such as flavonol mono- and di-glycosides. The extract was able to increase the GMSC proliferation and the gene expression of stemness markers (SOX2, Oct4). GMSCs release growth factors and cytokines (TGF-β, IL-6, IL-10 and COX2) in response to an inflammatory microenvironment (TNF-α). The stimulation of GMSCs with high TNF-α concentration affected their secretome inducing a decrease of the endothelial cells migration and proliferation. Interestingly, the negative effect of TNF-α was counteracted by the ribes challenging that was able to restore the endothelial functionality.

In conclusion, these results highlight the *R. nigrum* bud extract as a novel tool to enhance tissue regeneration enhancing the new vascularization, by directly affecting endothelial cells, and through the modulation of GMSCs cytokine release. These data open the way to the development of these extracts as topic agents in wound healing and periodontal regeneration.

Founded: PRA_2017_30

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Taste Receptor Polymorphisms: The Hidden Side of Obesity

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Obesity is a major risk factor for cardiovascular diseases[1], type 2 diabetes mellitus, cancer[2] and musculoskeletal disorders, and is linked to an increased overall mortality relative to non-obese individuals[3]. The prevalence of overweight/obese people has doubled since 1980. Recent advances show that SNPs are associated to differences in taste perception and preferences. Therefore, increased understanding of taste biology and genetics may lead to new personalized strategies. With this aim, we analyzed in 85 overweight/obese patients and 69 non-obese subjects four different polymorphisms: TAS2R38 A49P (rs713598) and TASR38 V462A (rs1726866) located at TAS2R38 gene (NM_176817.4) which codifies for bitter taste receptor; and TAS1R3 C-1572T (rs307355) and TAS1R3 G-1266A (rs35744813) located at TAS1R3 gene (NM_152228.2) which codifies for sweet taste receptor. No variation in the frequency of rs713598 (TAS2R38 A49P) C allele among patients was observed in comparison with controls. However, statistical analysis revealed a significant association between rs713598 polymorphism and obesity in a recessive model of inheritance. The frequency of rs1726866 (TAS2R38 V262A) T allele among patients was lower than controls. The statistical analysis revealed a significant association between rs1726866 polymorphism and obesity in a recessive and co-dominant models of inheritance. The frequency of rs307355 (TAS1R3 C-1572T) T allele among patients was higher than controls. The statistical analysis revealed a significant association between rs307355 polymorphism and obesity only in a recessive model of inheritance. No significant association was found between rs35744813 (TAS1R3 G-1266A) A allele and obesity. Although further studies are needed to elucidate the impacts of these polymorphisms on human physiology, our results seem to suggest that these genetic variations are associated with obesity. In this way, in each person the genotypic variations should be investigated in order to design individual therapies for weight loss and maintenance

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Proteins (P)

Physicochemical, Mechanical and Antimicrobial Properties of an Edible Coating Based on Chitosan and Pea Protein Crosslinked with Transglutaminase

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Consumer´s growing demand for more natural foods and an interest in protecting the environment have led to the design of edible coatings. Transglutaminase can improve the mechanical and barrier properties of proteins based edible coatings. Moreover, the incorporation of antimicrobial agents like nisin may prevent food spoilage. The objective of this work was to study the mechanical, physicochemical and antimicrobial properties of an active edible coating based on chitosan and pea proteins crosslinked with transglutaminase and containing nisin. A full factorial design 2^3 was used, factors were chitosan, transglutaminase, nisin, with two levels (presence or absence). Filmogenic suspensions (FS) were cast on polystyrene petri dishes and dried at 25°C, 45% RH. The obtained results showed that nisin and chitosan decrease significantly the particle size, the polydispersity index (PDI) and the ζ potential of FS, although the best FS stability was observed in the absence of nisin. The particle size and PDI decreased for treatments including nisin. The thickness and the tensile strength of the films increased significantly in the presence of chitosan. Elongation at break was significantly affected by the interaction of chitosan with transglutaminase and chitosan with nisin. Films containing nisin showed good antimicrobial effect, but it was affected by transglutaminase reducing 19% the inhibition zone of *M. luteus*. Nisin incorporation decreased the physicochemical stability of FS, but did not affect mechanical and barrier properties. Transglutaminase addition did not significantly affect mechanical properties, although it contributed to smoother structure of films surface. The active coating obtained may be used to extend the shelf life of foods.

Acknowledgements

This work was supported by the Italian Ministry of Foreign Affairs and International Cooperation through the "Fourth Executive Program of Scientific and Technological Cooperation between Italy and US of Mexico", 2016.

An Epitope Grafting Approach to Meioidosis Vaccine Component Design

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Structural Vaccinology (SV) is a powerful strategy that combines 3D antigen (Ag) structure information with computational biology, to design vaccine components with improved immunological and/or biochemical properties (1, 2). We previously applied a SV strategy involving 3D structure-based epitope discovery and design, as an alternative to experimental epitope mapping, to protein Ags from the Gram-negative bacterium *Burkholderia pseudomallei* that causes melioidosis (a severe human infection that can lead to fatal sepsis, endemic in the subtropical regions) (3-5). Based on their crystal structures, we computationally mapped epitopes and synthesized them in peptide form. Several peptide epitopes displayed improved immunological properties, with respect to the recombinant protein, that hinted at either diagnostic or therapeutic applications (3-5). In particular, one epitope (PalEp3) induced bactericidal antibodies and triggered bacterial agglutination, two responses important for pathogen clearance from the body, whereas two flagellin (FliC) peptides were shown to possess both T-cell and B-cell stimulatory activities (4-5).

Given that multi-epitope presentation can induce stronger and durable immune responses, we will apply an epitope grafting strategy, which foresees the transplantation of reactive epitopes from one Ag onto conformationally-apt regions of a receiving Ag, to combine multiple-epitopes from Pal onto FliC and *vice versa*.

Our hypothesis is that such engineered multivalent Ag against *Burkholderia* will trigger both humoral and cell-mediated responses that lead to immune protection. Given the high phylogenetic conservation of *Burkholderia* and previous reports of conserved epitopes, the same approach will be applied to Pal/FliC homologs from *B. cenocepacia* that causes fatal lung infections in Cystic Fibrosis patients (6). To validate our efforts, we will display generated epitopes, Ags and grafts on Outer Membrane Vesicles, which will be exploited as multi-epitope presentation vessels for *in vivo* tests in mice. We present here our pipeline and results obtained to date.

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Members of the GADD45 protein family form toxic amyloid-like aggregates under physiological conditions

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The Growth Arrest and DNA damage-inducible 45 (GADD45) gene family encodes three related highly similar proteins, denoted as GADD45α, GADD45β, and GADD45γ [1]. These proteins are involved in fundamental physio-pathological processes which include growth arrest, cell cycle control, DNA repair, terminal differentiation and apoptosis. Of particular relevance is the recent discovery that the complex formed by the antiapoptotic factor GADD45β with the JNK kinase MKK7 represents an interesting therapeutic target in multiple myeloma [2]. Since a full understanding of the biological functions of GADD45β and its homologues requires a detail characterization of their biochemical/biophysical properties we have undertaken structural studies on these systems. During these investigations we serendipitously found that GADD45β denaturates by forming aggregates that show a high content of β-structure. Further analyses that included binding experiments with the Thioflavin T dye clearly indicate that GADD45β forms amyloid-like assemblies. Since this protein unfolds at moderated temperatures (Tm ≈ 42 °C), these findings suggests that the protein may undergo this transition in physiological conditions.

The extension of these analyses to the other members of the family highlights analogies and differences. Indeed, GADD45α exhibits the same structural transition upon unfolding, although it occurs at higher temperatures (Tm ≈ 55 °C). On the other hand, GADD45γ, which shares a limited thermal stability with GADD45β, exhibits a reversible (partial) unfolding without forming any aggregate. Interestingly, cytotoxicity assays demonstrate that the amyloid-like assemblies of GADD45α and GADD45β are highly toxic against SH-SY5Y cells. The implications of these findings for the biological functions of these proteins will be discussed.

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The archaeal lysogeny regulator F55: from discovery to *in vitro* and *in vivo* characterizations

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The archaeon *Sulfolobus solfataricus* hosts a variety of viruses, among which the UV-inducible *Sulfolobus spindle* shaped virus 1 (SSV1) is the best characterized[1]. While studying the gene expression of SSV1 in the absence of UV irradiation[2], a novel viral transcript (T_{lys}) was identified and found to encode for a transcription regulator, named F55, that folds into the ribbon-helix-helix DNA-binding motif. DNA band-shift assays demonstrated that F55 specifically recognises and binds to target sequences located in the promoters of the early induced SSV1 transcripts (i.e., T_5, T_6, and T_{ind}) as well as in its own promoter (T_{lys}). The strongest affinity was observed towards T_5 and T_6 promoters and an apparent cooperativity in binding was observed for the T_{ind} promoter[2]. Altogether, these *in vitro* evidences strongly indicated that F55 might be involved in the regulation of the SSV1 lysogeny and UV-induction.

To shed light on the regulative role of F55, an *in vivo* survey of the molecular events occurring at the UV-inducible region of the SSV1 genome was carried out[3]. Chromatin immunoprecipitation (ChIP) assays followed by semi-quantitative PCR (sqPCR) analyses showed that F55 stably binds *in vivo* to the promoters of T_{lys}, T_5, and T_6 as well as to that of the UV-inducible T_{ind}. Moreover, ChIP-sqPCR data indicated that F55 dissociates upon UV irradiation first from the promoter of T_{ind} (2 hours post UV-irradiation) and subsequently from those of T_5 and T_6 (4 hours post UV-irradiation); thus, allowing their transcription to proceed[3]. The protein/DNA dissociation occurs because, upon UV-irradiation, the intracellular concentration of F55 becomes progressively suboptimal to saturate all the regulative binding sites, as shown by western blot analyses[3]. Further studies are underway to identify the molecular partners of F55 involved in the transition from the lysogenic to the UV-induced state of SSV1.

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THE EFFECT OF CBX7/PRMT1 INTERACTION ON E-CADHERIN EXPRESSION

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Polycomb (Pc) group (PcG) proteins are a class of structurally diverse but functionally related epigenetic regulators involved in several cell processes, such as haematopoiesis, stem-cell self renewal, cellular proliferation, senescence and tumorigenesis. The Chromobox protein homolog 7 (CBX7) belongs to the Polycomb repressive complex (PRC1). In human malignant neoplasias the loss of CBX7 often correlates with an advanced cancer state and a poor survival expectation suggesting a role of CBX7 in cancer progression.

Indeed, it has been demonstrated that CBX7 is able to positively or negatively regulate the expression of genes involved in cell proliferation and cancer progression, such as E-cadherin, cyclin E, osteopontin, EGR1.

To deeper understand the molecular mechanisms that underlie the involvement of CBX7 in cancer progression, a CHIP-MS experiment was performed in order to identify novel CBX7 protein partners. Among the identified CBX7-interacting proteins we focused our attention on the Protein Arginine Methyltransferase 1 (PRMT1) whose critical role in epithelial-mesenchymal transition, cancer cell migration and invasion has been already reported. The physical interaction between CBX7 and PRMT1 was validated both *in vitro* and *in vivo* and this interaction was demonstrated to be crucial for the regulation of E-cadherin expression, thereby proposing a new mechanism by which CBX7 may contribute to cancer progression.

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The prokaryotic zinc finger domains: folding mechanisms and amyloid fibrils formation propensity

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We describe the amyloidogenic propensities of three iso-structural globular proteins belonging to the prokaryotic zinc finger family (1) that possess different folding mechanisms. Particularly, the metal-free $Ml4_{52-151}$ (2) folds via classic two-state cooperative transition while the metal-binding homologues, Ros87 (2) and $Ml1_{53-149}$, exhibit more complex folding pathways, including a barrier-less downhill scenario. The results, obtained by CD and fluorescence spectroscopies, DLS, transmission and scanning electron microscopies, show that within 168 hours amyloid formation has already started in Ros87, while $Ml1_{53-149}$ has formed only amorphous aggregates and $Ml4_{52-151}$ is still monomeric in solution. Overall, this study shows how different folding mechanisms, here induced by metal binding, significantly affect amyloid fibril formation propensity of highly homologous proteins.

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Transglutaminase-Crosslinked Protein Films Reinforced by Mesoporous Silica Nanoparticles

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The environmental impact of plastic wastes is escalating rising widespread global concern since disposal systems are inadequate. Therefore, it is crucial to find enduring plastic alternatives, especially in short-term food packaging and disposable applications. One possible solution is the production of bio-based (polysaccharide and/or protein-derived) biodegradable/edible materials. The major limit of hydrocolloid films in food packaging is their relatively poor mechanical and barrier properties which currently hinder their industrial use. The advancement of nanotechnology has boosted interest to new types of composites in which the filler has at least one dimension smaller than 100 nm (nanocomposites). These innovative biomaterials exhibit generally increased mechanical and barrier properties, as well as improved heat resistance compared to their neat polymers and conventional composites. We suggest here a new strategy to produce nano-reinforced biomaterials by using as polymer matrix protein mixture extracted from bitter vetch (BV) seeds and as filler the mesoporous silica nanoparticles (MSN) functionalized or not with (3-aminopropyl)-triethoxysilane (APTES). To improve the structural network, the nanoparticle containing film forming solution was incubated in the presence of transglutaminase (TG), a protein crosslinking enzyme. The obtained results showed that all the BV protein films reinforced with MSN or MSN-APTES showed improved mechanical and barrier properties to both gases and water vapor, and that TG addition further reduced film permeability values to the ones of the well known and widely commercialized starch-based MaterBi bioplastics.

Acknowledgements

This work was supported by the Italian Ministry of Foreign Affairs and International Cooperation through the "Fourth Executive Program of Scientific and Technological Cooperation between Italy and the U.S. of Mexico ", 2016.

Isolation of a novel ribosome-inactivating protein from seeds of *Araujia sericifera* Brot

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Araujia sericifera Brot. (family Apocynaceae) is an evergreen climbing plant, native of South America, introduced as ornamental plant in Southern Italy and in several European countries (i.e. France, Greece, Portugal and Spain) where it has become invasive. This species is famous for catching both diurnal and nocturnal Lepidopteran flower visitors, giving rise to the common name of "cruel plant" (1). A remarkable plant invasiveness has been associated with the presence, in plant tissues, of biomolecules endowed with antipathogenic activity against viruses, bacteria, and pests (2). Therefore, the aim of this study was to verify the presence, in *A. sericifera* tissues, of proteins that might be responsible of plant resistance to attacks from pathogens.

In this framework, our research has been addressed to detect enzymatic activities such as ribosome inactivating proteins, RNases or ribotoxins, considering their role in plant defense against pathogens. In particular, we found that crude extract from *A. sericifera* seeds inhibited protein synthesis in a rabbit reticulocyte lysate system. In addition, with the aim to identify the possible peptide/protein responsible of this effect, a purification procedure of basic proteins has been performed (3). The last purification step on cationic exchange chromatography, revealed the presence of one peak with protein synthesis inhibitory capability. The SDS-PAGE analysis showed that a single protein band, with electrophoretic migration of about 28 kDa, was present. In addition, the Endo fragment, diagnostic of RIPs action, was detected only after acid aniline treatment from rabbit ribosomes (4). Further studies will be performed to purify and better characterize the protein responsible of this peculiar enzymatic activity.

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Quinoa Protein/Chitosan Films Crosslinked with Transglutaminase

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Hydrocolloid edible films represent an alternative to plastic packaging to preserve food quality and organoleptic properties. These biodegradable materials may be used as moisture and gas barrier, to improve sensory perceptions and to confer antimicrobial protection to the coated foods. Recent research on hydrocolloid films has been focused on minimizing some their disadvantages, such as the poor mechanical and water vapor barrier features . The aim of the present work was to prepare and characterize quinoa protein/chitosan (QP/CH) blended films. In order to choose appropriate QP/CH ratios and film processing conditions, the stability of the film forming solutions (FFSs) was evaluated by determining their zeta-potential and particle size at different pH values. QP/CH (5:1 and 10:1, w/w) films were then prepared in the absence or presence of transglutaminase (TG), a well known protein crosslinking enzyme. TG addition to FFS resulted in an increase of the diameter of the particles, with the highest value determined at 10:1 QP/CH ratio. This result suggested that the enzyme-mediated QP crosslinking in the presence of CH led to the formation of protein/polysaccharide aggregates with increased particle size obtained at higher QP concentrations. Finally, the physicochemical and mechanical properties of the crosslinked QP/CH films were determined and compared to the control ones prepared in the absence of enzyme.

Acknowledgements

This work was supported by the Italian Ministry of Foreign Affairs and International Cooperation through the Fourth Executive Program of Scientific and Technological Cooperation between Italy and the U.S. of Mexico , 2016.

Butyrylcholinesterase Inhibitors: Structure-Activity Relationships of 2-Phenylbenzofuran derivatives

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Alzheimer's disease (AD) is an irreversible and progressive brain disorder which is characterized by progressive memory loss and a wide range of cognitive impairments.[1] Although the precise cause of AD is not completely known, there are some factors that seem to play a significant role in the pathogenesis of AD. Since AD is characterized by a forebrain cholinergic neuron loss and a progressive decline in acetylcholine, a possible therapeutic strategy involves the use of cholinesterase (ChE) inhibitors to restore the neurotransmitter level and thus alleviate AD symptoms.[2]

Benzofuran scaffold has drawn considerable attention over the last few years due to its profound physiological and chemotherapeutic properties. Recent studies have also investigated their inhibitory activity towards ChEs.[3,4]

In this study, a series of 2-phenylbezonfurans compounds were synthesized and their inhibition activity towards the ChE enzymes were investigated. We further combined biochemical analysis and molecular modelling studies to identify selective butyrylcholinesterase (BChE) inhibition by benzofuran scaffold. In particular, two compounds exhibited the highest BChE inhibition with IC_{50} values better than the standard cholinesterase inhibitor compound. Molecular modelling studies highlighted the importance of catalytic and peripheral site residues in BChE inhibition. Subsequently, the biosafety of the two promising compounds was evaluated, in NSC-34 cells at the concentration in which BChE activity is inhibited, and no considerable cytotoxic effect was found.

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INVESTIGATIONS ON ONCONASE DIMERIZATION

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Onconase (ONC) is a 104 residues monomeric member of the secretory "pancreatic-type" RNase super-family, extracted from the frog *Rana Pipiens* oocytes [1]. ONC and the super-family proto-type bovine pancreatic RNase A share only 30% sequence identity with, but they display similar folds. Being able to evade the cytosolic RNase inhibitor, contrarily to most monomeric RNases, ONC shows a high cytotoxic activity and is in clinical trial for the treatment of some solid cancers.

We initially observed that ONC spontaneously forms traces of dimer, the amount of which increases after acid lyophilisation following the same protocol used for RNase A [2]. The dimer's structure has been modeled and spectroscopically characterized, and its enzymatic activity measured on yeast RNA. Furthermore, the cytotoxicity of the ONC dimer has been tested against pancreatic adenocarcinoma cancer cells, finding that the dimer is more cytotoxic than the monomer [3].

We ascertained that wt-ONC dimerizes through the domain swapping mechanism involving its N-termini [3], because the C-terminus is blocked by the C87-C104 disulfide. In order to increase the oligomerization tendency of ONC and possibly form different oligomeric conformers, as RNase A does [2], we designed two mutants: C87S-ONC and C104S-ONC to delete the C87-C104 disulfide and possibly allow the swapping of C-termini. Unfortunately, these mutants don't oligomerize at a higher extent than the WT. Anyway, C87S-ONC seems to self-associate more than C140S-ONC. To further investigate the aggregation propensity of ONC, other two mutants are now under production: the first depleting the C104 terminal residue, the other one depleting both S103 and C104 residues. The rationale is that the shortening of the C-terminus may facilitate its possibility to be swapped and induce the formation of additional ONC oligomers to be characterized and biologically tested.

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Identification and characterization of toxin-antitoxin systems in *Lactobacillus* species

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Toxin-Antitoxin systems (TASs) are specific genetic elements found in plasmid or chromosome DNA of bacteria which are activated as response to stress conditions, such as nutrient starvation, high salt concentration, phage infection and oxidative stress. These systems are composed by a stable toxin, a protein or a peptide able to inhibit vital cell processes leading to cell dormancy or death, and an unstable antitoxin, a protein or a RNA that prevents the activity of the toxin. TASs are well studied for pathogenic bacteria while only few information are available for lactic acid bacteria. In this work we report *in vivo* experiments aimed to elucidate the mechanism of action of the '*Lactobacillus* plasmid toxin' recently identified in plasmid DNA of *L. rhamnosus*[1]. Furthermore, we describe the identification in *Lactobacillus* strains of the TAS proteins RelB/E and MazE/F by bioinformatics analyses and PCR-based screening. DNA sequences identified by means of different bioinformatics tools, such as TADB[2] and RASTA[3] were used as a query to search NCBI nucleotide database. Multiple alignments and phylogenetic tree analysis allowed us to gather *Lactobacillus* strains in different groups and to design multi-species primers for toxin identification. A preliminary PCR-based screening of 20 strains belonging to *L. casei, L. paracasei, L. rhamnosus and L. plantarum* identified different *relE* and *mazF* genes, whose characterization is in progress. The great distribution of TASs observed in *Lactobacillus* genus suggest for them a notable role in lactic acid bacteria physiology. Moreover, TAS activation can affect the composition of the viable bacterial population strictly correlated to technological performance.

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This project is supported by Ministero degli Affari Esteri e della Cooperazione Internazionale, Direzione Generale per la Promozione del Sistema Paese.

TtSmtB, an arsenic sensing transcriptional repressor, mediates cadmium resistance in Thermus thermophilus HB27

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The ArsR/SmtB family of transcriptional regulators consists of metal responsive DNA binding proteins sharing a dimeric structure, a helix-turn-helix (HTH) or winged HTH DNA binding domain, and a metal binding box ELCV(C/G)D located within the HTH region. In microbial cells binding of these proteins to different heavy metals determines protein dissociation from target promoters and transcriptional activation. Metal binding mode and specificity vary very much among family members [1].

In a previous study we characterized *Tt*SmtB from the thermophilic microorganism *Thermus thermophilus* HB27 through *in vivo* and *in vitro* approaches, showing that the protein is an arsenic responsive transcriptional repressor which regulates the downstream gene *TTC0354* encoding a Zn^{2+}/Cd^{2+} dependent membrane ATPase involved in arsenic transport outside the cell [2].

In the present study, we further characterized *Tt*SmtB by an array of biochemical and biophysical approaches to deeply analyse its interaction with metal ions (different from arsenic) and target DNA; we show that the protein possesses significant thermal stability and binds other metal ions, such as cadmium and antimony with different affinity. To study the contribution of *Tt*SmtB and *TTC0354* in cadmium resistance *in vivo*, we generated transcriptional fusions of *Tt*SmtB regulatory regions to *LacZ* in *T. thermophilus* HB27 wild type and a mutant strain in which *TTC0354* was inactivated. These findings revealed the contribution of the system in cadmium resistance opening up to the realization of robust *whole-cell* biosensors for cadmium detection.

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Preparation and characterization of grass pea-based bioplastics prepared in the presence of transglutaminase

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The aim of this work was to prepare bioplastics from renewable and biodegradable molecules. Bioplastics are alternative to petroleum-based plastics, the latter extremely pollutant since their combustion contributes to the CO_2 enrichment in the atmosphere. In particular, we produced bioplastics by using as biopolymer source the grass pea (*Lathyrus sativus)* flour, the proteins of which were structurally modified by means of microbial transglutaminase (mTG), an enzyme able to catalyze isopeptide bonds between glutamines and lysines. mTG has been widely proposed for improving technological features of several protein-based edible films (1,2). We demonstrated that proteins from grass pea flour are endowed with glutamine and lysine residues able to act as effective acyl donor and acceptor substrates for mTG, as demonstrated by the formation of high molecular weight protein polymers following flour enzymatic treatment. After analyzing the film forming solutions by means of zeta-potential determination, the bioplastics, produced by casting, were characterized according to their mechanical, gas barrier and optical properties. The presence of mTG allowed to obtain films more mechanically resistant. On the other hand, the permeability and optical properties were not affected by the enzyme treatment. The visualization by Scanning Electron Microscopy (SEM) demonstrated that the enzyme-modified films possessed a more compact and homogeneous structure. In addition, digestion experiments under physiological conditions (1), performed in order to obtain information useful for applying these novel biomaterials as carriers in the pharmaceutical sector, indicated that the mTG-treated coatings might allow the delivery of bioactive molecules in the gastro-intestinal tract.

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Carbohydrate Active Enzymes from hyperthermophilic microbial consortia for the plant biomass degradation: a metagenomic approach.

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Geothermal areas are widely spread across the world and represent a variety of different and unique environments for extremophilic microorganisms. Among these, hyperthermophiles that thrive in solfataric fields at temperatures >80°C, are source of a variety of thermophilic glycosidases with high stability to extremes of pH and temperatures [1] and very promising candidates for the biotransformation of lignocellulosic materials for second and third-generation biorefineries [2].

We report here a metagenomic approach, aimed to discover novel carbohydrate active enzymes (cazymes) within the microbial consortia populating two neighbouring mud/water pools (Pool1 T=85°C and pH 5.5; Pool2 T=94°C and pH 1.5) in the Pisciarelli solfataric field (Naples, Italy) [3]. The pools were mainly populed by the phylum of Crenarcheaota that possessed a large number of ORF encoding cazymes. In addition, we enriched in-lab the microbial consortia of Pool1 to select microorganisms able to grow on different raw lignocellulosic biomasses, observing novel and different cazymes including previously unknown α-glucosidase and glucanase activities. We show here that the mixed approach of metagenomics of extreme environments, in-lab enrichment on lignocellulosic biomasses, and detailed enzymatic characterization is a powerful tool to obtain novel thermophilic biocatalysts for industrial applications.

Acknowledgements

This work was supported by the projects PON01_01966 “ENERBIOCHEM” and PON03PE_00107_1/1 “BioPoliS” of the PON R&C 2007-2013 MIUR-Italy.

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Identification of Molecules Capable of Modulating the Activity of Enzymes Involved in the Extracellular Matrix Remodeling

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Matrix metalloproteinases are zinc-dependent endopeptidases involved in several diseases and in the last decades many research groups have dealt with the problem of the selective inhibition of specific MMPs. In this work attention has been paid in respect of three MMPs commonly involved in a wide range of pathological conditions including inflammation and cancer: MMP-2 (gelatinase A), MMP-9 (gelatinase B) and MMP-3 (stromelysin-1). Starting from a broad panel of synthetic and natural compounds, has been performed the choise of molecules with potential capacity of interaction with the selected MMPs. The attention was focused on natural compounds, specifically have been used salvianolic acid B and polyphenolic compounds belonging to the group of flavonoids: quercetin, kaempferol, galangin and hesperidin.

In the first part of the work it was evaluated the probability of such compounds to interact from a structural point of view with the selected MMPs, and this was done using bioinformatic tools for target prediction and molecular docking. Following the *in silico* evaluations, an inhibition assay has been performed with the aim to evaluate the ability of selected molecules to influence the enzymatic activity.

An *in vitro* study on MDA MB 231 human breast cancer cells this cell line is characterized by an high constitutive expression of a wide panel of proteolytic enzymes, in particular MMP-9 and MMP-3. After treating cells with each compound, enzymatic evaluations both on conditioned media and cell lysates, have been carried out with the aim to define the expression and the activities of the enzymes of interest. Concerning flavonoids was also assessed the role of these compounds to influence the activity of enzymatic targets belonging to the lipoxygenase pathway, that are crucial in the expression of MMP-9 and MMP-3. Specifically, the 5-lypoxygenase activity was considered as well as the role of Leukotriene A_4 Hydrolase (LTA_4H), which is responsible of the production of leukotriene B_4 (LTB_4), a potent proinflammatory mediator and inductor of MMP-9 expression.

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Molecular basis of Sorcin function in calcium homeostasis and multidrug resistance in tumors.

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Abstract

Sorcin is an essential penta-EF hand calcium binding protein, able to control calcium homeostasis thereby reducing Endoplasmic Reticulum stress (1) and able to confer the multi-drug resistance (MDR) phenotype to drug-sensitive cancer cells (2). The X-ray structures of Sorcin in the apo (apoSor) and in calcium bound form (CaSor) reveal the structural basis of Sorcin function in calcium homeostasis: calcium binding to the EF1-3 hands promotes a large conformational change, involving a movement of the long D-helix joining the EF1-EF2 sub-domain to EF3 and the opening of EF1 (1, 3). This movement promotes the exposure of a hydrophobic pocket, which allows Sorcin to interact with its molecular partners through the consensus binding motif identified by phage display experiments and thereby prevent the ER stress (4).

Sorcin is able to bind directly and with high affinity to many drugs used against cancer namely doxorubicin, vincristine, paclitaxel and cisplatin directly and with high affinity, and in H1299 cells Sorcin localization changes upon doxorubicin administration. The high affinity binding of doxorubicin to Sorcin has been demonstrated with different techniques, i.e. Surface Plasmon Resonance, fluorescence titration and X-ray crystallography. The X-ray structure of Sorcin in complex with doxorubicin, solved at low resolution, allows the identification of one of the doxorubicin binding sites, placed at the interface between the EF5 loop the G helix and the EF4 loop. We demonstrate that Sorcin is able to limit the toxic effects of the chemotherapeutic agent in the cell by a dual mechanism: i) by binding directly the drug ; ii) by increasing the doxorubicin efflux via MDR1 (a P-glycoprotein responsible for the ATP-dependent extrusion of a variety of compounds, including chemotherapeutic drugs, from cells) (5).

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Functional and structural comparison of human phosphomannomutases

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PMM2-CDG is caused by mutations in the gene *PMM2* encoding phosphomannomutase2. Yet a paralogous enzyme exists in humans. PMM1 has an additional phosphatase activity and is able to hydrolyze glucose and mannose 1,6 bisphosphate. This latter activity is enhanced by increased concentrations of Inosine monophosphate (IMP) [a].

The specific role of the two enzymes *in vivo* is not fully understood and in particular the effect of PMM1 mutations on hypomorphic PMM2 has not been evaluated yet and IMP could play an important part. For this reason we believe that it is worth elucidating the functioning of PMM1.

The first step towards protein N- or C-glycosylation requires the isomerization of mannose 6-phosphate into mannose 1-phosphate. In vitro however PMM1 and PMM2 are assayed measuring their phosphomutase activity in the opposite direction. Using ^{31}P-NMR spectroscopy, we tested both enzymes in the physiologically relevant direction, as well as the phosphatase activity of PMM1 in the presence or in the absence of IMP.

We identified the residues which are responsible for the sensitivity of PMM1 to IMP and we modelled the interaction with the nucleotide using a docking program that consents full flexibility of the protein as well as the ligand.

Acknowlegment: Research funded by Telethon-2012 (GGP12108)

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ALLOSTERIC BEHAVIOUR OF THE MAIN ENDOCANNABINOID-DEGRADING ENZYME FAAH

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Fatty acid amide hydrolase (FAAH) is a membrane-bound dimeric enzyme that receives hydrophobic substrates from the lipid bilayer and hydrolyses them to release (more) hydrophilic products. FAAH plays a critical role in the modulation the so-called “endocannabinoid tone” in mammals, degrading important neurotransmitters and neuromodulators such as anandamide and oleamide. The latter endocannabinoids are involved in a number of human diseases, thus holding therapeutic potential.

To investigate the possible functional interactions between FAAH monomers, we analyzed the catalytic properties of rat FAAH and human FAAH, in addition to two rat mutants. We demonstrate that similar inhibitions of enzyme activity occur when only one of the two active sites is occupied by the inhibitor. Consistently, kinetic analysis and molecular dynamics simulation demonstrate that rFAAH and hFAAH behave as allosteric enzymes. The Hill coefficient of rFAAH and its F432A mutant suggests positive cooperativity (n_{Hill}=1.6), whereas the W445Y mutant of rFAAH looses the sigmoidal shape (n_{Hill}=1.0), and better fits a canonical Michaelis-Menten curve. Therefore the W445 residue, but not F432, plays a key role in the inter-subunit communication that leads to functional cooperativity.

Acknowledgements

This investigation was supported in part by the BioStruct X-BAG project “A new player in the modulation of protein function: the biological membranes”, under the EU Framework Programme grant agreement n. 283570 (to MM and ED).

FUNCTIONAL CHARACTERIZATION OF L-CYSTATHIONINE Γ-LYASE FROM *TOXOPLASMA GONDII*

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Toxoplasmosis is a zoonotic disease of medical and veterinary relevance with worldwide distribution. It is caused by *Toxoplasma gondii*, an obligate intracellular protozoan parasite belonging to the phylum Apicomplexa. As an efficacious vaccine remains a challenge and treatment of toxoplasmosis is based on the use of drugs for which resistance is emerging, there is an urgent need to identify new targets against the pathogen.

PLP-dependent enzymes are intimately involved in the metabolic pathways of *T. gondii* and could represent attractive drug targets. In particular, the cysteine metabolism plays an important role in synthesis of essential biomolecules and sulfur homeostasis. Herein, we performed a detailed biochemical characterization of the putative pyridoxal 5'-phosphate (PLP)-dependent enzyme cystathionine γ-lyase (CGL) from *T. gondii*, which catalyzes the cleavage of L-cystathionine to L-cysteine, α-ketobutyrate and ammonia. We overexpressed the protein in *E. coli* and analyzed its molecular and kinetic properties.

Recombinant TgCGL exists as a tetramer in solution and shows spectral properties of enzymes containing PLP as cofactor. The enzyme possesses a specific CGL activity toward L-cystathionine and is able to catalyze α,β-elimination of other sulfur-containing amino acids, such as aminoethyl-L-cysteine and djencolic acid. In contrast to the human enzyme, TgCGL is strongly inhibited by L-cysteine.

We also determined the contribution of the active site residue Ser77 to the specificity of the TgCGL enzyme by mutating it to glutamate, as found in the well-characterized human and yeast CGLs. Notably, the substitution of Ser77 with Glu causes a complete loss of the enzymatic activity; however further studies, e.g., the substitution of serine residue with alanine, will be necessary to uncover a possible catalytic role of this residue.

The findings that TgCGL possesses parasite-specific structural features as well as differs in substrate specificity from its human homolog make it an attractive target for antitoxoplasmosis inhibitor design.

Crystallization of *Euphorbia characias* Latex Peroxidase

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Euphorbia characias latex peroxidase (ELP) is a class III peroxidase characterized by an enzymatic activity finely tuned by calcium ions. ELP is constituted by a single glycosylated polypeptide chain of 347 amino acid residues (GenBank accession number AY586601) with a relative molecular mass of 47 kDa and contains a ferric iron-protoporphyrin IX as heme prosthetic group. With the aim to study the function-structure relationship of ELP, the enzyme, previously isolated and deeply characterized, have been crystallized.

Sparse matrix crystallization trails were performed at the High Throughput Crystallization Laboratory. The crystallization screenings were carried out at 20 °C on Crystal Direct CD-1 plates (EMBL) by using the sitting-drop vapour-diffusion method. Diffraction data were collected at the ID30A3 beamline at the European Synchrotron Radiation Facility (ESRF).

ELP crystallized very easily in 91 conditions of the 576 tested, from PEGs-Ion screen, JCSG and PACT. Crystals grew in prisms, occasionally assembled in clusters, of varying length (10 to 500 µm) and thickness. Positive crystallization hits were obtained over a large pH range, from 5 to 9, while the best crystals were obtained at a pH of 7.0-7.5 in presence of different monovalent cations. Best diffracting crystals were obtained in 0.2 M sodium formate, 20% (w/v) PEG 3350 and 0.2 M potassium thiocyanate.

Data collected from all crystals were integrated in the $P6_1$ space group with unit cell *a*=163.3 Å *b*=163.3 Å *c*=83.8 Å. The search model was derived from the three-dimensional structure of barley grain peroxidase 1, which shares a 56% identity with ELP. Phaser successfully placed two copies in the asymmetric unit with translation Z-score of 12.7 and 24.4 respectively. Initial residual map analysis clearly indicated the presence of one porphyrin group per molecule. Model completion is currently in progress.

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RHA-P: a novel bacterial α-L-rhamnosidase of biotechnological relevance from *Novosphingobium* sp. PP1Y

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α-L-Rhamnosidases (α-RHAs) are a group of glycosyl hydrolases, whose biotechnological potential in industrial processes is gaining much interest, able to catalyze the hydrolysis of terminal residues of α-L-rhamnose from several natural compounds. Several dietary products are rich in glycosylated compounds, such as natural flavonoids, often showing the presence of α-L-rhamnose and β-D-glucose. The ability to hydrolyze rhamnosylated flavonoids has been used to mitigate the bitterness or enhance aroma in beverages [1, 2], and in general to improve flavonoids bioavailability [3]. A novel α-L-RHA activity was identified in the crude extract of *Novosphingobium* sp. PP1Y, a marine bacterium able to grow on a wide range of aromatic polycyclic compounds [4]. The *orf* PP1Y_RS05470 coding for the enzymatic activity was cloned in pET22b(+) vector in frame with the His-tag coding sequence, expressed in *E.coli* strain BL21(DE3) and purified. The recombinant protein, named rRHA-P, was characterized as an inverting monomeric glycosidase of ca. 120 kDa belonging to the GH106 family. Enzymatic activity assays performed on para-nitrophenyl-α-L-rhamnopyranoside (pNPR), allowed identifying optimal reaction conditions in terms of pH and temperature. [5]. In this work, rRHA-P ability to hydrolyze natural glycosylated flavonoids was evaluated; results showed that rRHA-P is able to hydrolyze α-L-rhamnose bound to β-D-glucose with either α-1,2 or α-1,6 glycosidic linkages in natural compounds such as naringin, rutin or neohesperidin dihydrochalcone [4]. Moreover, the putative catalytic residues were identified through an alanine-scanning site directed mutagenesis strategy, performed on five highly conserved residues of aspartic and glutamic acid. Enzymatic activity of mutants was evaluated on *p*NPR using alternatively whole recombinant cells or purified proteins. The homology model of rRHA-P was obtained using the crystal structure of the α-RHA from *Bacillus thetaiotaomicron* as template [6]. Data obtained strongly suggest that the mutagenized residues play an important, although differential, role

in the catalytic mechanism of the enzyme. The biochemical characterization performed so far encourage the potential use of rRHA-P as a biocatalyst for diverse biotechnological applications.

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Dependence of Oxidative Stress on the Oxygen Binding Properties of PEGylated Hemoglobin-Based Oxygen Carriers in Transfused Guinea Pigs

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Hemoglobin-based oxygen carriers (HBOCs) are an investigational replacement for blood transfusions and differ by oxygen binding properties, i.e. cooperativity and affinity [1]. HBOCs are known to cause oxidative damage to tissues, but the dependence of these effects on their oxygen binding properties has never been thoroughly investigated in the same experimental setting. To fill this gap, we produced two derivatives of hemoglobin by PEGylation either in the oxygenated state (PEG-Hb^{oxy}, high affinity and no cooperativity) or in the deoxygenated state (PEG-Hb^{deoxy}, low affinity and high cooperativity) [2,3]. Guinea pigs were used as pre-clinical model because, like humans and unlike mice and rats, they lack endogenous ascorbic acid. Groups of 7-9 animals underwent: i) control isovolumetric autotransfusion, ii) isovolumetric transfusion with PEG-Hb^{oxy}, and iii) isovolumetric transfusion with PEG-Hb^{deoxy}. The physiological parameters were recorded during the treatment. The surviving animals were sacrificed after 7 days and their organs harvested. Plasma samples were analyzed for biochemical markers of inflammation, tissue damage and organ dysfunction. Protein heart extracts were analyzed for oxidative damage, determining: i) carbonyl content, ii) S-nitrosylation; iii) S-glutathionylation; iv) adducts with 4-hydroxynonenal; v) adducts with malondialdehyde. Overall, significant differences were detected among the groups, with both HBOCs producing higher oxidative stress in comparison to controls. Some markers (i.e. S-nitrosylation, potassium, creatinine, creatine kinase, bilirubin, total plasma protein content, lactate dehydrogenase) were differentially altered between PEG-Hb^{deoxy} and PEG-Hb^{oxy}. This work indicates the suitable oxygen binding properties for HBOCs to be used as interventional clinical treatments.

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Proteomic analysis of bone marrow derived human MSC secretome stimulated with pro-inflammatory cytokines

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Mesenchymal stem cells (MSC) are very versatile and respond to specific environments by producing and releasing a variety of effector molecules. Although it is clear that upon transplantation MSC exert most of their therapeutic effects through the secretion of bioactive molecules, the effects of a pro-inflammatory recipient environment on MSC secretome have not been characterized. In this study, we used a label free mass spectrometry based quantitative proteomic approach to analyze how pro-inflammatory cytokines modulate the composition of the human MSC secretome.

Bone marrow derived hMSC were provided by Orbsen Therapeutics Ltd (Galway, Ireland). Stimulated (st-) or unstimulated (unst-) conditioned media (CM) were prepared in the presence of absence of a mixture of pro-inflammatory cytokines (IL1b, IL6 and TNFa). Following CM collection, protein were precipitated, reduced, alkylated and digested with trypsin. Mass spectrometric analysis was performed on a LTQ-Orbitrap Velos mass spectrometer (Thermo Scientific). Protein identification and label free quantification were performed using Max Quant (FDR 1% at peptide and protein level). Label free quantification based quantitative differential analysis of st- and unst-hMSC secretome resulted in the identification/quantification of 465/457 proteins in st-/unst-hMSC CM, respectively; 96 proteins were present only (39 proteins) or overrepresented (57 proteins, t-test difference cut-off at 1% permutation-based FDR) in st-hMSC CM. Functional analysis showed that 70% and 64% of up-regulated proteins in st hMSC-CM are involved in inflammation or angiogenesis, respectively. Moreover, stimulation resulted in increased levels of a number of proteases and protease inhibitors.

Results on st hMSC secretome strengthens our observation based on similar studied on mouse MSC secretome that a fine but complex tuning of proteolytic activity is a key mechanism regulating MSC effects on angiogenesis and tissue remodeling (1).

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COMPARATIVE CHARACTERIZATION OF YEAST AND HUMAN 3-KETOSTEROID REDUCTASE, TWO MOONLIGHTING PROTEINS THAT SHARE A JOB

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Yeast and human 3-ketosteroid reductase (ERG27 and human HSD17B7, respectively) catalyze the reduction of 3-keto-intermediates of sterol biosynthesis into the corresponding beta-hydroxy compounds[1]. Besides this shared function, each of the two proteins possess an additional ability that up to now has not been reported to be shared with the other protein: yeast ERG27 behaves as a chaperonine-like protein towards oxidosqualene cyclase (OSC), an upstream enzyme of ergosterol biosynthesis; HSD17B7 is highly effective in transforming estrone into estradiol. In mammals, the 3-ketosteroid reductase is not required for OSC[2]. To date, no structures of 3-ketosteroid reductases are known.

In order to distinguish the intrinsic properties of the proteins from those depending on cellular/tissue environment, yeast ERG27 and human HSD17B7 were expressed in *E. coli*, and a series of parallel experiments with cell homogenates were performed: (i) proteins were assayed for their 3-ketoreductase activity or their estrone reductase activity through separate incubation of cell homogenates with 4-methyl zymosterone or estrone, respectively; (ii) the inhibitory effect of molecule designed as inhibitors of estrogenic activity of human HSD17B7 were assayed. In addition, to better understand the enzyme structure-activity relationship, a series of human HSD17B7 mutants was designed and produced.

Both proteins proved to be active in reducing 4-methyl zymosterone, whereas only the human enzyme displayed the estrogenic activity. This result poses an interesting question of when and thanks to which aminoacidic residues the protein 3-ketosteroid reductase gained its second job, the estrone-reductase activity. In this respect, it is interesting that some human HSD17B7 mutants showed differences between the two reductase activities. Furthermore, inhibitors of the estrogenic activity of the human HSD17B7 resulted scarcely active against the 3-ketosteroid reductase activity of the same enzyme, suggesting the existence of two (at least partially separated) active sites in the human HSD17B7.

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Differentially expressed muscle proteins are associated with healthy longevity effects of lifelong football training

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Football is an intermittent team sport characterized by multiple high-intensity anaerobic actions interspersed with periods of low-intensity recovery. Football training improves cardio-respiratory fitness and the oxidative component of muscle fibers in healthy untrained individuals (1, 2). Emerging studies are focused on the effects of long-term football training on the expression of healthy longevity-related muscle molecular markers (3, 4).

Here, we explored the proteomic changes in muscle tissues of Veteran Soccer Players (VSP) compared to healthy age-matched untrained subjects (control group, CG) in order to cast light on the molecular mechanisms underlying the healthy longevity effects of lifelong football training.

Muscle biopsies were obtained from the Vastus lateralis of 12 male volunteers (65–75 years), 6 belonging to VSP and 6 to CG. Protein expression profiles from VSP vs CG were analyzed by label-free approach using liquid chromatography-tandem mass spectrometry (LC-MS/MS). Protein Discoverer platform was used for quantitation analysis. Proteomic data were further analyzed by bioinformatic tools to classify identified proteins according to gene ontology (GO) terms and to unravel relevant molecular networks.

Quantitative analysis revealed 170 differentially expressed proteins in VSP vs CG with fold changes ≥ 1.50 or ≤ -1.50 (75 overexpressed and 95 underexpressed proteins).

Classification based on GO terms showed that the top significantly enriched biological processes involved “Amino acid metabolism” and “Proteasome-dependent protein degradation”. We also identified protein interaction networks related to “Fatty acid metabolism” and “Oxidative phosphorylation”.

In elderly lifelong football training modulates the expression levels of muscle proteins involved in crucial pathways related to healthy longevity.

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A strategy to find a cell surface receptor for the alpha-gliadin peptide 31-43

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Celiac disease is a widespread enteropathy affecting 1:100 individuals in western population. Alpha-gliadin peptide 31-43 (p31-43) is considered the main responsible of the innate immune response in CD patients. Recent evidences indicate that p31-43 rapidly enters cells and interacts with the early endocytic vesicular compartment. However little is known about molecular mechanism underlying p31-43 uptake and about the possible involvement of a membrane receptor for this. Interestingly, evidences has been reported on a role of cell surface type 2 transglutaminase (TG2) in modulate p31-43 uptake by Caco-2 cells; indeed, antibodies to TG2 specifically reduced both p31-43 uptake by cells and its biological activity. Here we investigated whether TG2, or another membrane protein, could be directly responsible of p31-43 translocation into cells of intestinal epithelial origin, acting as a specific receptor or carrier for p31-43.

We used a chemical cross-linker to block p31-43 on cell surface proteins, and pulled-down peptide-proteins complexes using antibodies raised against p31-43. By this experimental approach, we could not visualize any specific complex between cell proteins and p31-43 on the Coomassie-stained denaturating gel or by western blot. We also found that that TG2 was not necessary for p31-43 internalization, even if it had a regulating role in the process. Finally, we demonstrated that p31-43 did not behave as a classical ligand, indeed the labelled peptide did not displaced the unlabelled one in a competitive binding assay.

On the bases of these finding and on previous evidences demonstrating that p31-43 is able to interact with a membrane-like environment *in vitro*, we conclude that membrane composition and organization, instead of a specific receptor protein, may have a major role in p31-43 internalization by cells.

KCTD1-Ap2α interaction: a crucial point in adipocyte differentiation

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Literature data reported the involvement in adipogenesis of KCTD15 and of the transcription factor Ap2α, a well-known differentiation repressor [1]. KCTD1 is an homologue of KCTD15 and its interaction with Ap2α has been previously demonstrated in transfected HEK293 cells[2]. KCTD1 and KCTD15 are both members of an emerging family of potassium channel tetramerization domain-containing proteins[3]. This research aimed to investigate a potential role of KCTD1 in the process of adipogenesis.

KCTD1 and Ap2α localization has been firstly evaluated by fluorescence microscopy in 3T3-L1 cells. Their interaction has been analysed “in vivo” by PLA technology and “in vitro” by SPR methodology. Kctd1 and *Ap2α* genic expression were monitored by qPCR in 3T3-L1 adipocytes during the differentiation. As a result of KCTD1 silencing, mRNA levels of different adipogenesis markers, as well as C/ebpα, Pparγ2, Glut4 and Adipoq, were examined. The data collected showed that the induction of Kctd1 expression during differentiation could be correlated with the regulation of Ap2α transcription factor. All the results will be widely discussed proposing KCTD1 as a novel "target" to modulate adipogenesis.

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COMBINATION OF REDUCED MRNA SPLICING AND PROTEIN SECRETION/FUNCTION DETERMINES COAGULATION FACTOR VIII EXPRESSION IN HEMOPHILIA

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Nucleotide exonic changes that translate into amino acid substitutions represent ideal models to dissect key determinants of pre-mRNA splicing and protein biology, which are hidden in the co-evolved and overlapped splicing and amino acid codes.

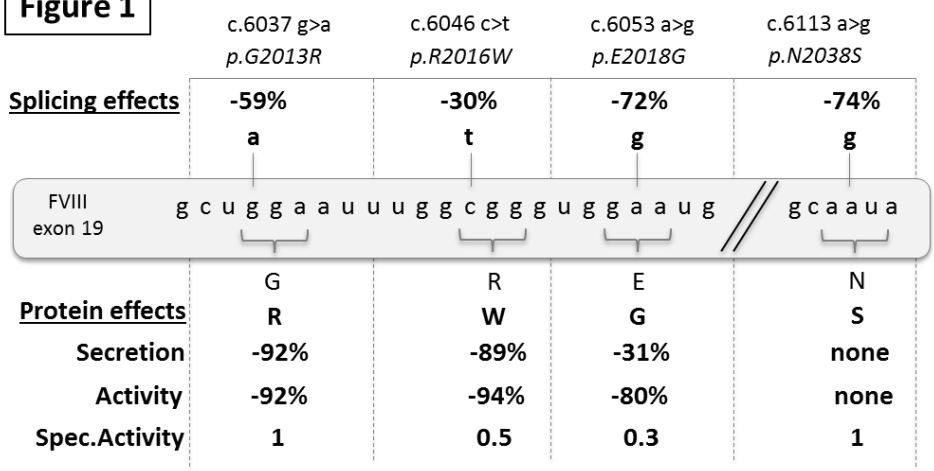
As a model we chose the *F8* exon 19 harbouring the frequent p.Arg2016Trp/c.6046C>T mutation, suggested to induce exon 19 skipping, which we confirmed in leukocytes from several patients' (74±15% of correct transcripts).

The splicing mechanism was addressed by F8 minigenes expression in hepatoma cells, which demonstrated that the mutation impairs the definition of the intrinsically weak exon 19 (70±5% of correct transcripts), and its effect was vanished by strengthening the 5'ss. We showed that masking the affected region by an antisense U7snRNA induced complete exon skipping, thus unravelling an exonic splicing enhancer, potentially affected by a panel of other *F8* missense mutations. Strikingly, the c.6037G>A (p.Gly2013Arg) reduced exon inclusion to 41±3% and the c.6053A>G (p.Glu2018Gly) to 28±2% of wt, similarly (26±2%) to the c.6113A>G, (p.Asn2038Ser), a variant affecting the 5'ss.

To investigate effects of substitutions clustered in the FVIII A3 domain on protein features, we expressed recombinant (r)FVIII in Chinese Hamster Ovary (CHO) cells through lentiviral expression vectors, chosen to overcome the intrinsically low FVIII expression. The rFVIII-2016Trp and the rFVIII-2013R displayed reduced secretion (ELISA; FVIII:Ag 11.0±0.4% and 7.0±0.9% of wt, respectively) and cofactor activity (chromogenic assay; FVIII:C 6.0±2.9% and 8.4±0.8%) whereas the rFVIII-2018Gly showed dysfunctional features (FVIII:Ag, 69.0±18.1%; FVIII:C, 19.4±2.3%). The rFVIII-2038Ser displayed normal FVIII:Ag and FVIII:C values.

These data highlight a F8 exonic region that has evolved in relation to both mRNA maturation and protein constraints, a feature potentially shared with other exons. They demonstrate that differential combinations of effects on mRNA and protein (Figure 1) accounts for a gradient of residual FVIII expression, thus providing the molecular bases of hemophilia A.

Figure 1



The recognition mechanism of the coactivator NCoA-1 by STAT6

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STAT6 belongs to a family of transcription factors known as the signal transducers and activators of transcription (STAT). STAT family members share a similar protein structure, which is essential for their activation and function. STAT proteins mediate signaling from activated cytokine receptors to the nucleus[1]. After phosphorylation at a specific tyrosine by a receptor associated Janus kinase, STATs form homo- or heterodimers and translocate into the nucleus where they modulate transcription by specific DNA sequence elements [1]. STAT6 becomes activated in response to IL-4 and IL-13 and mediates most of the gene expression regulated by these cytokines. By direct interaction with specific parts of its transactivation domain, STAT6 recruits the co-activators p300/CDP and NCoA1, which are essential for transcriptional activation by IL-4[2]. In particular, the interaction between STAT6 and NCoA1 is modulated by a short region of the transactivation domain that includes the motif LXXLL (where X is any amino acid). The crystal structure of a STAT6-derived peptide (Leu794-Gly814) in complex with the NCoA1 PAS-B domain$^{257-385}$ revealed that the Leucine side-chains of the motif (Leu802, Leu805 and Leu806), are deeply embedded into a hydrophobic groove of the surface of NCoA1[3]. More recently, it has been demonstrated by a fluorescence polarization binding assay that additional residues (Leu794, Pro797 and Thr798), flanking the LXXLL motif in STAT6, play an important role in stabilizing the protein binding to NCoA1[4]. Here, we report the structural characterization of the complex between a STAT6-derived peptide encompassing the region from Gly783 to Gly814 and the NCoA1 PAS-B domain257-385 using Nuclear Magnetic Resonance (NMR) and X-ray crystallography. The structural characterization of the STAT6$^{783-814}$/NCoA1$^{257-385}$ complex demonstrates that STAT6$^{783-814}$ peptide binds the NCoA1 PAS-B domain$^{257-385}$ by additional amino acid interactions from its N-terminal region resulting in a more extended binding interface with NCoA1 compared to that identified before in the crystal structure with the STAT6$^{794-814}$ peptide.

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Zn(II) to Ni(II), Hg(II) and Pb(II) replacement in the prokaryotic zinc-finger domain

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Zinc ion binding to the proteic domain is a principal event in the achievement of the correct fold in the classical zinc fingers domain since the motif is mainly unfolded in the absence of the metal cofactor. However, in the case of prokaryotic zinc finger the bigger □□□□□ domain shows a hydrophobic core larger than the one found in eukaryotic zinc fingers and that plays a more relevant role in the folding mechanism. For these reasons, as great attention has been devoted to unveil the effect of metal ion replacement in zinc fingers and in zinc-containing proteins in general, the prokaryotic zinc finger domain appears to be a good model to study the interaction of exogenous metal ions with metallo-proteins.

We here explore the structural and functional consequences of the native Zn(II) substitution by Ni(II), Pb(II) and Hg(II) in Ros87, the DNA binding domain of the prokaryotic zinc finger protein Ros. Our findings will complement and extend previous results obtained for different eukaryotic zinc fingers, contributing to the evaluation of whether metal substitution in zinc fingers may be a relevant mechanism in the toxic and/or carcinogenic effects of metal ions.

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Nanosubstrates modulate mechanotransduction processes in human islets of Langerhans

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Cells are competent to perceive biophysical signals of their microenvironment and to convert them into biochemical responses, a process called mechanotransduction. We study the influence of mechanotransduction in human islets of Langerhans to identify regulators of cell growth and maturation and to accelerate development of therapies for diabetes since there is significant evidence that β-cells depend on extracellular cues to replicate, survive and differentiate but the physical interactions are not completely understood. We used nanoscaffolds with different roughness to support long term culture of human islets and we evaluated the molecular mechanisms involved by a proteomic approach[1].

Zirconia surfaces nanoroughness were produced by supersonic cluster beam deposition (SCBD). Human pancreatic islets were isolated using collagenase digestion and density gradient purification from beating-heart organ donors with no medical history of diabetes or metabolic disorders donors by using the procedure already described. Shotgun mass spectrometry and label free quantification were carried out on a nanoLTQ Orbitrap Velos (Thermo Fisher Scientific)[2].

Nanostructured substrates preserve β-cells differentiation in long term cultures (up to 25 days). NanoLC-ESI tandem mass spectrometry allowed comparing the proteome of pancreatic islets grown on zirconia nanostructures (nrZr), flat zirconia (flZr) and matrigel (Gel); 65, 66 and 50 proteins are exclusively expressed in nrZr, flZr, gel, respectively, while 101 out of 1406 common proteins differ with statistical significance. The current study provides a first quantitative proteome comparison of human islets grown on a nanosubstrate and on matrigel. Though the quantitative architecture of their core proteome is highly conserved, cells show remarkable differences unraveling some interesting candidates for more detailed analysis of mechanotransduction processes induced by nanostructured surfaces.

Conclusions

Tailored nanostructured substrates may provide a unique strategy to identify novel hints for tissue engineering and molecular /pharmacological targets of intervention to treat diabetes.

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Exploring the kinetics of the self-assembly of an engineered archaeal ferritin protein

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Human recombinant ferritin (HFt) has been proposed as privileged protein carrier due to its cage-like structure and transferrin receptor (TfR1) binding ability, ideal properties for drug/diagnostics encapsulation and drug-delivery [1]. However, the most relevant limitation to its application is a stiff 24-meric assembly that can be dissociated only at very acidic pH, a process that could impair both drug and protein stability. In this context, the archaeal ferritin from A. *fulgidus* (Af-Ft) has emerged as especially promising, due to its unique 23 point-group symmetry typical of smaller 12-mer ferritin-like proteins resulting in the appearance of four large triangular pores (~45 Å) in the protein shell, combined with a reversible association in the presence of $MgCl_2$ [2]. Thus, the properties of HFt have been merged with those of Af-Ft, creating a mutated chimeric protein (HumAfFt) capable of high affinity binding to TfR1 and subsequent internalization, accompanied by preservation of the unusual assembly properties of Af-Ft [3].

The three-dimensional structure of HumAf-Ft has been obtained, and current research aims to characterize its assembly mechanism to guide further development of Af-Ft as a universal scaffold for drug and diagnostic delivery. The assembly kinetics of HumAfFt triggered by the rapid mixing of MgCl2 at physiological pH have been explored with time-resolved Small Angle X-ray Scattering (SAXS) revealing a very fast association at low salt concentrations. Nevertheless further experiments aimed at the clarification of the oligomerization mechanism and the role of cations in the assembly are essential to propose a relevant kinetic model for the assembly reaction. Detailed understanding of the mechanism of the 24-mer complex formation and its kinetics are still in progress and will be utilized to guide further engineering of Af-Ft based nanocarriers.

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Altered protein *O*-GlcNAcylation profile revealed by proteomics in AD: Novel insights on protein signalling mechanisms and potential therapeutic targets

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Background: PET scan analysis demonstrated the early reduction of cerebral glucose metabolism in AD patients that can make neurons vulnerable to damage *via* several mechanisms including the alteration of the hexosamine biosynthetic pathway (HBP). In turn, defective HBP lead to flawed protein *O*-GlcNAcylation coupled, by a mutual inverse relationship, with the increased protein phosphorylation on Ser/Thr residues (Hart et al., 2011). Impaired *O*-GlcNAcylation of Tau and APP have been reported in AD and are closely related with pathology onset and progression (Lefebvre et al., 2003; Kang et al., 2013; Yuzwa et al., 2014 and Förster et al., 2014). Previous studies from our laboratory (Di Domenico et al., 2010) demonstrated that aberrant O-GlcNAc of proteins occur in AD brain affecting, among others, metabolic, synaptic and others proteins known to be involved in pathways associated with cellular insults present in neurodegeneration. As well, type 2 diabetes patients show altered GlcNAcylation/phosphorylation balance together with an increased risk to develop AD. Therefore, altered protein *O*-GlcNAcylation might represent a link between metabolic defects and AD progression.

Methods: Our study aim to decipher the status of HBP pathway, the role of total *O*-GlcNAcylation reduction and the specific protein targets of altered *O*-GlcNAcylation in brain of 12 months-old 3xTg-AD compared with age-matched wild-type mice. Hence, we analysed: 1) the Global *O*-GlcNAc levels, as well as, the levels and activity of *O*-GlcNAc transferase (OGT) and *O*-GlcNAcase (OGA), the enzymes controlling its cycling; 2) Specific O-GlcNAc levels of proteins by 2D proteomic approach coupled with ESI-MS/MS; 3) The mutual relationship between *O*-GlcNAcylation and phosphorylation on our targets of interest;

Results: Our data demonstrate altered enzyme activity and expression levels of OGT and OGA, together with the decrease of total *O*-GlcNAcylation levels in 12 months-old 3xTg-AD compared to non-Tg. Data from proteomics analysis led to the identification of several proteins with differential *O*-GlcNAcylation levels, between transgenic and wild-type animals, which belong to key pathways involved in the progression of AD

such as neuronal structure, degradation processes and energy metabolism. Interestingly, the majority of proteins identified by MS analysis show the concomitant alteration of phosphorylation levels suggesting that the unbalanced *O*-GlcNAcylation/phosphorylation levels may lead to altered functionality of these proteins and contribute to early cognitive defects of AD.

Conclusions: Our findings may contribute to understand the effects of altered protein *O*-GlcNAcylation profile during AD, identifying novel mechanisms of disease progression related to glucose hypometabolism. In addition, our findings may lead to the identification of novel therapeutic targets and strategies to slow or delay AD progression.

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Biochemical and biophysical characterisation of calmodulin-like protein 19 (CML19), the Centrin 2 of Arabidopsis thaliana

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Centrins are calcium (Ca^{2+})-binding proteins that play key roles in centriole duplication and DNA repair mechanisms in organisms ranging from algae and yeast to humans. However, plant centrins remain poorly characterized [1]. Calmodulin-like proteins 19 (CML19) is one of the two centrin isoforms of Arabidopsis thaliana. CML19 has an important role in the Nucleotide Excision Repair mechanism (NER), by interacting with RAD4, the homolog of human xeroderma pigmentosum group C protein (XPC) [2]. Although the role of CML19 as a part of the RAD4 plant recognition complex for functional NER is known at a cellular level, the molecular and structural properties of CML19 and of its interaction with RAD4 have not been described so far. In this work we analysed the Ca^{2+}-binding affinity and Ca^{2+}-dependent functional properties of recombinantly produced CML19 using a combination of biochemical and biophysical techniques. Isothermal titration calorimetry analysis showed that CML19 possesses four Ca^{2+}-specific binding sites, two of high and two of low affinity. Circular Dichroism, Nuclear magnetic resonance, and fluorescence spectroscopy revealed that the binding of Ca^{2+} induces an increase in the alfa-helix content, stabilizes the tertiary structure and triggers a conformational change, resulting in the exposure of hydrophobic surfaces essential for target protein recognition. Moreover, through in vitro binding experiments we identified the CML19-binding site located at the C-terminus of RAD4 and described the interaction between a 17-residue peptide representing this site and CML19. We found that CML19 strongly interacts (Kd of ~ 54 nM) in a strict Ca^{2+}-dependent manner with this peptide, which possesses detectable sequence identity to the centrin binding region of human XPC. Taken together these data support a role for CML19 as Ca^{2+} sensor and provide information on the Ca^{2+}-CML19/RAD4 complex assembly, expanding the knowledge of the structural and biochemical properties of plant centrins.

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Other (O)

Molecular mechanisms for maintenance of genomic imprinting in mouse embryonic stem cells

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In mammals, about a hundred of genes, crucial for growth and development, are monoallelically expressed. The gamete-of-origin-dependent expression, known as genomic imprinting, is controlled by differential DNA methylation, established on Imprinting Control Regions (ICRs) during male and female gametogenesis, transmitted to zygote and maintained despite the wave of genome-wide demethylation in pre-implantation. The protein ZFP57, that binds the methylated allele (imprinted) at the ICRs, has been shown to be required for imprinting maintenance in early mouse embryo. This binding is essential for the recruitment of the co-repressor KAP1, DNA methyltransferases (DNMT1, 3A and 3B) and the histone H3 lysine 9 methyltransferase SETDB1. Inactivation of ZFP57 in mESCs results in loss of the repressive epigenetic marks mCpG and H3K9me3, acquisition of the permissive marks H3K9ac and H3K4me3 at the ICRs.

Nuclear Transcription Factor Y (NF-Y) is one of the transcription factors binding the CCAAT box of various gene's promoters. NF-Y's nucleosome-like properties seems to provide a stable binding for pioneer factors Oct4/Sox2 allowing and/or promoting other TFs binding. mESCs lacking one or more of the NF-Y subunits exhibit severe self-renewal defects and undergo differentiation.

It has been reported that NF-Y positively regulates the antisense transcription of Kcnq1ot1, suggesting a crucial role for NF-Y in the organization of the parent-origin-specific chromatin conformation at Kcnq1 ICR.

ChIP allele-specific performed in hybrid mESC lines showed that NF-Y binds the Kcnq1 ICR only on the non-imprinted allele. We also found other 2 ICRs bound by NF-Y in the same allele-specific way.

The aim of the project is to investigate what is preventing NF-Y from binding the imprinted allele : the ZFP57 binding, the methylation of DNA or the presence of H3K9me3.

Employing mESC lines lacking ZFP57 and SETDB1, we are going to study what is the link between NF-Y transcriptional factor and imprinting maintenance.

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Scale up of the biotechnological process to produce *two recombinant thermophilic phosphotriesterases of potential use in pesticide treatments*

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This work was supported by the Italian Ministry of the University and Research (MIUR) and by the European Union Commission (project PON_01585 to Giuseppe Manco)

Bacterial phosphotriesterases able to degrade organophosphate molecules, highly toxic compounds widely used as pesticides, are nowadays particularly interesting from scientific and industrial points of view because potentially employable as decontaminating agents in environmental bioremediation procedures. In our previous experimental work a new biotechnological process for the production of recombinant thermostable phosphotriesterases (PTE) *SsoPox* has been investigated coupling fermentation technologies and membrane-based downstream purification strategies (Restaino et al. 2017). Instead, in this study two engineered, recombinant thermostable phosphotriesterases: *SsoPox* C258L/I261F/W263A, whose wild type genes were originally isolated from *Sulfolobus solfataricus* (Del Giudice et al. 2015), and *Sac*Pox isolated from *Sulfolobus acidocaldarius* (Porzio et al. 2007) were produced using a boosted biotechnological coupled fermentation/purification process. New induction strategies, alternative to IPTG ones allowed to reach high cell density cultures for all the two bacteria. Enzyme concentrations in the range from 2900 ± 200 to 6700 ± 150 $U \cdot L^{-1}$ were obtained, according to the different strains, and the production processes resulted scalable from 2.5-L to 150-L reactors. A new downstream purification scheme was designed starting from one step of thermal precipitation, followed by an ultrafiltration/diafiltration membrane based purification protocol, after enzyme extraction from biomass by using a mechanical homogenizer. This procedure resulted robust enough to be applied to both strains allowing to achieve a final enzyme recovery that ranged from 75.0 to 80.0 ± 4.5% and a purity grade from 77.0 to 83.0 ± 5.0%, according to the enzyme.

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Erythrocytosis due to a quantitative decrease of VHL protein levels: clinical and molecular features

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Erythrocytosis is a condition characterized by an absolute increase in the red blood cell mass, hemoglobin and hematocrit levels. It is usually classified either as primary or secondary; each form can be either familiar or acquired (1). Familiar erythrocytosis can be caused by mutations in genes involved in the Oxygen Sensing Pathway (VHL, EPAS1, EGLN1) (2). ECTY2 is a congenital erythrocytosis with an autosomal recessive inheritance, caused by missense mutations in VHL and leading to an impaired HIF-degradation (3). VHL was initially identified as a tumor suppressor (4) and the inactivation or loss of both alleles causes the VHL syndrome and has been widely demonstrated in sporadic clear cell renal carcinomas and cerebellar hemangioblastomas (5). We identified two patients (C.G. and P.M.) affected by erythrocytosis caused by a synonym VHL-mutation, p.Val74Val (c.222 C/A). C.G. carries the mutation in association with an already described one, p.Met54Leu; the other patient (P.M.) has p.V74V in homozygosis. These patients are affected by a severe erythrocytosis with a very early onset (P.M. had an important pre-natal bradycardia and severe hypoglycemia at birth). The p.Val74Val mutation causes a new alternative splicing, inducing a 121bp-skipping and introducing a premature stop codon; the blunt VHL protein produced, loses its function. Moreover, the expression levels of wt-VHL are reduced by 5-folds, compared to a healthy control. In P.M., muscle mitochondria show significant anomalies in their oxygen-consumption. Both patients have polymorphic clinical features; in particular, P.M. shows severe hypoglycemia, growth retardation and short stature caused by IGF1-deficiency, prolonged prothrombin time and deficiency of vitamin K-dependent clotting factors, bradycardia and alterations of the cardiorespiratory dynamics.

Up-to-date (21- and 45-years old), neither of the two patients have neoplasms. C.G. and P.M. are the first patients with Erythrocytosis due to a quantitative decrease in VHL protein.

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α-LIPOIC ACID REDUCES IRON OVERLOAD INDUCED BY OXIDATIVE STRESS

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Iron overload describes a condition in which total body iron stores are increased, with or without organ dysfunction. Inherited primary iron overload syndromes mainly include hemochromatosis while secondary iron overload syndromes are due to hematological disorders such as thalassemia syndromes, transfusion-dependent anemias, aplastic anemia and myelodysplastic syndrome. Several organs are affected by iron overload including liver, heart, joints and endocrine glands. Additionally, bone alterations, such as osteoporosis and fractures, have been documented in hematological iron overload diseases and in animal models. Furthmore, increasing data indicate that ROS and oxidative stress are involved in the pathology of iron overload diseases. ROS also play an important role in the proliferation, differentiation and senescence of MSCs, important elements of hematopoietic microenvironment. (+)Lipoic acid (ALA) is a protective antioxidant agent able to act as scavenger of ROS, to regenerate endogenous antioxidants and also stimulating the glutathione synthesis and chelating metals. In the current work, we have evaluated the effects of ALA coadministrated with SFG (Sodium Ferric Gluconate) both *in vitro* and *in vivo* model. Our preliminary data indicate that ALA acts as a potent antioxidant and chelanting agent. ALA reduces oxidative stress on HS-5 cell line induced by SFG decreasingROS and HO-1 levels and increasing the GSH too. Also, the autophagy induced by iron overload, that probably acts as mechanism of cellular damage, reverts to the basal condition when ALA is coadministrated with SFG. Concerning in vivo model, zebrafish individuals treated with SFG shown that, after acute administration, Fe3+ crowds round in liver and that ALA coadmnistrated with SFG reduces the Fe3+ stores and downregulates two of the main proxidant genes such as HMOX1B and MTT1.

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Variation of *Hypoxia Inducible Factor 1 alpha* subunit (HIF-1α) and *Prolyl Hydroxylase Domain 2* (PHD2) transcripts in *Toxoplasma gondii* infection: a preliminary comparative analysis

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Toxoplasma gondii infects nearly one-third of the world population [1]. Virtually all species can be infected, but the parasite spreads more easily among some species (e.g. Pig) that infect humans more often than others (e.g. Horse). Investigating which host pathways are modulated by *T. gondii* and if there is an interspecies variation is interesting both for human and animal medicine. Experiments performed in cultured cells have shown that *T. gondii* activates the host HIF-1 to persist inside the cell. This activation is not due to an increased synthesis of HIF-1α coding mRNA, but to the stabilization of the protein subunit, via down regulation of PHD2 abundance and/or activity [2]. Since those data came from the analysis of experimentally infected cells, the aim of this study was to confirm results in naturally infected animals and compare species which differ in susceptibility to Toxoplasmosis. *T. gondii* target tissues (brain stem and diaphragm) were collected post mortem from pigs and horses. Infected or uninfected condition was assessed by immunoenzymatic assay. HIF-1α and PHD2 mRNA synthesis was measured by Real-time PCR. Results were processed byΔΔCt method and expressed as fold change among the target mRNAs, normalized to β-actin. Performed analysis revealed that *T. gondii* doesn't significantly alter neither HIF-1α nor PHD2 transcripts levels. Comparing uninfected animals from a naturally resistant species (Horse) and a naturally susceptible one (Pig), a significantly higher level of HIF-1α transcripts was unexpectedly detected in brain stem of the first one compared to the latter, while no difference was observed for PHD2. Further analysis should be performed to assess if this variation of brain HIF-1α transcripts in Horse compared to Pig influences the protein abundance.

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NOVEL 1,2 BENZOQUINONE-BASED DERIVATIVES SUPPRESS 5-LIPOXIGENASE AND INDUCE ENDOPLASMIC RETICULUM STRESS-MEDIATED CELL DEATH IN HUMAN GLIOBLASTOMA

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Human brain tumors are characterized by high expression of 5-Lipoxygenase (5-LOX), a versatile class of oxidative enzymes involved in arachidonic acid metabolism, which promotes the proliferation of glioma cells.[1] Several studies show that Embelin (2,5-dihydroxy-3-undecyl-1,4-benzoquinone), a well-known inhibitor of 5-LOX, is able to suppress human glioma cell growth and induce apoptosis by activation of caspases and NF-κB signaling pathway.[2] Taking into account that 5-LOX can represent an attractive therapeutic target, we investigated whether novel 1,2-benzoquinone derivatives (EA100C and EA100C red) were able to hinder glioblastoma (GBM) cancer cell growth by inhibiting 5-LOX. The in vitro antitumor effects of EA100C and EA100C red on two different GBM cell lines (U87-MG and LN229) were evaluated by MTT assay. We found that EA100C was more potent on U87-MG, while EA100C red had a higher antiproliferative effect on LN229. The effect of the two compounds on cell cycle, apoptosis, mitochondrial potential and autophagy was assessed by flow cytometry (FACS). Both the compounds induced a significant increase of apoptosis and autophagy and a block of the cell cycle, but only EA100C red induced a significant variation in the mitochondrial potential. The mode of action was confirmed by real time PCR and western blot analysis. Both the compounds induced apoptosis through a caspase-dependent mechanism, but only EA100C red led to the activation of CHOP, JNK, Beclin and NFKB, which are involved in the mechanisms of cell death related to the ER stress. Confocal microscopy analysis confirmed that EA100C red was able to induce ER stress. These results indicate that novel benzoquinone derivatives can be useful in inhibiting in vitro GBM cell growth and represent promising compounds for designing a new class of anti-cancer treatment.

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LIGNANAMIDE ENRICHED EXTRACT FROM HEMP SEED: QUALI- AND QUANTITATIVE LC HRMS ANALYSIS AND CYTOTOXICITY ASSESSMENT

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Hemp (*Cannabis sativa* L.) is an ancient crop, cultivated worldwide until the early twentieth century.[1] The interest in this multipurpose crop delivering fibers, shives, and seeds has been recently renewed, thanks to the increasing demand for natural fibers and/or for seeds with high content and quality of protein and oil. In this latter context, the abundance of essential fatty acids, proteins and fiber, along with the current awareness about their nutritional and functional role in human diet, has claimed hemp seed as a nutritious superfood.

The understanding of the (poly)phenol heritage of these seeds is not fully available.[2] To this purpose, commercial hemp seeds underwent ultrasound assisted maceration using hexane first and then ethanol as extracting solvents. The fractionation of ethanol extract, through SiO_2 column chromatography, provided a fraction, which was chemically characterized by Ultrahigh-Performance Liquid Chromatography coupled with High-Resolution Mass Spectrometry (UHPLC-HRMS) techniques. In particular, full negative mode scan and data-dependent tandem mass spectrometry, employed for the first time for the analysis of these molecules, considerably resolved the fraction complexity highlighting its richness in hydroxycinnamoyl amine conjugates and lignanamides.

The fraction, whose scavenging capability was assessed by DPPH and ABTS methods, was evaluated for its cytotoxicity by means of MTT, SRB and LDH assays. Data obtained highlighted that it exerted a dose- and time-dependent anti-proliferative efficacy towards all the tested cell lines (human keratinocyte HaCaT cells, hepatoblastoma HepG2 cells, epithelial colorectal adenocarcinoma Caco-2 and neuroblastoma SH-SY5Y cell line). In particular, upon treatment with fraction dose levels higher than 25 µg/mL clear cell morphological changes were observed. In fact, the cells shrunk and became round.

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An example of dual nature: characterization of SIRC rabbit corneal cells

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Aim of the present study is to investigate, in Statens Seruminstitut Rabbit Cornea (SIRC), the presence of both epithelial and fibroblastic markers, comparing their levels with those in Human Retinal Pigmented Epithelial cell (ARPE-19) and Human Keratocyte (HK), respectively. SIRC cells, often described as of epithelial origin, are principally used as a corneal epithelial barrier model in order to study the permeability of ophthalmic drugs. However, they exhibit a phenotype that is more consistent with a fibroblastic cell phenotype such as the corneal keratocyte. Our results show that cytokeratin expression level (evaluated by differential extraction and Coomassie-blue staining) and tight junctions' protein expression level (evaluated by western blot immune-detection), were lower in SIRC than in ARPE-19. Moreover, SIRC cells resulted positive by immunostaining for keratocyte transition markers such as lumican, α-SMA and vimentin. In SIRC cells, significantly lower levels of both lumican and vimentin were detected, whereas α-SMA protein levels were comparable in both cell lines. In conclusion, our hypothesis is that SIRC cells exhibit a hybrid nature between epithelial and keratocyte cells.

S-Adenosylmethionine Inhibits the Growth of Human Breast Cancer Cell by Promoting Autophagy and Apoptosis

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S-Adenosyl-L-methionine (AdoMet or SAM) is a natural biomolecule, which plays a pivotal role in cellular metabolism. A variety of pharmacological effects are attributed to the molecule in the treatment of liver diseases, depression, and other pathological conditions. Recently, we have shown that AdoMet enhances the anti-tumor activity of Doxorubicin on hormone-dependent breast cancer CG5 cells, inducing apoptosis by the activation of the death receptor Fas (1).

Based on these results, we evaluated the antiproliferative effect of AdoMet on hormone-dependent breast cancer MCF-7 cells. MCF-7 cells were incubated with increasing concentrations of AdoMet ranging from 8 to 500 μM for 24, 48 and 72 hours and then cell proliferation was determined by MTT assay. AdoMet causes a dose- and time-dependent reduction of cell viability with EC50 value of 500 μM and 200 μM, after 48 and 72 hours, respectively. We demonstrated that AdoMet strongly inhibits the proliferation of MCF-7 by inducing both autophagy and apoptosis. In details, AdoMet enhances the levels of the autophagic markers beclin-1 and LC3B-II and causes a significant increase of pro-apoptotic Bax/Bcl-2 ratio, paralleled by poly(ADP ribose)polymerase (PARP) and caspase-9 and 6 cleavage. We also evaluated the combination of AdoMet and the autophagy inhibitor chloroquine (CLC) in order to assess whether inhibition of autophagy could modulate the antiproliferative effect of AdoMet. The results showed that autophagy block is synergistic in inducing both growth inhibition and apoptosis, with a parallel inhibition of AKT and MTOR activity and by increasing the cleavage of caspase-6 and PARP.

These data suggest, for the first time, that autophagy can act as an escape mechanism from the apoptotic activity of AdoMet, and that AdoMet could be used in combination with CLC or its analogs in the treatment of breast cancer.

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Effects of T3 and 3,5 T2 on insulin sensitivity and related metabolic changes in muscle cells treated with fatty acids

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T3 and its metabolite 3,5-T2 both increase skeletal muscle insulin sensitivity but it is unknown if they act directly on muscle or only indirectly through increased hepatic FA oxidation. Furthermore, the metabolic effects of thyroid hormones in animal models vary with the diet's FA composition or the animal model. To invstigate this, we studied whether T3 and 3,5-T2 prevent insulin resistance in myotubes induced by FAs with different saturation degrees.

Rat L6 myotubes were treated with 0.75 mM palmitate, oleate or linoleate and 100nM 3,5-T2 or T3. Cells treated were lysed for RNA isolation after 16h, or serum-starved for 2h, with a subset being treated with insulin (20nM) for 15 min. Lysates were assayed for phosphorylation of protein kinase B (PKB)/Akt at serine 473 and AMP-activated protein kinase (AMPK) at threonine 172. Gene expression analysis was performed using real-time PCR. Cell respiration was measured using an Oxygraph (Oroboros Instruments).

In the presence of insulin, the reduction of Akt phosphorylation in response to palmitate was fully prevented by 3,5-T2 (confirming data obtained in skeletal muscle of rats treated with diets based on saturated FAs), and only partially by T3. Oleate- mediated enhanced insulin sensitivity was normalized by 3,5-T2 and T3, instead linoleate-mediated enhanced insulin sensitivity was further enhanced by 3,5-T2 and T3. Cell respiration was reduced by palmitate and only increased by T3, through ATP synthesis-independent respiration. PGC-1alpha expression reflected insulin-induced Akt phosphorylation caused by the FAs and thyroid hormones.

These results show that 3,5-T2 and T3 can act directly on muscle cells by differentially modulating FA- induced insulin resistance, which indicates that dietary backgrounds profoundly alter the effects of thyroid hormones on muscle insulin sensitivity *in vivo*. This may be of importance for the design of insulin sensitizing compounds in general

A novel TetR-like transcriptional regulator is induced in acid-nitrosative stress and controls expression of an efflux pump in mycobacteria.

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Tuberculosis (TB) is still endemic in many low and middle-income countries and the high incidence of *M. tuberculosis* multi-drug resistant strains continues to plague the control of TB worldwide. *Mycobacterium tuberculosis* has the ability to survive in the macrophage under acid- nitrosative stress. The expression profile of *M. tuberculosis* and *M. smegmatis* exposed to acid-nitrosative stress shows up-regulation of *M. smegmatis MSMEG_3765* and of its ortholog, *M. tuberculosis Rv1685c* (1). Both genes are annotated as TetR transcriptional regulators. This family of proteins regulates a wide range of cellular activities, including multidrug resistance, efflux pumps, virulence and pathogenicity (2). Microarray and RT-qPCR analysis, conducted on *M. smegmatis* wild type and *ΔMSMEG_3765* strains, show that *MSMEG_3765* is a repressor of the *MSMEG_3762/63/65* operon. This was confirmed by GFP analysis performed on both *MSMEG_3762* and *Rv1687c* upstream regions. By electrophoretic mobility shift assay (EMSA) with the purified recombinant MSMEG_3765 protein we were able to confirm its binding motif, which had been previously identified by bioinformatics analysis. This 36 bp motif is located in the upstream regions of *MSMEG_3762/63/65* and *Rv1687c/86c/85c* operons, spanning into the coding sequences. *MSMEG_3762* and *MSMEG_3763* are annotated as ABC transporter ATP-binding protein and ABC transporter, respectively, as well as their orthologues in *M. tuberculosis.* These results suggest that the TetR repressor *MSMEG_3765/Rv1685c* controls expression of an efflux pump with an, as yet, undefined role in the mycobacterial response to acid-nitrosative stress. In this contest, strains carrying deletions in *MSMEG_3763* and *Rv1686c* are under investigation.

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Allelic variants in α and β-globin genes of the river buffalo *Bubalus bubalis L.* as markers of useful genotype differentiation

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The complex hemoglobin polymorphism of river buffalo (*Bubalus bubalis L.*) was addressed. Four α-amplicons extending 1311 bp were obtained from the genome of river buffalo using primers designed on the basis of α-globin gene sequences from goat; in the same way, five β amplicons extending from 1645 to 1803 bp were obtained using primers from β-globin gene sequences from sheep. All amplicons were sequenced and the multiple alignments of the β-globin genes pointed to a high degree of homology, the greatest difference being in intron 2 of a β allele called *HBBA*, containing a 119 bp insertion. Indeed, near to this region, all five β-genes contained sequences identified as transposable elements, belonging to the family of short interspersed nuclear elements. Among the five genes, two were easily identified as alleles of adult β-genes and called *HBBA* and *HBBT*, whereas the remaining genes were identified as embryonic (*HBE*), fetal (*HBG*) and pseudo gene (*HBψ*).

Concerning the four α-globin genes, the differences emerging allowed the characterization of the two haplotypes A and B even in river buffalo. In particular, haplotype A consists of two genes, called *HBA1A* and *HBA2A*, differing at codons 129 and 131, whereas haplotype B, with *HBA1B* and *HBA2B*, differs at codons 7, 10 and 11. Moreover, both genes of haplotype A differ from those of haplotype B at codon 64, where the triplet GCC for Ala changed to ACC for Asn. A multiple alignment definitively characterized their primary structures, thus contributing to highlight the different rate of gene homogenization. Finally, based on the alignments of the α-globin genes among some ruminant species, a phylogenetic tree was obtained through the Neighbor-Joining method, in order to analyse the evolutionary events occurring in the species.

Determining the keratan sulfate contamination in animal origin chondroitin sulfate

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Chondroitin sulfate is widely used as active principle of anti-osteoarthritis drugs and nutraceuticals, manufactured by extraction from animal cartilaginous tissues. During the manufacturing procedures, another glycosaminoglycan, like the keratan sulfate, might be contemporarily withdrawn, thus eventually constituting a potential contaminant. To satisfy the strict regulatory rules on the pureness of pharmaceutical grade chondrotin sulfate specific, sensitive and reliable methods to detect keratan sulfate are needed. In this research work a multi-analytical and preparative approach was developed by employing i) high performance anion-exchange chromatography with pulsed amperometric detection, ii) gas chromatography-mass spectrometry analyses, iii) size exclusion chromatography analyses coupled with triple detector array module, on iv) strong anion exchange chromatography separation and v) NMR. Analyzing seven pharmacopeia and commercial standards, as well as nine commercial samples of different animal origin and manufacturers, we determined a KS contamination varying in the range from 0.1 to 19.0% (w/w). Analyses revealed also an heterogeneous composition of both glycosaminoglycans in terms of sulfation grade and molecular weight as well as the presence glycosaminoglycan chains still partially linked to a proteoglycan core.

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Novel Selective $\alpha_V\beta_5$ Integrin Antagonist Hidden into an Anophelin Family Protein

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In the last decades, there have been significant advances in anticancer therapy. However, the development of resistance to anticancer drugs and the lack of specificity related to actively dividing cells leading to toxic side effects have weakened these feats. As a result, there is considerable interest in looking for alternative drugs with novel antitumor mechanisms, among which the anticancer peptides (ACPs) are to be included[1].

A structural and functional characterization was recently carried out on a potent salivary thrombin inhibitor cE5, belonging to anophelin family protein, from the major African malaria vector *Anopheles gambiae*[2]. A RGD motif, known for its involvement in binding to some integrins,[3] was identified in the N-terminal region of cE5. A peptide (APQ30), encompassing the first 30 amino acids of the protein and including the RGD tripeptide, was tested on tumor cell lines proving to be able to inhibit $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins mediated adhesion. To analyze in greater detail the sequence requirements for integrin binding we designed and synthesized a shorter version of APQ30, the APQ16 peptide, encompassing the first 16 amino acid residues and fully conserved in all cE5/anophelin family members from *A. gambiae* species complex. Adhesion assays demonstrated a newly acquired specificity of APQ16 only versus $\alpha_v\beta_5$, moreover migration and invasion assays showed its capacity to inhibit the invasiveness in two malignant cell lines. Altogether our data indicate APQ16 as a new promising candidate for theranostic applications.

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Acute tadalafil administration increases plasma fatty acids without changes in inflammatory response in healthy men

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The PDE5I tadalafil has been shown to reduce visceral adipose tissue in rabbit and to improve lean mass content in non-obese men. In order to clarify this effect in humans, in the present study we determined the impact of an acute oral tadalafil administration on lipolysis by evaluating plasma free fatty acids (FFAs) and glycerol. FFAs are potential modulator of inflammation response that we evaluated through tumor necrosis factor alpha (TNF), interleukin 6 (IL6), interleukin 8 (IL8) and interleukin 10 (IL10) plasma levels. Moreover, we determined whether the effects of tadalafil would be reflected in variation of plasma levels of cGMP and NO, two important molecules involved in PDE5i signalling.

Twelve healthy subjects were supplemented with tadalafil 20 mg or a placebo, in a double blind, randomized, cross-over design. Blood samples were collected immediately before, and at 2, 6, and 24 hours post ingestion, and assayed for biochemical analysis.

A condition effect was noted for FFAs and glycerol ($p < 0.0001$) with values higher for tadalafil compared to placebo group at 2 and 6 hours post ingestion. No statistically significant effects were noted for glucose, cGMP, nitrate and nitrite. No inflammatory response was induced by tadalafil.

Tadalafil, in human subjects, increases lipolysis as evidenced by a significant increase in circulating FFAs and glycerol without affecting plasma cGMP and NO levels, noticeable the increase in FFAs did not develop an inflammatory response. Further well-controlled studies are warranted to assess the impact of tadalafil administration on weight/fat loss.

The binding of MeCP2 and major satellite transcript to chromocenters is mutually dependent and critical for heterochromatin organization

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The methyl-CpG binding protein 2 (MeCP2) is an ubiquitous modulator of transcription predominantly expressed in the brain and mutated in Rett syndrome, a progressive neurodevelopmental disorder.

Since its discovery, MeCP2 has been linked to global chromatin organization. In neurons, MeCP2 genomic distribution tracks the methyl-CpG density and its absence results in large-scale changes in chromatin structures. In mouse cells, MeCP2 accumulates to pericentric heterochromatin (PCH) domains called chromocenters, closely resembling the distribution of major satellite DNA. These structures seems to be critical for the establishment of silent compartments. Several proteins and non coding RNAs (ncRNAs) seem to be relevant for the establishment and maintenance of PCH.

We previously highlighted a crucial role of MeCP2 in the PCH re-organization during neural differentiation, supporting the view of MeCP2 as a multifunctional chromatin organizing factor.

Our study aims to clarify the mechanisms underlying MeCP2-mediated PCH organization and maintenance during neural differentiation and to investigate the role of major satellite ncRNAs in this phenomenon.

Taking advantage of Mecp2$^{-/y}$ murine embryonic stem cells differentiating to neurons, we provide the first evidence for a role of MeCP2, and especially MeCP2B isoform, in the recruitment of major satellite forward (MajSat-fw) transcript to PCH. Moreover, through molecular and imaging assays we established the physical and spatial interaction between MeCP2 and MajSat transcripts. Furthermore, RNAseA treatment and knock-down experiments demonstrated that MajSat-fw transcript is, in turn, involved in MeCP2 recruitment to PCH.

Finally, we proved that MeCP2 is important for the proper enrichment of H3K9me3 and H4K20me3 histone modifications to PCH.

Altogether, our data support the hypothesis that MeCP2 and MajSat-fw transcript are mutually dependent for the PCH organization and maintenance and adds another piece to the molecular scenario that links lncRNAs with chromatin function. Finally, our findings strengthen the role of MeCP2 in the regulation of chromatin architecture.

Effects of Neu3 sialidase activation on cardiac fibrosis

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Cardiac fibrosis is a physiological response to tissue injury with deposition of extracellular matrix proteins by activated myofibroblasts. Interestingly, the process protects the heart from wall rupture, but in the long run its progression increases heart stiffness, causing a decrease in heart contractility (1). In this context, TGF-β signaling pathway is responsible for myofibroblasts activation, although other pathways have been shown to be involved (2, 3). Moreover, it has been reported that gangliosides play a role in the process, as their biosynthesis is altered in hepatic myofibroblasts differentiation (4) and ganglioside GM3 is involved in the TGF-β pathway (5). Actually, we reported the involvement of sialidase NEU3, which is known to modulate GM3 content in the cell lipid rafts, in cardiac and skeletal muscle cell response to hypoxia, revealing the link between NEU3 and the hypoxia inducible factor (HIF), the master regulator of cell response to oxygen deprivation (6,7).

Therefore, the aim of the study was to assess the role played by NEU3 in myofibroblast activation in response to pro-fibrotic stimuli.

TGF-β treatment of human cardiac fibroblasts caused their activation, as they acquired a myofibroblast phenotype, confirmed by α-SMA, smoothelin, transgelin, collagen I and III increase. Moreover, treated cells showed higher levels of α-SMA and collagen I protein and formation of stress fibers, hallmarks of active myofibroblasts. Alterations of genes involved in ganglioside synthesis were also observed: in particular, Neu3 significantly decreased both in expression and enzymatic activity after TGF-β treatment. Finally, upon Neu3 overexpression, we observed a decrease in myofibroblast activation, counteracting the TGF-β-induced increase of α-SMA and collagen I.

In conclusion, these preliminary results unveil the involvement of sialidase Neu3 in myofibroblast activation. In particular, we found that Neu3 activation seems to play an important role in fibrosis reduction, suggesting a new possible target for cardiac fibrosis modulation.

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Effects of sex differences in response to inflammation under cellular stress-mimicking conditions[a]

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Sex differences have been demonstrated in inflammatory processes and in chronic inflammatory diseases (1). The stress hormone Cortisol modulates immune activity and showed different levels in male and female populations; men seem to have higher basal cortisol levels than women, nevertheless in response to acute stressors, cortisol levels were comparable between men and women (2,3).

As little is known how the different levels of cortisol are able to modulate cytokine release, the purpose of this study was the investigation, in human lymphomonocytes from male and female donors, of sex-related differences in release of inflammatory mediators under stress-mimicking conditions. In order to achieve this goal, the effects of cortisol acute exposure on the production of cytokines and other molecules involved in the inflammatory pathway, such as the protein complex NFkB, IDO and kynurenine, have been determined. Lymphomonocytes were stimulated *in vitro* with drugs able to induce a strong immune response in mammalian cells, lipopolysaccharide (LPS) or Lectin (PHA). The cortisol sensitivity has been defined as the concentration of cortisol required to inhibit by 50% the LPS or PHA stimulated cytokine release (4). Appropriate statistical tests were performed to analyse the obtained data.

Under physiological condition, the results evidenced that the levels of released cytokines and kynurenine were different between women and men. Following the inflammatory insults, these sex differences were maintained, nevertheless the stress responsiveness in release of immune mediators was slightly different between men and women.

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COMPARATIVE PROTEOMIC APPROACH OF MITOCHONDRIAL PROTEINS IN A MOUSE MODEL FOR CREATINE TRANSPORTER DEFICIENCY

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Mutations in the creatine (Cr) transporter (CrT) gene lead to cerebral Cr deficiency syndrome-1 (CCDS1), an X-linked metabolic disorder characterized by cerebral Cr deficiency causing intellectual disability, seizures, movement and autistic like behavioural disturbances, language and speech impairment. The lack of knowledge about the effects of Cr deficiency on neuronal circuits derives at least partially from the paucity of studies on animal models.

Evidence of mitochondrial dysfunction in animal models of Cr deficiency suggests that mitochondrial function may also be abnormal. In this study for the first time we investigate the mitochondrial proteome in a CCDS1 mouse model with the aim to show the potential protein alterations induced by CCDS1. Mitochondria were obtained by differential centrifugation from brain of wild type (CrT +/y, n=3) and knock-out (CrT -/y, n=3) mice sacrificed at 12 and 30 days-old. Mitochondrial proteins of CrT +/y and CrT -/y samples were separated by 2DE and proteomic profiles compared by Same spot software. Fifty-five spots resulted differentially expressed in significant manner in CrT -/y with respect to CrT +/y at 30 days-old, in particular 14 spots showed fold > 2. All these spots were increased in CrT-/y deficiency mice. Only 4 spots showed a significant difference of expression after comparison of mitochondrial maps of mice at 12 days-old with fold of increase in CrT -/y with respect to CrT +/y ranging from 1.2 to 1.5. Spots of interest were cut and analyzed by mass spectrometry. Identified proteins belong to mitochondrial respiratory chain, and to oxidative stress. Finally, Ingenuity Pathways analysis was also performed and the network generated involves developmental and hereditary disorder and metabolic disease. Overall our results suggest that the lack of uptake of creatine actives the processes of ATP production and as a consequence the increase of systems involved in controlling oxidative stress.

3,5-diiodothyronine (T_2) induces browning of white adipose tissue through different pathways

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White adipose tissue (WAT), in response to appropriate stimuli, can undergo a process known as Browning where it takes on characteristics of brown adipose tissue (BAT). Many molecular factors involved in this processes have been identified, such as, among the others, miRNA and irisin. As it has been shown that the 3,5-diiodo-L-thyronine (T_2), an endogenous metabolite of thyroid hormones, stimulates energy expenditure, the aim of this study is to verify whether T_2 could induce browning and to investigate the underlying mechanism. In subcutaneous white adipose tissue (SAT) of HFD-T_2 rat, we observed an increase of the uncoupling protein 1 (UCP1) expression and a modulation of the expression of miR-133a and miR196a, when compared to N and HFD animals. Associated to reduction of miR-133a there was an increase of Prdm16 expression, a critical regulator of brown adipocyte development, and associated to an increase to miR-196a there was a reduction of Hoxc8, a repressor of adipogenic marker C/EBPβ, which resulted increased. Furthermore, T_2 increased serum levels of irisin, a myokine acting via the stimulation of ERK pathway and that regulates positively white-to-brown adipocytes conversion.

The present data demonstrate that T_2 is able significantly to affect important pathways underlying the browning processes such as, among others, miR133a, miR196a and irisin. These effects may constitute part of the mechanism by which T_2 exerts the well-known stimulatory effect on metabolism.

Non-Coding RNAs Regulated by S-Adenosylmethionine: New Strategies for Pharmacological Treatment of Breast Cancer

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The naturally-occurring sulfonium compound S-Adenosyl-L-methionine (AdoMet) is an ubiquitous molecule exerting a series of well-documented biological functions in all living cells (1). It has been demonstrated that AdoMet exerts cell-type specific antiproliferative effects and is involved in tumor suppression through the regulation of multiple cell processes, such as cell cycle regulation, proliferation and apoptosis. Several epigenetic modifications have been linked to cancer, including post-translational modification of histones, DNA methylation, and the most recently discovered non-coding RNAs. Recently, a new class of small endogenous non-coding RNAs, known as microRNA (miRNAs), has been associated with several human diseases including cancer.

The aim of the work is to evaluate the regulation of miRNA expression profiling after AdoMet-treatment in MCF-7 breast cancer cell line. Microarray analysis after 48 hours of MCF-7 treatment with 500 µM AdoMet revealed 3 significantly modulated miRNAs (miR-34a, miR-34c and miR-486-5p), compared to controls. In particular, miRNA 486-5p was significantly 2-fold downregulated and this result was then confirmed by quantitative real-time PCR (qRT-PCR). Thereafter, we transfected MCF7 cells with either miR-486-5p mimic or inhibitor in free medium or medium supplemented with 500 µM AdoMet, for 48 and 72 hours. In these experimental conditions, miR-486-5p inhibitor alone induced autophagy and inhibited MCF7 cell proliferation that was potentiated by the addition of AdoMet. On the other hand, miR-486-5p mimic caused opposite effects. Autophagy occurrence was confirmed by Western blot analysis of the main autophagy markers including LC3BI-II, Beclin and p62.

Overall, this study contributes to better understand the mechanism of action of AdoMet in breast cancer cells, highlighting a miRNA-based pathway that supports the antiproliferative activity of the sulfonium compound.

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Synergic effect of curcumin and ellagic acid on genomic stability of human amniotic cells

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Amniotic cells constitute the corpusculated fraction of the amniotic fluid. Pregnancy is susceptible to oxidative stress closely related to the pathogenesis of many fetal diseases. It is known that many natural antioxidant molecules are able to counteract oxidative imbalance, so this study has been focused on evaluating the individual antigenotoxic effects of ellagic acid (EA) and its combination with the curcumine (CUR) on human amniotic cells *in vitro*. Both polyphenols are known to have antioxidant effects, anti-inflammatory action, and a significant antitumor potential occurring through the inhibition of cell proliferation thanks to the arrest of malignant G1 cells.

The study presents new data on the DNA damage in amniotic cells exposed *in vitro* to a known oxidant agent H_2O_2 (15 µM) and to the curcumine (40 µM) plus ellagic acid (100 µM) for different times (48 and 72 hours). The genotoxicity has been highlighted by using two different experimental approaches (TUNEL test and RAPD-PCR technique).The results of the TUNEL test showed a statistically significant increase of the DNA fragmentation after 48 hours of exposure. The co-exposure to the antioxidants shows DFI% values comparable to that of the negative controls already starting from the minimum exposure time. The RAPD-PCR analysis showed a variation of the polymorphic profiles of the amniotic cells DNA exposed to H_2O_2 with respect to the control amniotic cells DNA. The evidence from the value of GTS showed statistically significant increase of the damage to DNA caused by H_2O_2, as opposed to an increase in the genomic stability of the template in the samples treated with the combination of the two molecules. The results provide a starting point for investigation on the potential protective rule that the two antioxidants may have in maintaining maternal oxidative balance

MICRORNA-449A AS POWERFUL BIOMARKERS AND A THERAPEUTIC TARGET OF NODAL METASTASES IN LARYNGEAL CANCER PATIENTS

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Laryngeal cancer (LCa) is a leading cause of death worldwide. Treatment of advanced LCa still remain challenges despite recently improved therapeutic strategies. Therefore we urgently need to prepare new methods for accurate diagnosis, prediction response to therapies, and more effective treatment options.

MicroRNAs (miRNAs) are a class of small, highly conserved non-coding RNAs that negatively regulate mRNAs at a post-transcriptional level. MiRNAs may work as either oncogenic promoters or tumor-suppressors. Increased evidence has shown that specific miRNAs are aberrantly expressed in various diseases, including LCa. Hence miRNAs are emerging as powerful biomarkers as well as therapeutic molecular targets of the malignancies.

Deregulated miRNA in LCa patients suffering from metastases remains to be elucidated[1]. In this study, we investigated characteristic miRNA signatures in LCa patients with nodal metastases to identify candidate miRNAs which are excellent tools as biomarkers or targets of LCa with metastases.

We first performed global microarray analysis for the evaluation of miRNA levels in both LCa tissue and serum. Clinical specimens were taken from LCa patients with either metastasis (N+) or no metastasis (N-). Our microarray study showed that 4 miRNAs (miR-133b, miR-223, miR-449a, and miR-652) were significantly deregulated in N+ comparing to N-. We subsequently validated the miRNAs levels using quantitative real-time PCR. Only miR-449a was much aberrantly downregulated and was chosen as a candidate for further biological studies.

We predicted potential targets of miRNA-449a with bioinformatics tools. Among predicted targets, we focused on Notch2, which is a Notch family member and is known an association with cancer metastatic potential in LCa. Hep2, a laryngeal carcinoma cell line, was used for studies in vitro.

Concluding that miR-449a may be suitable for biomarkers to diagnose and to prognosticate and a promising therapeutic target of LCa with metastases.

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β-Sitosterol Reduces The Expression of Chemotactic Cytokine Genes in Cystic Fibrosis Bronchial Epithelial Cells

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Extracts from *Nigella arvensis* L. seeds were able to inhibit the expression of the pro-inflammatory neutrophil chemokine Interleukin (IL)-8 in Cystic Fibrosis (CF) bronchial epithelial IB3-1 cells exposed to *Pseudomonas aeruginosa*. CF is a severe genetic disease due to defects of the CF Transmembrane Conductance Regulator (CFTR) gene. Chronic pulmonary disease is the leading cause of reduced expectancy of life. It is well established that chronic infection sustained by *P. aeruginosa* is a hallmark of CF lung disease, associated with an excessive lung inflammation and characterized by huge infiltrate of neutrophils in the bronchial lumen, mainly due to the release of IL-8. The research regarding modern therapies to neutralize the inflammation in CF patients is aimed at finding new putative anti-inflammatory drugs. The extracts chemical composition led to the identification of three major components, β-sitosterol (BSS), stigmasterol, and campesterol. BSS was the only compound that significantly reproduced the inhibition of the *P. aeruginosa*-dependent expression of IL-8. BSS (100 nM) was tested in CF airway epithelial CuFi-1 cells infected with *P. aeruginosa* showing a consistent inhibitory activity on expression of the *P. aeruginosa*-stimulated expression chemokines IL-8, GRO-α GRO-β, which play a pivotal role in the recruitment of neutrophils in CF inflamed lungs. Preliminary mechanistic analysis showed that BSS partially inhibits the *P. aeruginosa*-dependent activation of Protein Kinase C isoform alpha, involved in the transmembrane signaling activating IL-8 gene expression. These data indicate BSS as a promising molecule to control excessive inflammation in CF patients[1].

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Alternatively spliced EDA fibronectin plays a key role during bone marrow regeneration

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Abstract: In the bone marrow (BM), the hematopoietic stem cells (HSCs) give rise to all blood cell lineages. The maintenance, differentiation and proliferation of HSCs are regulated in both cell-autonomous and non-cell-autonomous niche-mediated fashions. Understanding the mechanisms that govern HSC niche maintenance is fundamental for BM regeneration[1].

Prompt deposition of Fibronectin (FN)-rich extracellular matrix is a critical feature of tissue healing and the host-response to injury. Tissue FN appears in different isoforms, due to alternative mRNA splicing of the EDA, EDB, and IIICS regions, and subsequent post-translational modifications[2].

Alternatively spliced EDA FN isoforms are absent in adult tissues but highly expressed during physiological or pathological tissue remodelling, particularly in sites of injury, chronic inflammation and solid tumors. Although its function is not well understood, EDA FN has been previously linked to a variety of *in vitro* cellular events and *in vivo* molecular outcomes, including cell cycle progression, myofibroblast differentiation and protection of vessel walls in conditions of disturbed flow. However, effects of EDA FN variant on HSC behaviour are largely unknown. We recently demonstrated that during physiological hematopoiesis a small fraction of BM derived FN contains the EDA domain and that mice constitutively including (EIIIA+/+) or excluding (EIIIA-/-) the EDA exon present comparable levels of HSCs, myeloid and lymphoid progenitors in the BM[3]. In this work we demonstrated that, during 5-Fluorouracil-mediated myelosuppression, EDA FN is expressed by endothelial and stromal cells and sustains the release of matrix metalloproteinases and pro-inflammatory cytokines through engagement of Toll Like Receptor 4 and downstream activation of NF-κβ. Exclusion of EDA exon in mice leads to a delayed BM recovery and increased lethality due to abnormal vascular niche healing, as demonstrated by reduced HSC differentiation, stromal activity and vascular integrity. In conclusion, we delineated a new physiological role of EDA domain of FN in supporting the

inflammatory and fibrotic responses that precede the endothelial/hematopoietic rebound during BM regeneration after chemotherapy.

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ANNURCA APPLE POLYPHENOLS INDUCE CELL CYCLE ARREST AND APOPTOSIS IN HUMAN BREAST CANCER CELLS BY INCREASING OXIDATIVE STRESS

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The potential of dietary components, in particular polyphenols as antiproliferative agents has been evidenced in the literature, and growing scientific interest is focused on identifying the biological mechanisms and the signal transduction pathways related to the chemopreventive activities of these compounds. Most of the advantageous effects of natural polyphenols have been ascribed to their ability to scavenge free radicals endogenously generated or formed by radiation and xenobiotics. However, emerging evidences indicate that the anticancer and chemopreventive properties of polyphenols are mainly related to their pro-oxidant activity (1). The antiproliferative properties of apple polyphenols have been described extensively by *in vitro* studies and great interest has been paid to *Annurca* apple, one of the most important cultivars of southern Italy which is characterized by an extremely high content of polyphenols. The pro-oxidant, antiproliferative, and pro-apoptotic effects of *Annurca* apple polyphenol extract (APE) in human breast cancer MCF-7 cells have been investigated and the potential underlying molecular mechanisms have been explored. We show that APE at concentrations higher than 100 µM catechin equivalents induces lipid peroxidation and strongly inhibits the proliferation of MCF-7 cells causing G2/M cell cycle arrest and apoptosis. Immunoblot analysis demonstrated that APE treatment increases the levels of p53 and p21, down-regulates the expression of the cell cycle regulatory protein cyclin D1, and inhibits ERK1/2 phosphorylation. Moreover, APE treatment causes a marked increase of proapoptotic Bax/Bcl-2 ratio paralleled by caspase 9, caspase 6, caspase7, and poly(ADP ribose)polymerase cleavage. Our findings suggest that APE-mediated ROS generation probably represents the central trigger for the antiproliferative activity of these compounds in MCF-7 cells and allow to propose *Annurca* APE as a promising target for further investigations finalized to the design of innovative adjuvant therapies in breast cancer treatments.

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Urtica dioica: a new promising tool in chemotherapies strategies against non-small cell lung cancer (NSCLC)

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Urtica dioica is a medicinal plant native form America, India, Malaysia and tropical countries. Principal profile constituents of the plant include formic acid, tannis, mucilages and phytosterins.

The use of *U. dioica* has been observed since ancient times in alternative medicine, different pharmacological properties like: antioxidant, antimicrobial, antiulcer and antiproliferative, these latter recently documented. Infact, the effects of an aqueous extract of *U. dioica* against the MCF-7 (3), prostate cancer tissues and HeLa cells (5) have been specifically reported.

Non-small cell lung cancer (NSCLC) is the principal cause of cancer-related deaths worldwide (1). Until few years ago the main therapeutic option for patients with advanced NSCLC was platinum-based chemotherapy witch result in a limited improvement of median overall survival (OS) and a 5-years survival rate <1% (2). For this reasons new robust preventive and therapeutic strategies in case of lung cancer are strongly needed.

Pursuing the assessment of the pharmacological properties of *Urtica dioica*, in the current work we have combined an extensive 2D-NMR (HSQC, TOCSY, CIGAR-HMBC, H2BC, HSQC-TOCSY) investigations (2) of hydroalcholic extract of *Urtica dioica* with evaluation of cytotoxic effect against on panel of NSCLC cancer cells as well as on the normal bronchial epithelial cells alone and in combination with cisplatin to evaluate the synergistic effects. NMR data allowed to identify different compounds belonging tethahydrofuranc lignans, flavonol glycosides, oxilipins classes as principal constituent of active extracts.

Cell cycle distribution and apoptosis, as well as combined effects of *Urtica dioica* extract and cisplatin on selected NSCLC cell lines, in order to evaluate the synergistic effects, have been investigated.

These effect would be a promising strategy to reduce adverse effect and improve cancer chemotherapy efficiency.

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Placental expression of nitric oxide synthase and vascular endothelial growth factor in obese pregnant women

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Angiogenesis is strictly related to gestational success, since an adequate placental vascularization allows the interaction between maternal and fetal tissues throughout gestation, and consequently a proper fetal development. A number of factors regulates normal angiogenesis, among which the vascular endothelial growth factor (VEGF). In addition to its role in promoting angiogenesis, VEGF induces the synthesis of nitric oxide (NO), which has a crucial role in maintaining a low vascular resistance in the feto-placental circulation (1). Nevertheless, at high concentrations it may combine with excess superoxide to produce peroxynitrite, which can rapidly react with proteins, giving rise to nitrotyrosine.

Placental NO synthase (NOS) expression has been evaluated in both normal and high-risk pregnancies (2). However, there is lack of evidence about its expression in placental tissue from obese women, although the number of women of reproductive age who are overweight or obese is growing worldwide.

For this reason the aim of our study was to evaluate the expression of VEGF, eNOS, iNOS and nitrotyrosine in placentas of both normal weight and obese women by means of immunohistochemistry and to assess placental NO levels in the same groups of subjects.

eNOS immunoexpression was significantly higher in the placentas from obese women compared to the controls, while iNOS showed a similar expression in the two groups. Finally, VEGF and nitrotyrosine expression, as well as NO production were significantly increased in the placental tissue from obese women in comparison with controls.

Our results suggest that the up-regulation of both VEGF and eNOS may act as a compensatory mechanism for changes in placental blood flow. These alterations in placental vascularization may result from an increased nitrative stress caused by obesity.

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Antiproliferative Effect of the Methyl Donor S-Adenosylmethionine in Oral Tongue Cancer Cells

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S-adenosyl-L-methionine (AdoMet) is a naturally-occurring compound found in almost every tissue and fluid in the body, involved in important biochemical process. Indeed, AdoMet is the link to three key metabolic pathway: polyamine synthesis, transmethylation and transsulfuration (1). In recent years, several new metabolic functions have been assigned to this important and widely occurring sulfonium compound that exerts pleiotropic effects on signal transduction in many and different cell-types (1). AdoMet is now considered a key regulator in different cellular processes including proliferation, apoptosis and autophagy.
In this study we have explored the antitumoral effects of AdoMet in Cal33, a oral tongue squamous cell carcinoma. We have firstly evaluated the antiproliferative effect of AdoMet using concentrations ranging from 3 µM to 500 µM for 24 and 48 hours treatment and then cell viability was determined by MTT assay. AdoMet causes a dose- and time-dependent reduction of cell viability with EC50 value of 350 µM, after 48 hours treatment. In order to assess cell death mechanisms, we have evaluated the apoptotic and autophagic process by flow cytometric analysis. The solfonium compound at the 300 µM concentration strongly inhibits the proliferation of Cal33 cells by inducing both autophagy and apoptosis. To better understand the molecular pathways underlying these processes, we have performed Western blot at 200 and 300 µM AdoMet concentrations. We have also investigated the endoplasmic reticulum (ER) stress signalling pathway by immunofluorescence after staining with lysotracker blue. AdoMet at 300 µM concentration induced ER-stress in Cal33 cells at 24 and 48 hours.

Taken together these data suggest a new antitumor process triggered by AdoMet and contribute to identify new putative prognostic markers and opportunities for targeted therapies in oral tongue cancer.

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Effects of HDAC Inhibitors on Glioblastoma Cells

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Glioblastoma Multiforme (GBM) is a high-grade glioma (grade IV) and is the most common and aggressive form of brain cancer. Treatment options for GBM are limited to surgery, chemotherapy and radiation with a poor survival outcome. Rapid migration and aggressive invasiveness are major pathobiological characteristics of GBM. GBM depends on vascular networks to supply blood, oxygen, and nutrients. Tumor blood vessels can either be formed from pre-existing blood vessels (neo-angiogenesis) or from tumor cells (vasculogenic mimicry) due to a process of epithelial-mesenchymal transition; vascular mimicry provides a mechanism whereby GBM could escape anti-angiogenic therapies.

Epigenetic mechanisms are increasingly recognized as implicated in glioblastoma pathogenesis. Unlike genetic mutations, epigenetic changes are reversible and can be targeted by drugs.

We evaluated whether different Histone Deacetylase Inhibitors (HDACis) are able to affect migration, invasion and vasculogenic mimicry in GBM cells.

It has been tested SAHA (Varinostat) and trichostatin A (TSA) as inhibitors of class I and II HDACs, MS275 (Entinostat) as selective inhibitor of class I HDACs (specifically of HDAC 1 and 3) and MC1568 as selective HDAC class II inhibitor.

We observed that TSA, MS275 and MC1568 HDACis significantly decrease U87MG directional cell migration in Boyden chamber assays and we found that HDACi MS275 is able to impair U87MG matrigel invasion.

In Tube Assay, HDACis inhibit significantly vasculogenic mimicry both in U87MG and C6 cell lines, without affecting cell viability.

Our results suggest that HDACis may be promising candidates for GMB therapies.

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Human Cryptic Host Defence Peptides: identification and analysis of their antimicrobial, anti-biofilm and immunomodulatory properties

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Antimicrobial peptides (AMPs), known also as host defense peptides, are fundamental evolutionarily conserved components of innate immunity. Constitutively or inducibly expressed in response to invasion by pathogens, they operate synergistically with other defence molecules to combat infections. Despite differences in their size and sequence, many of them share a net positive charge at neutral pH, and fold into amphipathic structures, often after contact with bacterial surfaces. AMPs are attractive alternative candidates for antibiotic treatment, because they offer several advantages over the currently used drugs. They combat pathogens by targeting bacterial membranes, thus impairing essential membrane-related functions, and, in some cases,also target intracellular components. Due to their peculiar mechanism, the resistance towards these peptides would be difficult for the bacteria to develop. Several proteins, including proteins apparently not involved in immunity, can behave as sources of AMPs hidden in their primary structures and released by the action of host and/or bacterial proteases. Recently we developed a bioinformatic tool allowing to identify such “cryptic AMPs” [1]. Analyzing a library of four thousands secreted human proteins, we have identified and studied several novel human cryptic HDPs. Among these, three peptides (GVF27, $ApoE_{(133-150)}$ and IMY47) show pharmacologically relevant properties like significant antimicrobial activity on a broad spectrum of bacteria (including some clinical isolates), very promising antibiofilm properties (both on pre-formed and attached biofilm), strong affinity for endotoxins as LPS and LTA and immunomodulatory properties on LPS induced murine macrophages [2,3,4]. These AMPs offer several advantages compared to other antimicrobial agents: (i) they are non-immunogenic due to their

human origin; (ii) specifically target bacterial strains, (iii) are not hemolytic and contribute to enhance anti-inflammatory response without impairing cell viability. Overall our data suggest that these new human cryptic AMPs, could serve as leads for the design of innovative antimicrobials with immunostimulating and immunomodulatory properties.

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A new tool to profile the UDP-sugar precursors of microbial capsular polysaccharides

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Capsular polysaccharides (CPSs) are produced by both gram-positive and gram-negative bacteria as camouflage to protect the cells from mechanical and physical stresses as well as from attack of the immunosystem of host organisms. Some of these CPSs have structures that resemble the mammalian glycosaminoglycans and are interesting as potential pharmaceutical active molecules; as for example the chondroitin and heparosan-like structures of the capsular polysaccharides of *Escherichia coli* K4 and K5, respectively. CPS biosynthesis is performed in the cytoplasm by glycosiltransferase enzymes that extend progressively the nascent chain by addition of uridine di-phosphate sugars (UDPs); UDPs are synthesized according to specific pathways starting from the carbon sources present in the growth medium. Profiling the nucleotide sugar pools of wild type and recombinant capsulated microbial strains could provide a metabolic fingerprint and it could help to understand and eventually sort out the bottlenecks in the synthetic pathways, like a limiting concentration of precursors or an unbalanced ratio between them. In this research work a new analytical tool was developed by capillary electrophoresis for the simultaneously determination of five nucleotide sugars (UDP-Glc, UDP-Gal, UDP-GalNAc, UDP-GlcNAc, UDP-GlcA) as extracted from bacterial biomasses, and commonly present in microbial CPS synthetic pathways. The method was used to profile the UDP pools of *Escherichia coli* K4 and K5 strains and it also revealed how the UDP concentrations change at the variation of the growth conditions.

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Optimization of Hyaluronan-based Eye Drop Formulations

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Hyaluronan (HA) is widely used in the eye drops formulation: it plays a key role in corneal hydration, regeneration and prolongs precorneal residence time due to its viscosifying and mucoadhesive properties.. A hydrodynamic and rheological characterization of several commercialized HA-based eye drops was firstly provided here revealing that most of the available preparations are not optimized. Then, a study aiming at optimizing HA-based eye drops efficacy, by maximizing mucoadhesiveness and viscosity was performed. HA samples with molecular weight in the range 250-1100kDa, and hybrid cooperative complexes (HCC), based on high and low molecular weight HA were considered for the study.

Hydrodynamic analyses were accomplished using a SEC-TDA system. Rheological measurements were performed using an oscillatory rheometer. Mucoadhesiveness was evaluated by means of viscosity measurements . Primary porcine corneal epithelial cells were used for biological studies.

For each HA sample, rheological studies were performed to identify the concentration maximizing viscosity and mucoadhesiveness. Compared to commercialized products, the formulations set here allow to deliver far higher biopolymer amounts without exceeding the viscosity limit for the intended application. Further, they proved enhanced viscosity and capacity to interact with mucin, especially in conditions simulating *in vivo* blinking. This allow to predict longer retention on the ocular surface and, therefore, higher efficacy. A biological characterization was performed to evaluate the formulations' ability to protect corneal epithelial cells against desiccation. For the HCC-based preparation, wound healing experiments were also performed. The set formulations overperformed conventional products for the ability to preserve corneal hydration. Time lapse experiments proved the HCC-preparation superior also in hastening corneal cell wound repair. The results obtained suggest the developed formulations as promising topical ophthalmic medical devices.

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A NEW STRATEGY NANOPARTICLE-BASED FOR PERSONALIZED LYMPHOMA THERAPY

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Nanomedicine represents a promising strategy for the development of new cancer treatments. Nanocarriers, thanks to nanoscale size, can target specifically tumor cells, avoiding toxicity to normal cells, accumulate in tumor environment through EPR effect, protect sensitive therapeutic agents from degradation, improve bioavailability, enhance therapy efficacy and increase drug tolerability. Furthermore, nanocarriers give the possibility to overcome the MDR, maintaining the drug level below a cell-killing threshold[1]. B-cell lymphoma is a clonal expansion of neoplastic cells that can have a fatal outcome. Tumorigenic B-cell lymphomas are sensitive to conventional anticancer treatments; however, the disease is associated with incomplete response to clinical treatment, resulting in a minimal residual disease, where some undetected cancerous cells supply the cancer cell reservoir *in vivo*. Despite considerable efforts in lymphoma therapy, the development of an effective strategy to target therapies to tumorigenic B cells remains challenging.

In this study, an active targeting strategy for Bcl2 siRNA delivery was developed to specifically target aggressive murine A20 lymphoma cells, using as ligand an idiotype-specific peptide endowed with high-affinity toward the B cell receptor (homing device). The idiotype determinants of the immunoglobulin B-cell Receptor of B cell malignancies are unique for a given clonal population and function as patient-specific tumor antigen that may be exploited for active therapeutic targeting in personalized cancer therapy. The choice of Id-peptides was based on the following properties: i) binding to tumor target cells with high specificity and sensitivity, both *in vitro* and *in vivo*, and ii) internalization into target tumor cells by BCR-mediated endocytosis, which overcomes drug resistance. Based on these considerations, diatomite nanoparticles conjugated with an Id-peptide and loaded with siRNA directed against *Bcl2,* highly expressed in B cell lymphoma, were explored as a ligand-mediated delivery platform.

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MiR-34a : a new potential therapeutic in Multiple Myeloma to enhance antitumor activity of anticancer drugs

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In the last decade, the outcome of patients with Multiple Myeloma (MM) has markedly improved due to the introduction of novel agents such as proteasome inhibitors and immunomodulatory drugs. However, MM commonly acquires drug resistance leading to relapse of disease[1]. Recently, miRNAs emerged to have a key role in MM pathophysiology and the replacement of oncosuppressor miRNAs provides a promising strategy against tumors[2,3]. MiR-34a acts as tumor suppressor microRNA (miRNA) in several cancers, including multiple myeloma (MM), by controlling the expression of several target proteins involved in cell cycle, differentiation and apoptosis[4]. In this study, we focus on the molecular mechanisms of miR-34a-mediated tumor suppression in RPMI 8226 MM cell line and its role in modulating responsiveness to anticancer drugs, in particular γ-secretase inhibitor (γSI), Sirtinol or zoledronic acid (ZOL). The rationale of the work was to enhance the inhibitory action of this miRNA on its canonical targets such as Notch1 and SIRT1, and on Ras/MAPK-dependent pathways. Our data demonstrate that miR-34a ectopic expression in MM RPMI 8226 cells enhances significantly the anti-tumor effects of all three anticancer agents. In details, we found that γSI potentiated the miR-34a antitumor effects overcoming a cytoprotective autophagic mechanism by activating apoptotic extrinsic pathway. Moreover, the combination between miR-34a and γSI induced an increase of calreticulin (CRT) surface expression that in turn triggers an anti-tumour immunological response. The combination between miR-34a and Sirtinol induced the activation of an intrinsic apoptotic pathway along with increased surface expression of CRT. Regarding ZOL we found a powerful growth inhibition after enforced miR-34a expression which was not likely attributable to neither apoptosis or autophagy modulation. Based on our data, the combination of miR-34a with other antitumor agents could be a promising anti-cancer strategy in MM.

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