WebPro

Proteins on the Web 2021

Scientific Program and Abstract book

Venue: online, 20-21 May 2021

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Scientific Program

Thursday, May 20

14:00 - 14:10 - Opening and Welcome address

Session 1 - Protein Stability: the importance of being folded

(Chairs: Chiara Giacomelli, Giulia Faravelli, Mariafrancesca Scalise)

14:10 - 14:50 – Opening Plenary Lecture: Patrizia Polverino de Laureto *(University of Padova)*

Probing proteins that do not undergo conformational change upon ligand binding

14:50 - 15:10 – Barbara Cellini *(University of Perugia)*

Intrinsically disordered regions of alanine:glyoxylate aminotransferase shape its fitness and function

15:10 - 15:30 – Silvia Errico *(Università degli studi di Firenze)*

The composition of lipid membranes affect their affinity for toxic and nontoxic misfolded protein oligomers

15:30 - 15:50 – Riccardo Ronzoni *(University College London)*

The molecular species responsible for α1-antitrypsin deficiency are suppressed by a small molecule chaperone

15:50 - 16:10 – BREAK with **Poster Session 1**

Session 2 - The role of proteins in infectious diseases

(Chairs: Riccardo Miggiano, Angelo Toto)

16:10 - 16:50 – Opening Plenary Lecture: Andrea Carfì *(VP & Head of Research, Infectious Diseases at Moderna)*

Development of the Moderna COVID-19 vaccine (mRNA-1273) and preparing for SARS-CoV-2 variants of concern

16:50 - 17:10 – Serena Rinaldo *(Sapienza, University of Rome)*

L-Arginine in *Pseudomonas aeruginosa* controls C-DI-GMP levels and biofilm formation

17:10 - 17:30 – Omar De Bei *(Università degli Studi di Parma)*

Identification of PPI disrupters that bind human hemoglobin and block its interaction with bacterial hemophore IsdB

17:30-17:50 – Marco Lolli *(Università degli Studi di Torino)*

Targeting dihydroorotate dehydrogenase (DHODH) in anti-infective research: focusing in the design of anti-malarial and anti-tuberculosis agents

17:50 - 18:00 – First Poster winner awarded by Dr. Davide Ferraris (CEO of Ixtal)

18:00 SOCIAL EVENT

Friday, May 21

9:00 - 9:10: Opening and Welcome address

Session 3 - Protein-mediated organelles dynamics

(Chairs: Elisa Maffioli, Matteo Becatti, Lisanna Paladin)

9:10 - 9:50 – Opening Plenary Lecture: Fabrizio Chiti (*University of Firenze*)

Liquid-liquid phase separation (LLPS): an important behavior of proteins involved in the formation of membraneless organelles and human pathology

9:50 - 10.10 – Flora Cozzolino *(University of Naples Federico II)*

ADAM10 hyperactivation acts on piccolo to deplete synaptic vesicle stores in Huntington's disease.

10:10 - 10:30 – Simona Nonnis *(Università degli Studi di Milano)*

Nanostructure alters the inner mitochondrial membrane dynamics of β-TC3 cells and the interplay with other organelles.

10:30 - 10:50 – Chiara Tremolanti *(University of Pisa)*

Translocator Protein (TSPO, 18 kDa) in neuroinflammation: investigation of its functional role in a human microglial cell line

10:50 - 11:10 – BREAK with **Poster Session 2**

Session 4 - Structure-function relationship: an evergreen topic in proteins

(Chairs: Francesco Marchesani, Giulia Babbi, Maria Monticelli)

11:10 - 11:50 – Opening Plenary Lecture Tom Blundell *(University of Cambridge)*

Understanding Structure-Function Relationships in Proteins and Exploiting the Information in the Design of New Medicines

11:50 - 12:10 – Martina Bevilacqua *(Università degli Studi di Padova)*

RepeatsDB: extending the classification of protein tandem repeat structures

12:10 - 12:30 – Tiziano Mazza *(University of Calabria)*

Unexpected transport of glutamate by a membrane carrier of neutral amino acids

12:30 - 12:50 – Marco Mangiagalli *(University of Milano - Bicocca)*

Role of quaternary structure in cold adaptation of proteins

12:50 - 13:00 Second Poster winner awarded by Dr. Giuseppe Scotti (CEO of Falc)

13:00 Closing Remarks

Abstract Book

Plenary Lectures

1)Probing proteins that do not undergo conformational change upon ligand binding

Patrizia Polverino de Laureto

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Proteins interact with various small molecules generally through noncovalent bonds to form complexes that play core roles in cellular processes. Such interactions are important also in relation to the activity of many pharmaceutical compounds, which bind proteins affecting their function as a part of their mechanism of action. Proteins react to this binding and often, but not necessary, undergo conformational change upon ligand binding. A special case concerns the structure-less proteins. They do not have a single conformation, but appear as a dynamic ensemble of conformations. They exhibit ability to fold differently upon interaction with different partners, or only partially fold or even bind without any binding-induced folding. Some of these proteins are involved in neurodegenerative diseases and their amyloid aggregation correlates to the disease onset. The evidence of the role of these proteins in the diseases has led to the development of targeted therapeutic strategies, with the aim to interfere with their amyloid pathway. In this context, α-synuclein represents a significant example. It is the prototype of structure-less proteins and its amyloid aggregation represents a major pathogenic process in Parkinson disease, PD. Several strategies are underway for the prevention of its toxicity. A level of intervention might be hampering the formation of fibrils and oligomers, associated to PD toxicity. The study of the interaction of α-synuclein with small molecules revealed that some proposed structures share common characteristics. They interact weakly and transiently with the protein, inducing a scarce conformational change, and inhibit the formation of fibrils. They also affect the interaction of α-synuclein with membranes. By using a limited proteolysis approach, we provided evidence that some ligands interact differently with the interconverting conformers of monomeric α-synuclein in solution, stabilizing the best-fitting conformation and selecting the most prone-to-aggregation one, confining it into a non-toxic off-pathway oligomer.

2) Liquid-liquid phase separation (LLPS): an important behavior of proteins involved in the formation of membraneless organelles and human pathology

Fabrizio Chiti

Department of Experimental and Clinical Biomedical Sciences "Mario Serio", Section of Biochemistry, University of Florence, Italy.

Proteins have a generic ability to assemble into liquid droplets that constitute a different phase, well separated from the solution in which they are suspended, similarly to what is being observed when "olive oil" droplets phase-separate from water. This phenomenon, generally referred to as liquid-liquid phase separation (LLPS), has been well known by protein chemists for many years, but it was not until 2009 that it started to attract the attention of a larger scientific community in the life sciences, after the discovery that a membraneless organelle formed in cells actually consists of proteins and RNAs in a liquid state phase-separated from the cellular milieu. Today, examples of membraneless organelles recognized to form from LLPS include P-bodies, stress granules, speckels, paraspeckles, Cajal bodies, just to cite some of the most widely investigated. LLPS has therefore a physiological role *in vivo,* but is also extremely important in pathology. Indeed, it is increasingly recognized that a liquid droplet of proteins can serve as a sub-cellular microenvironment with a high concentration of protein molecules that can convert into pathological solid-phase fibrillar aggregates, such as those associated with human neurodegenerative disease like Parkinson's disease, amyotrophic lateral sclerosis and many others. In this lecture I will provide an introduction to the fundamentals of LLPS and how this process can occur *in vitro* and *in vivo*. I will then show a survey of the membraneless organelles recognized to form as a result of LLPS and the physiological role of the LLPS process within them. I will finally show the importance of LLPS in human pathology, focusing on data of TDP-43, which is the main proteins involved in amyotrophic lateral sclerosis and also one of the proteins studied in the laboratory at our University.

3) Understanding Structure-Function Relationships in Proteins and Exploiting the Information in the Design of New Medicines

Tom L Blundell

Cambridge University
Department of Biochemistry, Tennis Court Road, Cambridge, CB21GA, UK

Knowledge derived from genome sequences of humans and pathogens has the potential to accelerate diagnosis, prognosis and cure of disease. We are moving quickly into an era of precision medicine, not only in familial diseases where an inherited mutation in a human gene is important, but also for understanding somatic mutations in cancer. Equally important, the genome sequences of pathogens, for example in tuberculosis, leprosy or SARS CoV-2, can give clues about the choice of protein targets including those of existing drugs, repurposing of others, and the design of new ones to combat the increasing occurrence of drug resistance.
Structure-guided approaches, both in academia and large pharma, have informed drug discovery for five decades. For these we require extensive and detailed knowledge of the high resolution structure of a target protein, and an understanding of how this relates to function. I will discuss the evolution of structure guided-methods over five decades, including the development of fragment-based screening that has proved effective over the past two decades, not only for classical enzyme targets such as protein kinases in our company Astex, but also for less "druggable" targets such as protein-protein interfaces. Initial screening involves small fragments with very low, often millimolar affinities, and biophysical methods, including cryo-EM since the resolution revolution a few years ago. Progress has been made not only for targets in cancer but also in mycobacteria tuberculosis, abscessus and leprae and SARS CoV-2 infections.
I will also review our computational approaches using both statistical potentials (SDM) and machine learning/ artificial intelligence (ML/AI) methods (mCSM) for understanding mechanisms of drug resistance. We have demonstrated that resistance does not only arise from direct interference of the resistance mutation to drug binding but can also result allosteric mechanisms, often modifying target interactions with other proteins. This has led to new ideas about repurposing and redesigning drugs, which can also take advantage of new ML/AI methods.

Oral Presentations

Session 1 - Protein Stability: the importance of being folded

1) Intrinsically disordered regions of alanine:glyoxylate aminotransferase shape its fitness and function

Mirco Dindo[1], Giorgio Giardina[2], Stefano Pascarelli[1], Davide Chiasserini[3], Silvia Grottelli[3], Gen-Ichiro Uechi[1], Claudio Costantini[3], Gioena Pampalone[3], Sharon Spizzichino[2], Paola Laurino[1], Barbara Cellini[3]

1 Okinawa Institute of Science and Technology Graduate University, 1919-1 Tancha, Onna-son, Kunigami, Okinawa 904-0495, Japan.
2 Department of Biochemical Sciences "A. Rossi Fanelli", Sapienza University of Rome, Italy.
3 Department of Medicine and Surgery, University of Perugia, P.le L. Severi 1, 06132 Perugia, Italy

Intrinsically disordered regions (IDR) play a key role in shaping the plasticity of proteins and often define their function. However, how protein evolution co-opts IDRs to impact on the population of native protein conformers and their individual fitness has remained unexplored. Alanine:glyoxylate aminotransferase (AGT) is a liver pyridoxal 5'-phosphate-dependent enzyme involved in the detoxification of glyoxylate and the cause of primary hyperoxaluria type I (PH1) when dysfunctional. In Caucasian population, AGT is present in two allelic forms, the major (AGT-Ma) and the minor (AGT-Mi) alleles, the latter increasing the susceptibility of AGT to PH1-causing mutations. By solving the crystal structure of AGT-Mi we identified three distinct regions exposed to the solvent that have a defined structure in AGT-Ma but are disordered in AGT-Mi. Molecular dynamics showed that AGT-Mi samples more flexible conformations than AGT-Ma supporting the hypothesis that IDRs originate from an enhanced plasticity of the entire structure. Characterisation of variants from a library of these three regions shed light on their effect on enzymatic activity and intrinsic stability of AGT. In addition, the analysis of the behaviour of selected hits from the library in human cells, paired with determination of the interactome of AGT-Ma and AGT-Mi, revealed the impact of IDRs on protein fitness and function at a cellular level. This work establishes that naturally occurring conformers generating by taking advantage of subtle instability of a protein can modulate its function and intracellular fitness.

2) The composition of lipid membranes affect their affinity for toxic and nontoxic misfolded protein oligomers

Silvia Errico[1,2], Hassan Ramshini[1,3], Claudia Capitini[4,5], Michael Zasloff[6,7], Michele Vendruscolo[2] and Fabrizio Chiti[1]

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Many neurodegenerative diseases, including Alzheimer's disease, Parkinson's disease (PD), and many others are associated with the self-assembly of specific polypeptide chains into fibrillar aggregates. It is increasingly recognized that small misfolded oligomeric assemblies formed during the aggregation process, or released by mature fibrils, play a crucial role in neurodegeneration through their interaction with the membrane of neurons, and are thought to represent the principal pathogenic species. In this study we have used liposomes and a pair of toxic and nontoxic protein oligomers obtained from HypF-N, named "OA" (toxic) and "OB" (nontoxic), to measure quantitatively the affinity of the two oligomeric species for lipid membranes. To this aim, we quantified the perturbation to the lipid membranes caused by the two oligomeric species by using the fluorescence quenching of two probes embedded in the polar and apolar regions of the lipid membranes, and a well-defined protein-oligomer binding assay using fluorescently labelled oligomers, to determine the Stern-Vomer and dissociation constants, respectively. With both approaches we observed that toxic oligomers have a membrane affinity significantly higher than that of nontoxic oligomers. Using circular dichroism, intrinsic fluorescence and FRET as optical probes, we found that neither oligomer type changes its structure upon membrane interaction. Using liposomes enriched with trodusquemine, a small molecule known to penetrate lipid membranes and make them refractory to toxic oligomers, we found that the oligomer-membrane affinity was remarkably lower and, at protective concentrations of the small molecule, oligomer-membrane binding was fully prevented. Furthermore, the oligomer-membrane affinity was found to increase and slightly decrease with GM1 ganglioside and cholesterol content, respectively.

3) The molecular species responsible for α1-antitrypsin deficiency are suppressed by a small molecule chaperone

Riccardo Ronzoni[1], Nina Heyer-Chauhan[1], Annamaria Fra[2], Andrew C. Pearce[3], Martin Rüdiger[3], Elena Miranda[4], James A. Irving[1]* and David A. Lomas[1]*

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The formation of ordered Z alpha-1 antitrypsin polymers within the endoplasmic reticulum (ER) of hepatocytes is central to liver disease in alpha-1 antitrypsin deficiency. Although in vitro experiments have identified an intermediate conformational state (M*) that precedes polymer formation, this mechanism and the cellular fate of polymers have yet to be completely characterised. In this work we aim to advance our understanding of the kinetics of formation of Z alpha-1 antitrypsin polymers within the ER of hepatocytes, their secretion and the proteostatic degradative mechanisms that protect against the burden of misfolded proteins. Using cell models of disease in conjunction with conformation-selective monoclonal antibodies and a small molecule inhibitor of polymerisation, we demonstrated the in vivo presence of the folding intermediate M*, defining the kinetics and fate of two populations of polymers of Z alpha-1 antitrypsin and their different solubility in non-ionic detergents. Polymers partitioning with soluble cellular components are secreted efficiently, whilst those that accumulate in the insoluble fraction are generally longer-chain and exhibit a significantly longer half-life. These polymers are also subject to different degradation pathways: soluble polymers are degraded by the proteasome while insoluble polymers are degraded by a specific lysosomal-related system. Blocking either proteasomal or lysosomal degradation increased intracellular polymers and their secretion into the extracellular medium. Our polymerisation inhibitor prevented the formation of M*, abolished the formation of polymers within the cell and increased the secretion of Z alpha-1 antitrypsin restoring M-like kinetics of retention and secretion. Our data allow us to propose a model for the handling of Z alpha-1 antitrypsin within the ER of cells in which the nascent alpha-1 antitrypsin polypeptide folds via M* to native monomer or becomes incorporated into a polymer in the soluble fraction of the cell. This polymer can in turn become insoluble through mechanisms that have yet to be elucidated, can be secreted or degraded. As these secreted polymers are a product of the intracellular processes of expression, M* formation, oligomerisation, soluble-insoluble partition and secretion, they may represent a useful reporter of intrahepatic polymerisation for polymer-blocking therapies in individuals with alpha-1 antitrypsin deficiency.

Session 2 - The role of proteins in infectious diseases

4) L-Arginine in *Pseudomonas aeruginosa* controls C-DI-GMP levels and biofilm formation

Chiara Scribani Rossi[1], Federico Mantoni[1], Giorgio Giardina[1], Alessandro Paiardini[1], Adele Di Matteo[2], Francesca Cutruzzolà[1], Serena Rinaldo[1]

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2 Istituto di Biologia e Patologia Molecolari, Consiglio Nazionale delle Ricerche, Rome, Italy.

Biofilm lifestyle allows bacteria to resist to antimicrobial treatments and host defenses. The second messenger c-di-GMP controls biofilm formation and dispersion in response to environmental cues, including NO and nutrients [1]. Among nutrients, arginine is associated to chronic infections, biofilm/virulence and antibiotic resistance, being at the crossroad of many metabolic processes and acting as a substrate for NO production by the host immune system. We recently found that P. aeruginosa is able to perceive environmental arginine to decrease the intracellular levels of c-di-GMP via the RmcA (Redox regulator of c-di-GMP) protein [2, 3], a multidomain membrane protein. Moreover, we show that RmcA may perceive the metabolic status of the cell via FAD/FADH2 sensing via a redox-based switch. This evidence widens the impact of this nucleotide also in the re-shaping of the central metabolism. 1. Rinaldo et al. (2018). FEMS Microbiol Lett., 365(6). 2. Paiardini et. al. (2018) Proteins. 86(10): 1088-1096. 3. Mantoni et al. (2018) FEBS J. 285(20): 3815-3834.

5) Identification of PPI disrupters that bind human hemoglobin and block its interaction with bacterial hemophore IsdB

Omar De Bei[1], Eleonora Gianquinto[2], Luca Ronda[1,3,4], Serena Faggiano[4,5], Marialaura Marchetti[1], Monica Cozzi[3], Roberta Giaccari[5], Barbara Campanini[1,5], Stefano Bettati[1,3,4], Loretta Lazzarato[2], Mariacristina Failla2, Francesca Spyrakis[2]

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4 Consiglio Nazionale delle Ricerche, Istituto di Biofisica, Via Moruzzi 1, 56124 Pisa, Italy.
5 Dipartimento di Scienze degli Alimenti e del Farmaco, Università degli Studi di Parma, Parco Area delle Scienze 27/A, 43124 Parma, Italy.

Nutritional immunity is a strategy developed by many organisms aimed at blocking bacterial proliferation by limiting nutrient availability, especially iron. Indeed, in the human body iron is mainly sequestered inside hemoglobin (Hb). In turn, pathogens have developed efficient strategies to circumvent iron limitation and these mechanisms have the potential to be exploited as antibacterial targets. Staphylococcus aureus relies on Hb as the preferred iron source during infection and the expression of the hemophore IsdB on the cell wall grants for heme supply. IsdB is a specific receptor for human Hb that also catalyses efficient heme extraction by the coordinated action of two specialized NEAT domains [1,2].
Here we have developed an integrated in-silico/in-vitro platform for the identification of disrupters of Hb-IsdB PPI targeting a pocket on the Hb surface involved in the complex formation. Structure-based virtual screening and molecular docking were applied to identify potential inhibitors in commercial compound libraries. LogD filters were applied to select molecules likely able to remain in the blood vessels and not pass red blood cells membranes, with the aim of restricting targeting to cell-free Hb.
The identified molecules were purchased from commercial suppliers and the positive hits re-synthesized in house. Molecules were tested at 1 mM concentration for their ability to disrupt Hb-IsdB interaction by an in-house developed ELISA assay that exploits immobilized IsdB and blood-purified Hb. The Hb bound to IsdB is detected by an HRP-conjugated anti-Hb antibody. Nine out of 55 molecule tested were able to decrease by 50% or more the amount of Hb bound to IsdB. Five of the selected molecules specifically bound Hb, as demonstrated by STD-NMR. Structural investigations and modifications of the identified hits to improve potency are currently underway.

[1] Ellis-Guardiola K et al. Front Microbiol. 2021 6;11:607679.
[2] Gianquinto E et al. Sci Rep. 2019 9;9:18629.

6) Targeting dihydroorotate dehydrogenase (DHODH) in anti-infective research: focusing in the design of anti-malarial and anti-tuberculosis agents

Marco L. Lolli, [1] Agnese C. Pippione[1], Riccardo Miggiano[2], Davide Ferraris[2], Marta Giorgis,[1] Marta Alberti[2], Noemi Villella[1], Stefano Sainas[1], Salam Al-Karadaghi[3] and Donatella Boschi[1]

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2 Department of Pharmaceutical Sciences - University of Piemonte Orientale, Novara, Italy
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Pyrimidine building blocks are synthesised via both de novo biosynthesis and salvage pathways, the latter of which is an effective way of recycling pre-existing nucleotides. As many parasitic organisms, as for example Plasmodium species causing Malaria, lack pyrimidine salvage pathways for pyrimidine nucleotides, blocking de novo biosynthesis is seen as an effective therapeutic means to selectively target the parasite without effecting the human host. Dihydroorotate dehydrogenase (DHODH), an enzyme that plays a key role in the de novo biosynthesis of pyrimidines, is attracting in recent years a growing interest as validated target either in leukemia/COVID-19 [1, and ref herein] as well as in anti-infective drug research.[2] In this occasion, recent the advances in the DHODH microorganism field will be discussed, focusing in particular in the development of in house PfDHODH inhibitors [3] based on hydroxyazole substructure as antimalarial agents. Preliminary results on the design of tuberculosis DHODH (MtDHODH) inhibitors will be also presented.

[1] a) Stefano Sainas, et al Targeting Acute Myelogenous Leukemia using potent human dihydroorotate dehydrogenase inhibitors based on the 2-hydroxypyrazolo[1,5-a]pyridine scaffold: SAR of the biphenyl moiety. J Med Chem 2021, in press. b) Calistri, A. et al The new generation hDHODH inhibitor MEDS433 hinders the in vitro replication of SARS-CoV-2. bioRxiv 2020, 2020.12.06.412759.
[2] Boschi D., et al. Dihydroorotate dehydrogenase inhibitors in anti-infective drug research. Eur J Med Chem. 2019, 183, 111681.
[3] Agnese C. Pippione, et al Hydroxyazole scaffold-based Plasmodium falciparum dihydroorotate dehydrogenase inhibitors: synthesis, biological evaluation and X-ray structural studies European Journal of Medicinal Chemistry 2019, 163, 266-280

Session 3 - Protein-mediated organelles dynamics

7) ADAM10 hyperactivation acts on piccolo to deplete synaptic vesicle stores in Huntington's disease

Flora Cozzolino[1,2], Elena Vezzoli[3], Cristina Cheroni[4], Dario Besusso[5,6], Paola Conforti[5,6], Marta Valenza[5,6], Ilaria Iacobucci[1,2], Vittoria Monaco[2,7], Giulia Birolini[5,6], Mauro Bombaci[6], Andrea Falqui[8]§, Paul Saftig[9], Riccardo L. Rossi[6], Maria Monti[1,2], Elena Cattaneo[5,6], Chiara Zuccato[5,6].

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4 European Institute of Oncology, IRCCS, 20141 Milan, Italy; Department of Oncology and Hemato-oncology, University of Milan, 20122 Milan, Italy.
5 Department of Biosciences, University of Milan, 20133 Milan, Italy.
6 Istituto Nazionale di Genetica Molecolare "Romeo ed Enrica Invernizzi", 20122 Milan, Italy.
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8 King Abdullah University of Science and Technology (KAUST), Biological and Environmental Science & Engineering (BESE) Division, NABLA Lab, 23955 Thuwal, Saudi Arabia.
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Hyperactive A disintegrin and metalloproteinase domain-containing protein 10 (ADAM10) is involved in synaptic dysfunction and cognitive decline in Huntington's disease (HD). To identify the molecular mechanisms through which ADAM10 is associated with synaptic dysfunction in HD, we employed an IP-MS strategy focusing on endogenous ADAM10 in the brains of wild-type (WT) and HD mice (zQ175). In the normal brain, proteins implicated in synapse organization, synaptic plasticity, and vesicle and organelles trafficking interact with ADAM10, suggesting that it may act as a hub protein at the excitatory synapse. Importantly, for the first time, the ADAM10 interactome either on WT and zQ175 was investigated, finding that is enriched in presynaptic proteins. In particular, ADAM10 was found to interact in both conditions with piccolo (PCLO), a key player in the recycling and maintenance of synaptic vesicles (SVs). In addition, reduced ADAM10/PCLO complex formation occurs in the HD brain, with decreased density of SVs in the reserve and docked pool at the HD presynaptic terminal. Conditional heterozygous deletion of ADAM10 in the forebrain of HD mice reduces active ADAM10 to wild-type level and normalizes ADAM10/PCLO complex formation and SVs density and distribution. The results indicate that presynaptic ADAM10 and PCLO are relevant components of HD pathogenesis.

8) Nanostructure alters the inner mitochondrial membrane dynamics of β-TC3 cells and the interplay with other organelles

Simona Nonnis[1,2], Elisa Maffioli[1,3], Alessandra Galli[4], Algerta Marku[4], Armando Negri[1], Claudio Piazzoni[3,5], Paolo Milani[3,5], Cristina Lenardi[3,5], Carla Perego[4] and Gabriella Tedeschi[1,2,3].

1 Department of Veterinary Medicine, University of Milano, Lodi, Italy;
2 CRC "Innovation for Well-Beeing and Environment" (I-WE), University of Milano, Milan, Italy;
3 Centre for Nanostructured Materials and Interfaces; University of Milano, Milan, Italy;
4 Department of Pharmacological and Biomolecular Sciences, University of Milano, Milan, Italy;
5 Department of Physics, University of Milano, Milan, Italy.

Recently, using cluster-assembled zirconia substrates with tailored roughness produced by supersonic cluster beam deposition, we demonstrated that β-cells can sense nanoscale features of the substrate and can translate these stimuli into a mechanotransductive pathway capable of preserving β-cell differentiation and function in vitro in long-term cultures of human islets (1). Using the same proteomic approach, we focused on the mitochondrial fraction of β-TC3 cells, grown on the same zirconia substrates, characterizing the morphological and proteomic modifications induced by the nanostructure (2). The proteomic results suggest that, in β-TC3 cells, mitochondria are perturbed by the nanotopography and activate a program involving metabolism modification and modulation of their interplay with other organelles. In particular, the expression of the most abundant import machineries of proteins into the mitochondrial inner membrane is altered by the nanostructure. Indeed, we find that the substrate roughness alters the expression of proteins involved in vesicle-mediated transport, lysosomal transport and the protein network linked to the complex interplay between ER and mitochondria. Since it is well known that mitochondrial dysfunction and ER stress are involved in the development of type 2 diabetes, these studies could be helpful to understand whether the organelle crosstalk perturbation also participate in the development of this disease.

(1) Galli, A., et al. (2018). Cluster-assembled zirconia substrates promote long term differentiation and functioning of human islets of Langerhans". Sci Rep. 8:9979. doi: 10.1038/s41598-018-28019-3;
(2) Maffioli, E., et al. (2020). Proteomic Analysis Reveals a Mitochondrial Remodeling of βTC3Cells in Response to Nanotopography. Proteomic Analysis Reveals a Mitochondrial Remodeling of TC3 Cells in Response to Nanotopography. Front. Cell Dev. Biol. 8:508. doi: 10.3389/fcell.2020.00508

9) Translocator Protein (TSPO, 18 kDa) in neuroinflammation: investigation of its functional role in a human microglial cell line

Chiara Tremolanti[1], Chiara Giacomelli[1], Eleonora Da Pozzo[1], Barbara Costa[1], Claudia Martini[1].

1 Department of Pharmacy, University of Pisa, Via Bonanno 6 - 56126 Pisa, Italy

The mitochondrial translocator protein (TSPO, 18 kDa) is a ubiquitous protein implicated in the modulation of several cellular processes including steroidogenesis, respiration, apoptosis, and reactive oxygen species generation and metabolism [1]. Interestingly, it has been proposed to play a central role in neuroinflammation due to its overexpression in activated glia, a cellular phenotype frequently associated with diseases. Despite it has emerged as a promising target against neuroinflammation, the precise role of TSPO in the immunomodulatory mechanisms during active disease states is still unclear. In fact, conflicting data have been accumulated in years, mainly due to the large use of synthetic compounds on in vivo and in vitro murine models of neuroinflammation and neurodegeneration [2]. Here, the potential homeostatic role of TSPO during the inflammatory response was evaluated in a model of immortalized human inflamed microglia (C20 cells) [3]. Two different experimental approaches were exploited: 1) silencing of the protein by knocking down (KD) TSPO in C20 cells, and 2) amplifying the physiological function of TSPO by pharmacological stimulation. Our results showed that TSPO KD amplifies C20 microglia responsiveness to the inflammatory stimulus. Moreover, in the absence of the inflammatory stimulus, TSPO KD C20 cells showed a more inflamed phenotype, suggesting that TSPO is required to maintain the basal release of pro-inflammatory and anti-inflammatory cytokines. In parallel, the pharmacological stimulation of TSPO promoted the shift from pro- to anti-inflammatory microglial phenotype. In conclusion, the results obtained in our experimental setting suggested that TSPO contributes to the preservation of microglia well-being, exerting a negative regulation on neuroinflammatory mechanisms.

[1] Biochem Pharmacol, Costa et al., 2020, 177, 114015.
[2] Cells, Betlazar et al., 2020, 9(2), 512.
[3] J Neurovirol, Garcia-Mesa et al., 2017, 23:47–66.

10) RepeatsDB: extending the classification of protein tandem repeat structures

Martina Bevilacqua

Dep. Biomedical Sciences (University of Padova)

Almost twenty years ago the discovery of non-globular proteins has shaken the long-held structure-function paradigm where well-defined native protein structures are needed for function. The definition covers tandem repetitions, intrinsically disordered regions, aggregating domains and transmembrane domains. Tandem repeats proteins (TPRs) are composed of repetitions of these same or similar structural element modules. TPRs have been shown to act as an integral component of protein complexes and therefore to be involved in several biological functions, as well as neurodegenerative diseases. TPRs are diverse in their amino acid sequences, structural states and functions. On one hand, data structures of solved repeat structures are accumulating, providing new possibilities for classification and detection. On the other hand there is an increasing need to organize and distribute specialized information on TPRs in an efficient way. The RepeatsDB database (URL: https://repeatsdb.org/) (Fig.1) provides annotations and classification for TPRs from the Protein Data Bank (PDB). The major conceptual change compared to the previous version is the hierarchical classification combining top levels based solely on structural similarity (Class > Topology > Fold) with two new levels (Clan > Family) requiring sequence similarity and describing repeat motifs in collaboration with Pfam. In particular, 'Clan', is a subfold that groups protein structures having a common sequence motif within the repeat (or part thereof). 'Family', will accommodate structures that have a common ancestor based on sequence similarity. Family classification aims at joining the sequence- and structure-based TR classifications of RepeatsDB and Pfam and to support the transfer of evolutionary and functional information. Additionally, a new UniProt-centric view unifies the increasingly frequent annotation of structures from identical or similar sequences.

11) Unexpected transport of glutamate by a membrane carrier of neutral amino acids

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ASCT2 (SLC1A5) is one of the seven members of the SLC1 family. Despite the acronym (Ala, Ser, Cys Transporter 2), Cys is not a substrate of the protein but it acts as an allosteric modulator. It has been recently demonstrated that cholesterol exerts effects on the ASCT2 transport activity. It increases transport rate without affecting the Km for substrates. Gln is the preferred substrate, exchanged through the cell membrane with neutral amino acids such as serine or threonine in a Na+-dependent fashion. This mechanism underlies the main physiological role of ASCT2 in balancing the amino acid pools in several tissues. ASCT2 is over-expressed in human cancers, providing Gln for cancer cell development and growth. Recently, we employed ASCT2 over-expressed in Pichia pastoris and reconstituted in proteoliposomes to unveil a novel property for ASCT2; indeed, in this experimental model we demonstrated that ASCT2 catalyses a Na+-dependent antiport of Glu with Gln with a preferred sidedness in proteoliposomes: Glu is mainly inwardly transported in exchange for Gln. This corresponds to the transport of Glu from the extracellular to the intracellular compartment as confirmed also by transport experiments performed in intact HeLa cells. The competitive inhibition exerted by Glu on the Gln transport together with the docking analysis indicates that the Glu binding site is the same as that of Gln. Differently from the neutral amino acid antiport, the Glu ex/Gln in antiport is pH-dependent with optimal activity at acidic pH on the extracellular side. This phenomenon suggests the occurrence of a proton flux coupled to the Glu transport. To detect the proton flux, a spectrofluorometric method has been pointed out revealing a 1:1 stoichiometry H+: Glu. This novel antiport reaction may have a relevance in those body districts in which a glutamine/glutamate cycle occurs, such as in astrocytes and in placenta.

12) Role of quaternary structure in cold adaptation of proteins

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Cold-active enzymes exploit a repertoire of molecular solutions developed by psychrophilic organisms to survive at low temperatures. These enzymes owe their cold activity to their great flexibility, which often causes thermolability. The structural determinants of these properties remain partly elusive, and the role of the quaternary structure is even less clear. Oligomerization has been previously associated with adaptation to high temperatures, producing more compact and rigid structures in oligomers than monomers. Here, we report about two cold-active enzymes whose quaternary structure is characterized by a higher level of oligomerization than their mesophilic and thermophilic counterparts, suggesting an important role of quaternary structure in cold adaptation. The β-galactosidase from the Antarctic bacterium Marinomonas ef1 (M-βGal) is an atypical enzyme as it couples activity at 5 °C with unusual thermal stability. Structural analysis indicated that the hexameric structure of M-βGal is at the basis of the peculiar behavior of this enzyme1. Another example of the involvement of quaternary structure in cold adaptation emerges from the study of the acyl aminoacyl peptidase from Sporosarcina psychrophila (SpAAP). SpAAP is cold-active, but unlike M-βGal, it shows low thermal stability. X-ray crystallography and site-directed mutagenesis indicated that SpAAP is an oligomer with intertwined domains adopting a peculiar mechanism of dimerization, not shared among its thermophilic homologous2. Overall, our data hint that cold adaptation derives from a combination of structural solutions, and among these it is appropriate to include the quaternary structure as well. 1Mangiagalli M. et al. "The co-existence of cold activity and thermal stability in an Antarctic GH42 β-galactosidase relies on its hexameric quaternary arrangement." The FEBS Journal (2021). 2Mangiagalli M. et al. "The activity and stability of a cold-active acylaminoacyl peptidase rely on its dimerization by domain swapping. "Intern. J. of Biol. Macromol. (2021).

Poster Session 1 – May 20th

1) Targeting *Mycobacterium tuberculosis* Orotate Phosphoribosiltransferase for antitubercular drug discovery

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Mycobacterium tuberculosis (MTB) is the causative agent of tuberculosis in humans, an ancient disease which, still today, is considered a major threat worldwide. Purine and pyrimidine ribonucleotide metabolism represents a valid source of molecular targets for antitubercular drug discovery pipeline. MtPrsA enzyme catalyzes the synthesis of PRPP and, since it is essential for MTB survival, MtPrsA has been investigated for rational design of active compounds, even if it shares a high degree of homology with the human ortholog 1. Among several screened molecules, 14 were selected based on their activities towards MTB bacilli and tested for their inhibition and selectivity against MtPrsA and hPrsA. Unfortunately, they proved to be weak inhibitors of the mycobacterial enzyme, while they exhibited a relevant inhibition on the human enzyme. Considering that PRPP has a key role also in the de novo pyrimidine synthesis cascade, we are exploring alternative targets of the selected molecules along the pyrimidine biosynthetic pathway (PBP) by means of a drug repositioning approach. In particular, we focused on MtOPRT enzyme along the PBP, whose crystal structure has been solved by our group 2, as alternative target of the molecules described above due to its binding activity towards PRPP. In order to perform a comparative analysis with the human ortholog, we also produced a recombinant version of human UMPS bifunctional enzyme that includes the OPRTase domain, converting orotate into OMP using PRPP as ribose donor. Preliminary data showed a selective inhibition of MtOPRT with an IC50 value in the low micromolar range, suggesting a possible new interesting target. With the aim of analyzing the molecular details of the protein-ligand interaction, the mentioned compounds underwent co-crystallization trials with MtOPRT, so as to perform a structure-based optimization of the most promising molecules, which could result in interesting chemical scaffold for antitubercular drug design.

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2. Donini, S. et al. (2017) Scientific Reports 7

2) Iron-regulated surface determinant B (IsdB) mediates Staphylococcus aureus interaction with the von Willebrand factor (vWF)

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Endovascular infection is a highly critical complication of invasive Staphylococcus aureus disease. For colonization, staphylococci must first adhere to host extracellular matrix components. Von Willebrand factor (vWF) is a multimeric plasma glycoprotein mediating platelet adhesion at sites of endothelial damage. S. aureus is known to interact with vWF by protein A (SpA), a surface-associated protein. Here, we report that the iron-regulated surface determinant B (IsdB) protein, besides being involved in heme transport, plays a novel role as a receptor for vWF. In Western ligand assays of S. aureus lysates, IsdB was recognized by purified vWF. Recombinant IsdB bound vWF dose-dependently and its ligand-binding site was located in the A1 domain of vWF. Furthermore, we found that both near-iron transporter motifs NEAT1 and NEAT2 of IsdB individually bound vWF although with different KD values. Adherence to Human Umbilical Vein Endothelial Cells (HUVEC) exposing vWF by IsdB-expressing S. aureus cells was 3-fold higher compared to the level of adherence observed when an S. aureus isdB mutant was used. These findings suggest that, by mediating the binding between S. aureus and vWF, IsdB may contribute significantly to the pathogenesis of the endovascular staphylococcal disease.

3) Small molecules to treat protein misfolding diseases

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The disorders caused by destabilizing mutations are named conformational diseases. They count several rare genetic diseases among which TTR- amyloidosis (ATTR) and PMM2- congenital disorder of glycosylation (CDG). There is still no cure available to these patients. ATTR and PMM2- CDG are exemplary of the routes that unstable proteins can undergo: protein accumulation as toxic fibrils or protein elimination causing loss of function. Both outcomes can be prevented by acting upstream on protein stabilization. Native transthyretin (TTR) is a homotetramer. Missense mutations lead to unfolding into monomers followed by the formation of oligomers and then amyloid fibril deposition. Iodinated 4,4'-bipyridines were characterized as TTR stabilizers. The compounds' activity in vitro was assessed monitoring the fibril formation by a turbidity assay. Three ligands, named (M)-8, (M)-9 and (P)-9, resulted effective in preventing the change of wild type and mutants TTR conformation [1]. Mutations in the enzyme phosphomannomutase2 (PMM2) affecting N-glycosylation of protein lead to the most frequent glycosylation defect. -glucose-1,6-bisphosphate (βG16) was identified as a pharmacological chaperon as it enhances PMM2 stability preventing its degradation. The binding of βG16 to the protein causes a conformational change proved by in silico docking and by limited proteolysis. PMM2 stabilization in the presence of the ligand was measured using thermal shift assay [2]. Currently, we are refining a strategy to promote G16P bioavailability to be used for therapy. In our laboratory, we can analyze the ligand-protein interaction especially testing the effect of the compounds of interest on protein stability and activity. These approaches are useful in identifying potential drugs for protein misfolding disorders.

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[2] Monticelli et al. 2019, IntJMolSci. 20(17),4164.

4) Assessing charge patterning impact on synthetic IDP conformation through native-MS

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In recent years, native mass spectrometry (native-MS) - based on nano-electrospray-ionization- stood out as a powerful tool to investigate protein conformational transitions for intrinsically disordered proteins (IDPs)1. IDPs lack a stable tertiary structure, preferring a conformational ensemble of interconverting conformers. This structural promiscuity is far from being non-functional, as elucidated forbianchi fuzzy complexes and folding upon binding protein-protein interactions2. Primary structure plays a major role in determining IDP conformation3. Particularly, charge density and the linear distribution of opposite charges emerged as major determinants of conformational properties for IDPs. Charge patterning can be measured through k, which represents a normalised parameter for charge asymmetry4. Here, we employed three IDPs, measles virus Ntail, Hendra virus PNT4 and C-terminal region of human neurofilament medium subunit NFM, characterised by similar fractions of hydrophobic and charged residues but different in proline contents. For each IDP, we designed two synthetic k variants displaying the lowest and highest k value compatible with natural sequence composition5. Differences in terms of protein compaction between wild-type and synthetic variants have been investigated through native-MS and size-exclusion chromatography as an orthogonal approach. We proved a correlation between IDP conformation and charge patterning, observing a possible proline-mediated interference in structural response to charge clustering.

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5) Compound heterozygosis in AADC deficiency: a complex phenotype dissected through comparison among heterodimeric and homodimeric AADC species

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Compound heterozygosis is the most widespread and hardly to tackle condition in Aromatic Amino Acid Decarboxylase (AADC) deficiency, a genetic disease leading to severe neurological impairment [1]. Despite this, most biochemical studies have been undertaken on homodimeric recombinant AADC variants. Here we report a clinical case of a 16 years old heterozygous patient bearing two novel mutations, C281W and M362T. We provide the biochemical characterization of the homodimeric species and used a novel strategy to obtain the double tagged heterodimer C281W/M362T. Homodimeric C281W AADC is recovered as insoluble while M362T is poorly soluble respect to the wild-type (WT). Moreover, apoM362T exhibits a tendency to aggregate while C281W/M362T exhists in an equilibrium between dimeric and oligomeric species. Cys281 and Met362 map on the same Large Domain region of AADC where previously characterized variants E283A [2] and R285W show propensity to aggregate in their apo form. These observations reinforce the suggested importance of this region in protein folding. Despite this, both M362T and C281W/M362T retain a nearly good tertiary structure and catalytic efficiency respect to the WT while the heterodimer shows a great reduction of coenzyme affinity. The biochemical results are consistent with the related clinical picture reporting a mild phenotype. Patient shows responsiveness to the treatment with pyridoxine, MAO inhibitors, dopamine agonists. Overall, this integrated and cross-sectional approach enables proper characterization of pathogenic AADC variants and the functional result will help to elucidate the physio-pathological mechanisms in AADC deficiency

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6) Effect of D-aminoacid peptides on MHC-I biophysical properties

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Class I major histocompatibility complex (MHC-I) displays on the cell surface peptides derived from exogenous proteins for the recognition by appropriate T cell receptors (TCRs) and subsequent activation of an immune response (1). The potentialities of MHC-I/TCR system have been exploited for the development of vaccines. Indeed, the identification of MHC-I restricted epitopes for inclusion in peptide-based vaccines is used to design novel modified peptides that can increase the immune response against pathogenic cells (2). One of the main concern regards the stability and the immunogenicity of MHC-I bearing the administrated peptide. A powerful procedure to improve the effectiveness of this strategy is to increase the peptide half-life by inserting d-aminoacids within the sequence. Indeed, d-bearing peptide have been found to possess increased pharmacokinetics properties (2). Here, we investigated from a biophysical and structural view murine MHC-I displaying variants of the gp10025-33 antigen (gp33), an immunodominant epitope found in mice infected with the lymphocytic choriomeningitis virus (3). Our results revealed how the d-substitution has a low impact on MHC-I stability; in addition, the x-ray structural characterizations identified the atomic details of the peptide/MHC-I interactions. Finally, d-peptides bearing MHC-I were proved to bind efficiently TCRs. In conclusion, D-aminoacid does not have a strong impact on stability and affinity properties of MHC-I, remarking its potential role in peptide-based vaccine development.

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7) A proteomic approach to analyse Abyssinian renal amyloid deposits in FFPE histologic specimens

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The amyloidoses constitute a group of diseases occurring in humans and animals that are characterized by abnormal deposits of aggregated proteins in organs, affecting their structure and function. In the Abyssinian cat breed, a familial form of renal amyloidosis has been described. In this study, multi-omics analyses were applied and integrated to explore some aspects of the unknown pathogenetic processes in cats. Whole-genome sequences of two affected Abyssinians and 195 controls of other breeds were screened to prioritize potential disease-associated variants. Proteome analysis was performed on kidney FFPE blocks deparaffinized, rehydrated and then incubated in buffer with mini protease inhibitors cocktail. Hydrated tissue slices were harvested with a scalpel blade, homogenized and centrifuged. The supernatant containing proteins were quantified and extracted proteins were subjected to reduction, alkylation, and protein digestion. The proteolytic digest was desalted and directly inject into LC-ESI-MS/MS. MS spectra were searched against the Felis catus reference NCBI sequence database. The MS/MS data identify 215 and 56 proteins were identified as exclusive or overexpressed in the affected and control kidneys, respectively. With omics data integration, the general conclusions are: i) the familial amyloid renal form in Abyssinians is not a simple monogenic trait ii) amyloid deposition is not triggered by mutated amyloidogenic proteins but is a mix of proteins codified by wild-type genes iii) the form is biochemically classifiable as AA amyloidosis.

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8) Lscβand lscγ, two novel levansucrases of Pseudomonas syringae pv. actinidiae biovar 3, the causal agent of bacterial canker of kiwifruit, show different enzymatic properties

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Bacterial canker disease caused by Pseudomonas syringae pv. actinidiae (Psa) biovar 3 involved all global interest since 2008. We have found that in Psa3 genome, similarly to other P. syringae, there are three putative genes, lscα, lscβ and lscγ, coding for levansucrases. These enzymes, breaking the sucrose moiety and releasing glucose can synthetize the fructose polymer levan, a hexopolysaccharide that is well known to be part of the survival strategies of many different bacteria. Considering lscα non-coding because of a premature stop codon, we cloned and expressed the two putatively functional levansucrases of Psa3, lscβ and lscγ, in E. coli and characterized their biochemical properties such as optimum of pH, temperature and ionic strength. Interestingly, we found completely different behaviour for both sucrose splitting activity and levan synthesis between the two proteins; lscγ polymerizes levan quickly at pH 5.0 while lscβ has great sucrose hydrolysis activity at pH 7.0. Moreover, we demonstrated that at least in vitro conditions, they are differentially expressed suggesting two distinct roles in the physiology of the bacterium. Works are in progress to found inhibitors of levansucrases with the aim to detect the role of these enzymes in bacterium physiology and pathogenesis. Revealing the role of levansucrases in sucrose utilization and levan synthesis in Psa3 could suggest new targets to counteract the bacterial canker.

9) Targeting sulfur assimilation pathway for antimicrobial development: two birds with one stone

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Cysteine, the main precursor of sulfur-containing biomolecules, plays a key role in cell viability and redox state. Thus, the reductive sulfur assimilation pathway that leads to cysteine biosynthesis in bacteria and plants, absent in mammals, is an emerging target for the development of antimicrobials [1]. In bacteria, the last two steps of the cysteine biosynthesis are catalyzed by serine acetyltransferase (SAT) and O-acetylserine sulfhydrilase (OASS), that can associate to form a cysteine synthase (CS) complex which finely tunes both enzyme activities. In the quest for new antimicrobials or antibiotic enhancers, we carried out a drug discovery campaign that led to the identification of (1S,2S)-1-(4-methylbenzyl)-2-phenylcyclopropanecarboxylic acid (UPAR415) as a potent inhibitor of Salmonella Typhimurium OASS (StOASS), with a low nanomolar dissociation constant. The crystallographic structure at 1.2 Å resolution of StOASS with bound UPAR415 (pdb:6Z4N) indicated that the ligand binds in the active site and competes with the amino acid substrate [2]. Since SAT is known to bind to OASS by inserting its C-terminal sequence into the active site, we wanted to check if UPAR415, besides being an effective inhibitor of OASS, was also able to interfere with protein-protein interactions. We demonstrated by surface plasmon resonance and fluorescence spectroscopy that UPAR415 can displace the CS complex of S. Typhimurium and of the highly homologous Escherichia coli widening the spectrum of its mechanism of action and of its potential antimicrobial applications. Indeed, microbiologic assays demonstrated a synergic and/or additive action of UPAR415 with colistin, a last-line antibiotic, towards several bacterial strains, supporting the viability of our approach for the development of a new class of antimicrobial compounds.

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10) Protease-sensitive regions in amyloid light chains: a proteomic analysis of ex-vivo fibrils

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Systemic light chain (AL) amyloidosis is caused by deposition of immunoglobulin light chains (LC) as amyloid fibrils in target organs. Alongside the full-length monoclonal protein, abundant LC fragments are always present in AL deposits [1]. Herein we aimed to provide a detailed comparative description of the fragmentation sites of amyloid LCs in multiple organs of an individual patient (AL-55), deceased due to AL λ amyloidosis. To this aim, we combined high-resolution gel-based analyses with an LC-MS/MS-based "terminomics" approach, to define the amyloid LC cleavage sites in kidney and subcutaneous fat, and to compare them with the previously reported proteolytic pattern from the heart of the same patient [2]. Our data show that all tissues contain fragmented LCs along with the full-length protein and the pattern of LC fragments is substantially coincident across tissues, although microheterogeneity exists. Multiple cleavage positions can be detected; some of them are shared, whereas some are organ-specific, likely due to the action of a complex set of proteases. Cleavage sites are concentrated in specific "cleavage-prone" regions, which appear to be conserved in all tissues. A relevant number of proteolytic sites are not accessible on the native LC dimers, while they are compatible with the fibrillar assembly. Overall, our data suggest that LC fragments originate locally in tissues. In summary this work provides a unique set of molecular data on proteolysis from ex vivo amyloid deposits, which allows discussing available hypotheses on the role and the timing of proteolytic events occurring along amyloid formation and accumulation in AL patients.

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11) The interaction between the F55 virus-encoded transcription regulator and the RadA host recombinase reveals a common strategy in Archaea and Bacteria to sense the UV-induced damage to the host DNA

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Sulfolobus spindle-shaped virus 1 (SSV1) is a non-lytic and the only UV-inducible member of the virus family Fuselloviridae. Originally isolated from *Saccharolobus shibatae* B12, it can also infect Saccharolobus solfataricus. Like the CI transcriptional repressor of the bacteriophage λ, the SSV1-encoded F55 acts as a key regulator for the maintenance of the SSV1 carrier state, transcriptionally repressing viral genes. Particularly, F55 binds to tandem repeat sequences located within the promoters of the early and UV-inducible transcripts. Upon exposure to UV light, a temporally coordinated pattern of viral gene expression is triggered. In the case of the better-characterized bacteriophage λ, the switch from lysogenic to lytic development is regulated by crosstalk between the virus-encoded CI repressor and the host RecA, which regulates also the SOS response. For SSV1, instead, the regulatory mechanisms governing the switch from the carrier to the induced state have not been completely unraveled. In this study, we have applied an integrated biochemical approach based on a variant of the EMSA assay coupled to mass spectrometry (EMSA-MS) analyses to identify the proteins associated with F55 when bound to its specific DNA promoter sequences. Among the putative F55 interactors, we identified RadA and showed that the archaeal molecular components F55 and RadA are functional homologs of bacteriophage λ factor CI and *Escherichia coli* RecA system.

12) TTR mutations influence the kinetics of TTR fibril formation under the mechano-enzymatic mechanism

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The demonstration that proteolytic enzymes are involved in the mechanism of transthyretin (TTR) amyloidogenesis contributes to shed light on TTR related amyloidosis 1-3. The identification of the 49-127 TTR fragment in amyloid fibrils extracted from patients with systemic TTR amyloidosis 4 has originally led our research toward the discovery of a novel biocompatible mechanism of TTR amyloid fibrillogenesis. Here the mechano-enzymatic mechanism of fibril formation was applied to two highly amyloidogenic TTR variants, E51S52 Dup 5 and S52P 6.The effect of trypsin or plasmin in the presence or absence of amyloid seeds was specifically investigated. We have previously shown that both trypsin and plasmin cleave TTR into the same fragments putative for fibril formation in vivo, however several differences were observed between the two variants in terms of lag phase, rate of fibrils growth and final yield of amyloid at equilibrium. The kinetics of fibril formation of the two TTR isoforms was also influenced in a different manner by the presence of amyloid seeds. The differences observed between the two amyloidogenic TTR variants highlighted the complexity of fibril formation mechanism in the presence of activated proteases suggesting that two parallel processes may simultaneously occur. A degradative pathway provides non-amyloidogenic fragments whilst a partial cleavage of TTR molecule generates an amyloidogenic fragment. Any delay in reaching the critical concentration of the amyloidogenic fragment facilitates the degradative process. On the contrary, an early formation of fibrillar nuclei can favour fibrillogenesis over degradation. The kinetics of fibrillogenesis through the mechano-enzymatic mechanism is highly influenced both by the rate of specific amyloidogenic cleavages and the rapidity by which the first amyloid nuclei is formed.

13) Structural analysis of an anomalous form of TDP43 N-terminal domain

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Amyotrophic lateral sclerosis (ALS) is a fatal disorder characterized by a progressive degeneration of the upper and lower motor neurons of the central nervous system. In 2006 the transactive response (TAR) DNA-binding protein of 43 kDa (TDP-43) was identified as full length and C-terminal forms as the main element of poly-ubiquitinated, hyperphosphorylated protein aggregates found in ALS. This protein is physiologically a homodimer, and dimerization occurs through one of its four domains, i.e. the N-terminal domain (NTD). It has also been hypothesized that this domain may determine the formation of higher molecular weight assemblies that could play a crucial role in the pathogenesis of ALS. In this study we have purified the NTD and have obtained two forms, one that is fully folded and native and another that is “anomalous” and partially folded, respectively. We therefore decided to carry out a series of biophysical analyses, involving circular dichroism, fluorescence, Nuclear Magnetic Resonance, dynamic light scattering, analytical size exclusion chromatography, etc. in order to better study this new version of the domain. We have discovered in relation to the data obtained that the "anomalous" form of NTD it is a monomer that presents a different structure than its native form. In particular, this domain is partially folded and has pre-molten globule form. This suggests that this domain presents a certain structural plasticity that further places it as an interesting domain to understand the formation of TDP-43 assemblies and the pathogenetic process of ALS.

14) Biochemical and structural chracterization of proteins involved in DNA repair and replication pathways of *Mycobacterium tuberculosis*

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Mycobacterium tuberculosis (MTB) is the etiological agent of tuberculosis (TB) in human. Like most bacteria it encodes for several proteins which guarantee the correct replication and repair of the chromosomal DNA. Most of these proteins are essential and should be considered as source of novel drug targets in anti-TB drug discovery [1]. To this aim, we focused our attention on macromolecular protein complexes involved in the Nucleotide Excision Repair (NER) pathway and in the initiation phase of DNA replication. In particular, we obtained a low-resolution model of the UvrA-UvrB complex acting in the damage recognition step along NER pathway. The structural model allows us to map three residues involved in protein-protein interaction whose key role was further confirmed by site-directed mutagenesis experiments. On the other hand, we obtained preliminary results on the biochemical characterization of a novel helicase loader acting during DNA replication in MTB, called DciA [2]. In order to shed light on mechanistic aspects of DciA-dependent helicase in MTB, we investigated the secondary structure element of DciA domains protein using limited proteolysis coupled with native mass spectrometry. Moreover, we determined the kinetic constants of the interaction between DciA loader and the target helicase by means of Surface Plasmon Resonance (SPR).

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15) Lysozyme amyloid: evidence for the W64R variant by proteomics in the absence of the wild type protein

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Lysozyme is a bacteriolytic enzyme present in many different human tissues and body fluids. Single point mutations in lysozyme gene can lead to amyloid fibrils formation [1]. We report a case of a patient affected by lysozyme amyloidosis and carrying the mutation W64R. Two proteomics approaches were applied to characterise the molecular nature of a gastrointestinal (GI) amyloid positive sample. At first Dogan. et al procedure was applied using trypsin as proteolytic enzyme [2]. Peptides containing the W64R mutation were not detected since the tryptic cleavage site generated by W->R change resulted in the generation of fragments outside the range of the instrument. The miscleaved 51-64 peptide was detected with low confidence (Fig. 1A). The second approach allowed the identification of the peptide 53–66, containing the W->R substitution, by using Asp-N as digestion enzyme (Fig. 1C). There was no evidence of wild type (WT) peptides in both cases (Fig. 1B, 1D). To confirm these results WT lysozyme from human neutrophils was spiked into a amyloid negative GI tissue and analysed by proteomics. WT peptide was detected.
We highlight the importance of combining the use of different proteolytic enzymes to understand amyloid composition and fibrillogenesis mechanism. The reported study shows that WT lysozyme is not present in natural amyloid fibrils. These findings are in line with the resistance of WT lysozyme to the amyloidogenesis even in the presence of natural seeds of fibrils of the lysozyme variant. Lysozyme fibrillogenesis is deeply different from other types of amyloidosis such as that caused by transthyretin mutations. A potential explanation can be found in the difference in the stability of the WT and variant form of TTR compared to the variant and WT form of lysozyme.

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[2] Dogan, Amyloidosis: Insights from proteomics. Annu Rev. Pathol, 2017. 12: 227-304

16) Galectin-3: a multifunctional protein

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Galectins are a family of β-D-galactoside binding proteins characterized by a conserved carbohydrate recognition domains (CRDs)1. Members of this family are implicated in the onset of cancer, inflammation and fibrosis, and in the onset of bacterial and viral infections1. Considering the multifunctional role of galectins, a deep investigation on Galectin 3 (Gal3) was carried out to drive a rational design of molecules with an effective inhibitory action as anti-tumoral or anti-pathogenic agents. X-ray crystallography together with SAXS have been used in order to obtain detailed information on the Gal3CRD complexed with molecules of glycosidic nature whose potential anti-tumoral inhibitor activity have been previously demonstrated2. Bacteria such as Pseudomonas aeruginosa have been found to have galectin recognition ligands on their surface; some studies have in fact revealed that Gal3 interacts with the LPS of this bacterium3. Therefore, ITC studies were performed to analyse the interaction between Gal3 and LPS from P. aeruginosa in order to design inhibitors capable of displacing this binding and therefore with potential antimicrobial activity. Finally, considering the over-expression of Gal3 in SARS-CoV-2 patients, the interaction between Gal3 CRD and the RBD of the SARS-CoV-2 Spike was evaluated using the microscale thermophoresis technique. The formation of the complex has been experimentally verified and can represent a target for the screening of molecules able to displace their interaction and therefore to carry out an anti-inflammatory effect in therapy to struggle COVID194-5.

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3) Gupta S. K., et al. (1997) Infection and Immunity, vol. 65, n. 7, pp. 2747-2753.
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5) Di Gaetano S. et al. submitted to Frontiers of Chemistry

17) Targeting iron uptake pathway and virulence factors for the development of novel antitubercular multitarget drugs

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Tuberculosis is an infectious disease caused by M. tuberculosis and, according to WHO, is the leading cause of death worldwide caused by a microorganism (WHO, 2020). The development of new antitubercular drugs is nowadays a necessity since the insurgence of multi-drug resistant and extensively drug resistant strains. In our previous works we focused on blocking the salicylate synthase MbtI, the enzyme involved on the first step of the siderophores mycobactins biosynthesis, since iron is an essential factor for mycobacteria survival and infection. So far, we identified a series benzofuran-based compounds inhibiting MbtI and showing antitubercular activity2. Moreover, we found from literature a class of benzofuran salicylic compounds inhibiting the mycobacterial tyrosine phosphatase PtpB, a virulence factor that blocks macrophages phagolysosome maturation, with mechanism of action still not well known3. Based on the chemical similarities of the two classes of compounds, we aimed to develop multitarget compounds against these two enzymes, that may become new drug candidates to fight TB. To this purpose, we used two different approaches. Firstly, we screened our library of MbtI inhibitors against PtpB. Moreover, we performed an in-silico screening of molecules that may inhibit PtpB, that were synthetised and assayed against both MbtI and PtpB. Both approaches gave preliminary positive results, affording two series of nitrophenylfuran and quinolinic derivatives, active against both enzymes. These compounds will be then subjected to Structure Activity Relationships (SAR) studies for their development and improvement.

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2)Chiarelli LR, Mori M, Barlocco D, Beretta G, Gelain A, Pini E, Porcino M, Mori G, Stelitano G, Costantino L, Lapillo M, Bonanni D, Poli G, Tuccinardi T, Villa S, Meneghetti F, 2018. Discovery and development of novel salicylate synthase (MbtI) furanic inhibitors as antitubercular agents. Eur J Med Chem. Jul 15;155:754-763.
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18) Anti-aging and neuroprotective effects of *Vigna unguiculata* proteic extracts

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Aging and age-related neurodegeneration are among the major challenges in modern medicine and nutrition is an important way to prevent diseases and achieve healthy aging. α-Synuclein (α-syn) is a presynaptic neuronal protein and its aggregation is linked to a group of age-related neurodegenerative diseases called synucleinopathies, like Parkinson's disease (PD). Here, we show that an aqueous extract of V. unguiculata beans delays senescence both in Saccharomyces cerevisiae and Drosophila melanogaster, in a Snf1/AMPK-dependent manner. Moreover, in vitro aggregation of α-syn, its toxicity and membrane localization in yeast and neuroblastoma cells are strongly decreased in the presence of bean extract. Furthermore, in a Caenorhabditis elegans model of PD, V. unguiculata extract reduces the number of the degeneration of the cephalic dopaminergic neurons. This extract contains starch, free amino acids, peptides and proteins, that were analyzed by mass spectrometry. Further studies for the identification of the bioactive molecules (probably peptides or a small proteins) are currently in progress. In conclusion, our findings support the role of V. unguiculata beans as a functional food in age-related disorders.

19) Changes in thermodynamic stability of transthyretin fibrils made with different methods

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Methods suitable for the creation of amyloid in vitro may provide crucial clues to understand the biophysical basis of amyloid formation and offer important tools for drug discovery. A desirable method for amyloid formation in vitro should use biocompatible conditions to generate fibrils with structural characteristics closely resembling those of natural fibrils. Our aim was to compare the thermodynamic stability of V122I transthyretin (TTR) fibrils prepared in vitro at low pH (Colon & Kelly, Biochemistry 31:8654-60, 1992) or in physiological buffer containing traces of a protease (Marcoux et al, Embo Mol Med 7:1137-49, 2015) with the stability of natural fibrils. A remarkable difference in thermodynamic stability was observed between the two types of in vitro fibrils. Whilst the aggregates at low pH did not show any significant change in their stability compared with the precursor, the fibrils prepared by the mechano-enzymatic mechanism were much more stable than the native TTR and they appeared thermodynamically similar to those extracted from natural sources. Our data strongly suggest that the combination of proteolysis and biomechanical forces generates aggregates with thermodynamic features that more closely resemble the amyloid fibrils occurring in patients.

20) Interplay between structure, function and stability of RidA

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The Reactive intermediate deaminase (Rid) protein family is a group of enzymes widely distributed in all Kingdoms of life. RidA proteins prevents the accumulation of 2-aminoacrylate (2AA), a highly reactive enamine generated by pyridoxal phosphate-dependent serine/threonine dehydratases and cysteine desulfhydrases. 2AA and its tautomer, 2-iminopyruvate, spontaneously hydrolyse to the corresponding ketoacid (pyruvate) and ammonia. RidA accelerates the conversion to pyruvate thus preventing the accumulation of this reactive metabolite that could damage cellular components. RidA from goat presents a highly stable trimeric conformation with enzymatic activity against specific aminoacids. It was proposed that the high stability could be responsible for its immunomodular activity. In this study we expressed, purified and characterized mutants of goat RidA in order to unravel the peculiar features of this protein. All the mutations studied where focused on the catalytic residue R107 in order to abolish enzymatic activity or in interface residues, eg, K78 and E124, in order to disrupt the trimeric conformation and reduce protein stability. The enzymatic activity was almost abolished mutating the catalytic residue, while a significant reduction was observed in the mutants of interface residues. Indeed, the crystal structure revealed that the trimeric conformation was maintained in all the mutants even though we achieved a slight reduction of thermal stability. Moreover, we studied the differences underlying the two isoforms of RidA in salmon. The two proteins present a remarkable difference in substrate specificity and a different thermal stability. The comparison of the crystal structures provided us extensive comprehension of the features underlying the different behaviour of the two isoforms. All together these results could provide the basis to explore whether the immunomodulatory properties of RidA enzymes are related to their structure and/or catalytic activity.

Poster session 2 - May 21th

1) Study of structural and functional fibrinogen modifications in patients with Giant Cells Arteritis

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Giant cell arteritis (GCA) is a rare large vessel vasculitis complicated by thrombotic manifestations where the inflammatory, immunological and haemostatic mechanisms are closely linked. Recent reports indicate also a systemic redox imbalance characterized by altered ROS (Reactive Oxygen Species) production. Among plasma proteins, fibrinogen, which plays a key role in inflammatory response and in blood coagulation, represents a main target for ROS. Modifications in fibrinogen structure may influence fibrin network assembly, promoting haemostatic system alterations.
Here, we investigated fibrinogen structure and function, plasma redox status and leukocyte ROS production in 30 GCA patients and 30 age-matched healthy controls in order to highlight the mechanisms of inflammation-induced thrombosis.
Blood leukocyte ROS production was detected by FACS analysis. Plasma oxidative stress status was evaluated by Thiobarbituric Acid Reactive Substances (TBARS) and by Total Antioxidant Capacity (TAC). In fibrinogen purified from plasma of patients and controls thrombin-catalyzed fibrinogen polymerization and plasmin-induced fibrin degradation were assayed. Intrinsic fibrinogen fluorescence and dityrosine cross-links were assessed by fluorimetric methods. Furthermore, secondary structure was analyzed by circular dichroism spectroscopy (CD).
The obtained results clearly indicate that GCA patients are characterized by an overall imbalance in redox parameters associated with enhanced leukocyte ROS production. Moreover, impaired fibrinogen function and altered fibrinogen structure were observed in patients compared to controls. In particular, a marked fibrinogen oxidative modification and a reduction in alpha-helix content and intrinsic fibrinogen fluorescence were identified.
These results pave the way to new therapeutical approaches for cardiovascular events in GCA patients.

2) Cystathionine β-synthase from *Toxoplasma gondii* is involved in cysteine biosynthesis and H_2S generation and exhibits a unique domain organization

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Cystathionine beta-synthase (CBS), the first enzyme of the reverse transsulfuration pathway, catalyzes the pyridoxal-5´-phosphate-(PLP) dependent condensation of homocysteine with serine to produce cystathionine and H2O. Cystathionine is then hydrolyzed to cysteine, α-ketobutyrate and ammonia by the second enzyme of the route, cystathionine γ-lyase (CGL). We recently showed that *Toxoplasma gondii*, the causative agent of toxoplasmosis, includes both CBS and CGL enzymes. We also established the presence of an intact functional reverse transsulfuration pathway in this protozoon (1,2). Interestingly, TgCBS can use either serine or O-acetylserine to produce cystathionine, converting these substrates to an aminoacrylate intermediate as part of a PLP-catalyzed β-replacement reaction. Besides a role in cysteine biosynthesis, we found that TgCBS can also efficiently generate H2S, preferentially via condensation of cysteine and homocysteine. In contrast with human CBS, TgCBS lacks the N-terminal heme binding domain, and its activity is not stimulated by the allosteric effector S-adenosylmethionine (AdoMet) despite containing a C-terminal Bateman module. Structural analysis complemented with molecular dynamics reveal a basal-like fold that unexpectedly differs from the active conformations found in CBSs from other organisms and let explain why TgCBS is active and is not responsive to AdoMet. These results expand the knowledge on the general folding of CBS and its structure-to-function relationship across organisms and might have far-reaching consequences for the functional understanding of the molecular mechanisms involved in catalysis and regulation of other CBSs with similar domain distribution.

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2. Maresi, E*., et al. IJMS* 19, 2111 (2018).

3) Menin protein and its variants investigated by in silico approaches

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Menin is a mainly nuclear protein, involved in different biological processes as transcription regulation, genome stability, DNA repair, signaling and cell division. Mutations in the MEN1 gene, located on the chromosome in position 11q13 and made up of 10 exons, are responsible for multiple endocrine neoplasia type 1 (MEN1), a rare autosomal dominant disease, characterized by endocrine alterations that must be present in a combined manner for at least two of the following conditions: parathyroid glands, anterior pituitary gland and neuroendocrine tumors of the gastro-entero-pancreatic tract (GEP-NET). These conditions may occur in a non-hereditary form with no family history of MEN1 (sporadic MEN1) or in several members of a family (familial MEN1). Adrenal cortical tumors, carcinoid tumors and skin lesions such as facial angiofibromas, collagenomas and lipomas can also be associated. We investigated the structural properties of menin and the potential effects of 215 amino acid mutations known to affect the sequence of 615 amino acids. We obtained a classification of variants in terms of their potential effects in modifying structure, stability, and/or function. We have also created a web accessible database for browsing the results in detail, interactively visualizing and downloading the structure of the variant proteins.
This work was partly supported by ELIXIR IT research infrastructure project and by the project "RARE.PLAT.NET", CUP B63D18000380007 and SURF 17061BP000000012, POR Campania FESR 2014/2020.

4) Hydrogen-deuterium exchange reveals increased conformational fluctuations in the amyloidogenic V122I variant than in wild-type transthyretin

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The V122I transthyretin (TTR) variant causes cardiac amyloidosis1 and is more amyloidogenic than wild-type (WT) TTR.2,3 but there are no significant differences in the X-ray crystal structures of the two proteins. We investigated whether the decreased stability of V122I TTR is associated with larger conformational fluctuations detectable by H-D exchange after dissolving the protein in D2O and monitoring the disappearance of NH resonances by NMR spectroscopy. The rate of exchange of amide hydrogen with the solvent reflects steric shielding and/or hydrogen bond persistence and is related to the equilibrium between closed and open states.4 Our results showed that V122I TTR is intrinsically more flexible than WT TTR with a higher number of residues that completely exchange within the first 5 minutes after protein dissolution. The residues from the inner β-sheet close to the mutation showed the most reduced protection, indicating decreased strength in the inter-strand H bond network. Furthermore, residues involved in both strong and weak dimer-dimer interface interactions exchanged much more rapidly in V122I TTR, suggesting an increased tendency to dissociate. The mutation had more complex effects on the outer β-sheet H bond network, with protection increased in some amides and decreased in others. However, overall, V122I TTR showed more extensive conformational plasticity than WT TTR, consistent with the variant's greater amyloidogenicity.

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[2] Hammarström P et al Proc. Natl. Acad. Sci. USA (2002) 99:16427-16432
[3] Marcoux J et al EMBO Mol Med (2015) 7:1337-1349
[4] Bai Y et al Proteins (1993) 17:75-86

5) Establishment of new in vitro models to elucidate the effect of β-glucocerebrosidase loss of function on neuronal homeostasis in GCase-related pathologies

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Deficiency of the lysosomal β-glucocerebrosidase (GCase) and the consequent accumulation of the uncatabolized glycosphingolipid glucosylceramide (GlcCer), are responsible for the onset of GCase related pathologies, among there is Gaucher Disease (GD) when mutations in GBA, the gene encoding for GCase, are in homozygosity. Whereas heterozygous mutations in GBA, represent the major genetic risk factor for the development of GBA-dependent Parkinson's disease (PD). Nevertheless, the molecular mechanism underlying the relation between GBA mutations and the onset of neuronal damage in GD and PD remains unclear so far.
To identify which is the possible molecular mechanism linking GCase loss of function with the onset of neuronal damage, we developed two *in vitro* models of the neuronal form of GD represented by murine cerebellar granule neurons (CGN) treated for 17 days with conduritol B epoxide (CBE), a specific inhibitor of GCase and iPSCs-derived dopaminergic neurons, obtained from healthy subjects' fibroblasts and treated with CBE respectively for 15 and 30 days. In CBE-treated neurons we found a progressive and time-dependent accumulation of GlcCer. Upon reaching a threshold of GlcCer accumulation, CBE-treated neurons showed significant neuronal damage along with increased volume of intracellular acidic organelles, augmented lysosomal biogenesis and block of the autophagic flow in terms of reduced LC3II and augmented p62. In addition, we found that the accumulated GlcCer is not just confined to the lysosome but also affects the plasma membrane (PM), suggesting an enhanced lysosomal trafficking towards the PM.
In conclusion, these in vitro models help to investigate the onset of cell damage induced by GlcCer accumulation, with a particular focus on the role of lysosomal impaired biogenesis and augmented fusion towards the PM.

6) Characterization of human recombinant succinic semialdehyde dehydrogenase: from structure to function

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Succinic semialdehyde dehydrogenase (SSADH) deficiency is a rare disorder characterized by a flawed γ-aminobutyric acid (GABA) metabolism. Mutations in the *ALDH5A1* gene lead to an impaired function of SSADH, the NAD^+ dependent enzyme responsible for the oxidation of succinic semialdehyde (SSA). Alterations of this catabolic pathway lead to toxic accumulation of GABA and its metabolites, causing a wide range of neurological and motor symptoms.
To date, the poor knowledge of SSADH results in a lack of effective therapies, with treatments only addressed to symptoms mitigation. This contribution aims to characterize recombinant wild-type (WT) SSADH, to understand the molecular basis for the pathogenic variants.
To this purpose, different physical-chemical techniques have been employed. The purified protein is a homotetramer from 5 mg/ml to 0.1 mg/ml. Secondary structure determination, thermal stability at 222 nm, near UV circular dichroism, and intrinsic fluorescence reveal us that NAD^+ binds to the active site of SSADH forming a complex that causes a conformational change and renders the enzyme more thermally stable. The kinetic mechanism of the enzyme proceeds through a ternary complex (enzyme- NAD^+-SSA) of Bi-Bi ordered type with NAD^+ binding before the aldehyde. The measured catalytic parameters are kcat = 56 ± 1 s^{-1}, KM NAD^+ = 38 ± 3 µM, and KM SSA = 1.7 ± 0.2 µM. In addition, SSA exhibits a KI = 39 ± 5 µM leading to substrate inhibition. Enzyme activity shows an optimal temperature at 37°C. Log(kcat) versus pH identifies a pKa value of 5.6 ± 0.1, while log(kcat/KM) is pH independent leading to the attribution of the determined pKa to a dissociable group of the catalytic complex. This complete enzyme characterization is rather new, not only *per se* but also in the context of the family of aldehyde dehydrogenases.
Future investigations will be focused on pathological SSADH variants to provide an insight into the pathogenic mechanisms.

7) Proteomic analysis of ornithine aminotransferase deficient cells reveals deregulation of protein localization and stress response pathways

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Gyrate atrophy of the choroid and retina (GA) is a single-gene autosomal recessive disease characterized by a progressive degeneration of the choroid and of the retinal pigment epithelium (RPE) layer, leading to blindness. The disease is caused by mutations in the *OAT* gene encoding ornithine aminotransferase (OAT), but the molecular mechanisms explaining how OAT deficit leads to retinal degeneration are still elusive. OAT is a pyridoxal phosphate- dependent enzyme that converts ornithine (Orn) to glutamate-semialdehyde with the concomitant production of glutamate from α-ketoglutarate. Hyperornithinemia, the hallmark of GA, is considered one of the culprits of RPE degeneration. However, contrasting results have been reported, showing limited *in-vitro* and *in-vivo* effects of chronically elevated Orn concentrations on RPE viability. To understand how the lack of OAT may reshape the intracellular pathways of RPE, we analysed the global proteome of ARPE-19 cells, an established cell model of RPE, after OAT depletion (OAT-KD) through a specific siRNA. Using high-resolution mass spectrometry with label-free quantification, we compared protein expression in OAT-KD cells with control cells, treated with a non-target siRNA. We found 139 up-regulated and 217 down-regulated proteins in OAT-KD. Gene set analysis showed that OAT silencing: i) dysregulated intracellular protein trafficking across multiple compartments, as shown by the change in expression of several proteins involved in protein localization to the endoplasmic reticulum and mitochondria; ii) affected stress pathways, including the unfolded protein response and protein degradation. Interestingly, proteomic analysis did not evidence changes in the expression of enzymes involved in Orn metabolism, either upstream or downstream OAT. Our results show that the lack of OAT may induce cellular changes that transcend direct Orn metabolism and link OAT functions to new pathways possibly involved in GA pathogenesis.

8) Specific modulation of cellular pathways highlights Neuroglobin functions in breast cancer

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Neuroglobin (NGB) displays pivotal neuroprotective functions when its expression is induced by stress/hypoxia or molecules such as 17β-estradiol. Whereas the indications concerning NGB function are turning into robust evidences, instead its role in non-neuronal and cancer cells remains largely undefined [1]. In order to explore the cellular pathways strictly dependent on NGB function, NGB-deficient MCF7-breast cancer cells were treated with 17β-estradiol, H2O2, or 17β-estradiol+H2O2 to mimic *NGB* induction, and analyzed by label-free proteomics [2,3]. The proteins varying with the same trend of regulation in all the conditions enriched common features related to NGB function, namely response to stress, cytoskeleton organization and cell activation. Instead, the regulated proteome of each dataset revealed treatment-specific alterations. Particularly, the 17β-estradiol impaired carbohydrate and lipid metabolism, cell cycle and apoptosis. The effect of H2O2 increased the response to chemical stimulus, mostly affecting mitochondrial organization, transport, ATP synthesis, and oxidative phosphorylation. Finally, the combined treatment revealed imbalance of autophagy, proteolysis, cell division and apoptosis, suggesting alterations of mitochondrial and lysosomal compartments. These findings imply the direct/indirect involvement of NGB in a variety of pathways in breast cancer cells [4].

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9) Employment of recombinant angiogenin variants to clarify its role in the stress granules recruitment

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Angiogenin (ANG) is a 14-kDa ribonuclease, involved in different cell functions such as proliferation, survival and stress cell response depending on its intracellular localization [1]. Under physiological conditions ANG moves to the cell nucleus where enhances rRNA transcription by binding to the CT-rich angiogenin binding element (ABE) [2]; conversely, under stress conditions, ANG accumulates in cytoplasmic compartment and modulates the production of tiRNAs, a novel class of small RNAs that contribute to translational inhibition and recruitment of Stress Granules (SGs) [3]. To date, there are still limited experimental evidence relating to the ANG role in the epidermis, the outermost layer of human skin, continually exposed to external stressors. On the regard, our study is focused on clarifying ANG possible role in human keratinocytes (HaCaT) subjected to different stressors.
Our preliminary results indicate that endogenous ANG, as well as the recombinant ANG (rANG), are involved in the stress response of HaCaT; next step is focused on a new ANG variant, in which nuclear localization sequence (NLS) is modified (see figure), in order to verify whether the inability to move to the nucleus actively alters cell response to stress conditions and/or its potential activity in promoting transcription.

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10) Nitrobindins are RNS scavengers

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Nitrobindin (Nbs) form a new class of evolutionary conserved heme-protein characterized by a 10-stranded anti-parallel β-barrel fold. In Nbs, the heme is highly solvent exposed and the heme-Fe atom is stably in the ferric form. To date, crystal structures of Arabidopsis thaliana Nb (At-Nb), Mycobacterium tuberculosis Nb (Mt-Nb) and Homo sapiens Nb (Hs-Nb) have been solved. While Mt-Nb and At-Nb are single-domain proteins, Hs-Nb has been described as a domain of the human nuclear protein named THAP4, whose function is still unknown. Both in the ferric and ferrous form, Nb facilitates the detoxification of reactive nitrogen species (i.e., NO and peroxynitrite) by (i) reductive nitrosylation of the heme-Fe(III) atom, (ii) O2-dependent oxidation of the heme-Fe(II)-NO complex, and (iii) heme-Fe(III)-based conversion of peroxynitrite to NO3 − protecting free Ltyrosine against nitration. These processes depend on the redox state of the environment and are strictly related. The (pseudo)enzymatic properties of Nbs could reflect the weakening of the Fe-His proximal bond that is 0.10–0.17 Å longer than that observed in prototypical mammalian myoglobins. Moreover, EPR and absorbance spectroscopic properties of ferrous nitrosylated Nbs are typical of five-coordianted heme-proteins and heme-model compounds. Overall, Nbs play a role in RNS detoxification; their scaffold possibly representing a biotechnological frontier for the development of hybrid biocatalysts.

11) Ganglioside-protein interaction at plasma membrane level: the role of GM1 oligosaccharide

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Gangliosides are sialic acid-containing glycosphingolipids abundant in the neurons' plasma membrane (PM). They are composed by two portions: the ceramide, anchoring the molecule to the PM, and the oligosaccharide, protruding in the extracellular space. Among all, GM1 ganglioside is extremely relevant in the nervous system since it modulates the activity of different molecular partners within the PM including neurotrophins' Trk receptors.
Recently, we proved that the oligosaccharide alone (OligoGM1) is the bioactive moiety of GM1. Without entering the cells, OligoGM1 was able to perfectly replicate the GM1 activity, leading to neuronal differentiation, maturation and protection. All these events share the same activating mechanism at the PM: the direct interaction between OligoGM1 and NGF TrkA receptor. By exploiting radioactive and photoactivable GM1 derivatives, we proved that TrkA–GM1 interaction involves the GM1 oligosaccharide, but not the ceramide. Molecular docking analyses confirmed the presence of a specific pocket for OligoGM1 in the extracellular portion of TrkA and that OligoGM1 binding strongly stabilizes the NGF-TrkA complex by reducing the free energy. The biochemical analysis of isolated lipid rafts showed that TrkA localizes outside the detergent-resistant fractions where GM1 is found, suggesting that TrkA and GM1 belong to separate membrane domains and that their interaction probably occurs by a flopping down of the TrkA extracellular domain towards the OligoGM1. Finally, by using TrkA inhibitor or silencing its expression, the OligoGM1 modulatory activity was strongly reduced, further highlighting the importance of TrkA-NGF-OligoGM1 partnership. Altogether this evidence indicates that GM1 oligosaccharide can modulate neuronal homeostasis by a direct interaction with specific proteins at the cell surface, opening a new perspective on ganglioside action mechanism.

Chiricozzi 2017, 2019 J Neurochem – 2019 Mol Neurobiol; Fazzari 2020 Glycoconj J.

12) Reactive oxygen species effects on structural and functional properties of fibrinogen

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Fibrinogen, a 340-kDa soluble glycoprotein synthesized in the liver, plays a crucial role inhemostasis and represents the major structural clot component. Under physiological conditions, clotprevents blood loss at the sites of injuries, but under pathological conditions it can stuck bloodvessels causing thrombosis. It has been shown that fibrinogen is more susceptible to oxidation than most other plasma proteins; in particular, fibrinogen is 20× more susceptible to oxidation than albumin, as also reported in patients with MI, where it was found that total plasma protein carbonyls were formed preferentiallyon fibrinogen.
On the bases of these data, the aim of this study was to investigate the effects displayed by ROS on structural and functional properties of fibrinogen purified from healthy subjects. ROS generation was induced by 2,2'-azobis-2-methyl-propanimidamide, dihydrochloride (AAPH). Fibrinogen oxidation, thrombin-catalyzed fibrinogen polymerization and plasmin-induced fibrin degradation were assayed in AAPH-treated and untreated fibrinogen samples. Marked reduction in thrombin-catalyzed fibrinogen polymerization and plasmin-induced fibrin degradation, together with a strong increase in dityrosine formation were observed in AAPH-treated samples compared to untreated samples. Moreover, structural alterations were evaluated: a decrease in alpha-helix content (as suggested by circular dichroism spectroscopy) and an alteration in tertiary structure in AAPH-treated samples were evident. Atomic force microscopy analysis showed that AAPH treatment induces changes in fibrinogen morphology and aggregates formation.
In conclusion, our results strongly indicate that upon fibrinogen oxidation -an event which can occur in several pathophysiological conditions- fibrinogen structural alterations and functional activity modifications can occur giving rise to major pathological consequences.

13) A metabolomic approach to understand frataxin function: the Friedreich's ataxia associated protein

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Frataxin is a mitochondrial protein whose absence determines the development of Friedreich's ataxia (FRDA), a lethal neurodegenerative disease. FRDA patients show alterations in iron metabolism and in Fe-S clusters concentration leading to oxidative stress.
In bacteria, the biosynthesis of Fe-S clusters is ruled by *isc* operon, which encodes for a series of proteins involved in Fe-S cluster centers development and maturation. Surprisingly, although bacterial frataxin (CyaY) is not present on the *isc* operon, it is involved in Fe-S clusters production. The synthesis of Fe-S centers in bacteria implicates a first step in which the IscS enzyme extracts the thiol group from L-cysteine and transfers it to IscU protein scaffold, followed by iron-sulfur cluster transfer from IscU to the final effector.
This project focused on clarifying the impact of iron on the functionality of frataxin as a regulator of IscS activity *in vivo*. In this regard, with a holistic approach, IscS activity was evaluated by measuring the sulfide production via a colorimetric assay, using *E. coli* wild-type bacterial strain and a modified strain lacking the gene encoding CyaY.
The results suggest an effect of iron that modulates the IscS enzymatic activity in the presence of CyaY.

14) Anti-hyperglycemic effect of tea extracts is mediated by PTP1B inhibition and insulin receptor activation

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Type 2 diabetes (T2D) is a chronic pathology characterized by high blood glucose levels (hyperglycemia), as consequence of low insulin secretion by pancreas or the inability of the hormone to activate a correct physiological response (insulin resistance). Despite the abundance of oral hypoglycemic drugs, many patients have difficulties to maintain glycaemic control by time and experience a progressive worsening of their healthy condition. Many studies showed that bioactive molecule contained in food and beverages can have a positive impact human cells physiology. Tea is a very popular beverage in the world: it contains many bioactive molecules showing different biological properties, including antioxidant, antinflammatory, anticancer and antidiabetic activity. This study aims to evaluate the effects of tea extracts on both Low Molecular Weight Protein Tyrosine Phosphatase (LMW-PTP) and Protein Tyrosine Phosphatase 1 B (PTP1B), the two main negative regulators of the insulin receptor. We demonstrated that tea extracts are potent inhibitors of both these enzymes. Moreover, we identified galloyl moiety-bearing catechins as the tea components responsible for protein phosphatases' inhibition, showing IC_{50} values in the sub-micromolar range. Specifically, kinetic and docking analyses revealed that epigallocatechin gallate (EGCG) is a mixed-type non-competitive inhibitor of PTP1B and *ex vivo* assays confirmed that EGCG acts as an insulin-sensitizing agent. In addition, we highlighted that chronic treatment of liver cells with cold tea extracts improves both the expression of the insulin receptor and insulin sensitivity, whereas hot extracts do not. Altogether, our data suggest that tea components improve glucose homeostasis by regulating the insulin receptor expression levels and activation status.

15) New evidences of the cross-talk between fatty acid and glucose metabolism: inhibitory effect of fatty acids on enzymes that regulate the insulin signaling pathway

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Plasma free fatty acids (FFAs) play important physiological roles in several tissues, such as skeletal muscle, heart, liver and pancreas. Among these features, they play as energy resource: recent studies confirmed that, at rest, almost 70% of all fatty acids released during lipolysis were re-esterified, while, during the first 30 min of exercise, this value dropped to 25% and total fatty acid release via triglyceride hydrolysis, so a higher is quote available for oxidation. Since there is no insulin present, this coordinated mechanism must be regulated in some other way. Recent studies confirmed that FFAs can modulate the activity of different protein kinases and protein phosphatases, suggesting a their possible regulatory role. This study aims to evaluate the effects of several FFAs on both Low Molecular Weight Protein Tyrosine Phosphatase (LMW-PTP) and Protein Tyrosine Phosphatase 1 B (PTP1B), the two main negative regulators of the insulin receptor. We demonstrated that long chain FFAs such as palmitic and oleic, markedly inhibited PTP1B while shorter FFAs did not. On the other hand, our results confirmed that unsaturated long-chain FFAs could inhibit both PTP1B and LMW-PTP, indicating differential inhibition capacity that could bring to different effects in the organism. In addition, we highlighted that chronic treatment of liver cells with these FFAs, brought to phosphorylation of insulin receptor while Akt was not. Probably when these FFAs are present, the PTPs inhibition brings to insulin receptor activation resulting in lipogenesis since the Akt way is not active. That could explain the role of FFAs in controlling fat metabolism through PTPs inhibition.

16) Neurosteroidogenesis in human microglia: TSPO involvement in the control of microglial activities

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Dysregulated microglial activities are linked to the development of chronic neuroinflammation and related to neurodegenerative diseases [1]. Particular attention has been paid to the mechanisms underlying microglial activation for the identification of new therapeutic targets [2][3]. The mitochondrial 18-kDa Translocator Protein (TSPO) has been demonstrated to be high expressed in activated microglia, emerging as an interesting target for neuroprotection [4]. TSPO is involved in several cellular processes; however, the most peculiar and debated one is represented by its involvement in neurosteroids biosynthesis. In particular, TSPO permits the cholesterol transport into mitochondria, where the CYP11A1 enzyme promotes its conversion into pregnenolone [5][6]. In this context, the involvement of TSPO in human microglial neurosteroidogenesis was investigated in two human microglial cell lines, C20 and HMC3 [7]. C20 and HMC3 expressed the key members for neurosteroid production, comprising of TSPO, StAR, CYP11A1 and the enzymes involved in the neurosteroid biosynthetic pathway. Moreover, the investigated human microglial cells accumulate pregnenolone in a time-dependent manner and the pharmacological stimulation of TSPO by selective ligands drastically increased pregnenolone release. Notably, gene silencing of TSPO affected the time-dependent accumulation of pregnenolone and its release downstream TSPO ligand treatments. TSPO ligands increased also the BDNF release by human microglia, while the inhibition of neurosteroid production by AMG pre-treatment significantly reduced its production, unraveling a possible neurosteroid regulated mechanism for BDNF production. In conclusion, the microglial neurosteroidogenic pathway, coordinated by TSPO activity, seems to be an important autocrine/paracrine way to regulate microglial activities and could represent a crucial mechanism to exploit for the exogenous control of neuroinflammation.

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17) Computational study of the molecular interactions in the binding of coronavirus Spike-proteins with the human Angiotensin-Converting Enzyme 2 (ACE2) cellular receptor

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Due to the pandemic urgency of COVID-19, we investigated the molecular interaction of the Spike-proteins (Sp) from SARS-CoV and SARS-CoV-2 with the human Angiotensin-Converting Enzyme 2 (ACE2), recognized as the most relevant cellular receptor involved in both the coronavirus infections. SARS-CoV-2 Sp consists of a long sequence of 1273 amino acids, and the interaction with ACE2 receptor is due to its region 318-510, named Receptor-Binding Domain (RBD). On the other side, ACE2 consists of 805 amino acids and its physiological activity as peptidase regulates cardiovascular homeostasis. The interaction between ACE2 and Sp-RBD is described at atomic level by different crystallographic complexes obtained under different conditions, and available in the RCSB PDB Protein DataBank. We in silico analyzed the structural features of the Sp-ACE2 complexes and observed the surface interactions occurring in the different complex structures. The results of the comparative analysis highlight the details of the most peculiar interactions and the corresponding amino acids involved.

18) The multitasking role of GM1 oligosaccharide in modulating neuronal intracellular signaling

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Recently, we demonstrated that the GM1 differentiating properties in murine neuroblastoma cells N2a are mediated by its oligosaccharide chain, II3 Neu5Ac-Gg4 (OligoGM1) that, by interacting with the NGF receptor TrkA, leads to the activation of the ERK1/2 downstream pathway. To understand if TrkA activation by OligoGM1 was able to trigger other key biochemical pathways besides the neurodifferentiation, we performed a proteomic analysis on N2a cells treated with 50 µM OligoGM1 for 24 h. Over 3000 proteins were identified and, among these, 324 proteins were exclusively expressed in OligoGM1-treated cells. It emerged that several proteins only expressed in OligoGM1-treated cells are involved in biochemical mechanisms of neuronal differentiation and protection, such as suppression of proinflammatory molecules, inhibition of oxidative stress, mitochondrial bioenergetics, and regulation of calcium homeostasis. To support the proteomic data, biochemical analyses were performed. By calcium imaging experiments, we observed that OligoGM1 administration to undifferentiated N2a cells leads to an increased calcium influx, mediated by the activation of TrkA receptor. By immunoblotting analysis, we demonstrated PLCγ and PKC activation follows the TrkA stimulation by OligoGM1, leading to the opening of calcium channels both on the plasma membrane and on intracellular storages, as confirmed by calcium imaging experiments performed with IP3 receptor inhibitor. Moreover, we showed that N2a cells exposed to OligoGM1 displayed an increased mitochondrial density and an enhanced mitochondrial activity together with reduced reactive oxygen species levels. Taken together these results highlight that OligoGM1 triggers crucial pathways, starting at the plasma membrane level, aimed at maintaining neuronal homeostasis.

Refs: Chiricozzi et al. 2017 J. Neurochem.; Chiricozzi et. al 2019 Mol. Neurobiol.; Fazzari et al. 2020 Glicoconj. J.; Lunghi et al. 2020 Glicoconj. J.

19) Picosecond to millisecond insight into transthyretin dynamics

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Both wild type and genetic variants of the plasma protein, transthyretin (TTR), cause systemic amyloidosis but, among more than 300 crystal structures in the PDB, no amyloidogenic hotspots have been identified. Indeed, formation of authentic TTR amyloid fibrils under physiological conditions, *in vitro* and *in vivo*, requires the specific proteolytic cleavage of TTR when it is stressed by mechanical forces (1). To further elucidate the complex mechano-enzymatic mechanism of fibrillogenesis, we have thoroughly characterized wild type (WT) TTR dynamics by NMR. Different NMR experiments, R1, R1 , R 2, hetNOE and CPMG, were acquired at three magnetic fields, 16T, 21T and 22T and 25 and 37°C. Preliminary Model-Free analysis and Reduced Spectral Density Mapping (2) of NMR relaxation data enabled local mobility characterization on the ps-ns time scale. Interestingly, analysis of the Reduced Spectral Density, at 25 and 37°C, shows, for the first time, conformational exchange on the µs time-scale. The comparable exchange rates for all residues, suggest global protein motion in this temporal window, consistent with equilibrium, exchanging in the s timeframe, between a high and a low populated state, the latter in a random-coil like conformation. Moreover, residues in the β-strands G, H and F and around the dimer-dimer interface undergo slower motions in the ms regime, as previously reported (3). The global picture of WT TTR dynamics highlights motions on different timescales with potentially major effects on aggregation. Future studies on more amyloidogenic TTR variants will be performed to confirm and extend the correlation between µs-ms motions and amyloid fibrillogenic propensity.

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20) On the role of weak chemical interactions in the formation of beta turns

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Beta-turns are among the simplest secondary structures of proteins. They involve only four residues and, according to Venkatachalam [1], they are non-helical conformations of the backbone in which the carbonyl group of the first residue (i) forms a hydrogen bond with the nitrogen-bonded hydrogen of the fourth (i + 3) residue. Beta turns allow the peptide chain reversal and confer greater flexibility to protein structure, thus playing an important role in many biochemical processes. In the last 50 years the original definition by Venkatachalam widened to include 8 types of beta turns plus a miscellaneous category [2]. Several classifications have been proposed based exclusively on the evaluation of the phi and psi dihedral angles.
In this work we wish to discuss the influence of weak intramolecular interactions as n → π* interactions in the stabilization of beta turns [3].
To this purpose we identified repeated patterns of n → π* interactions between carbonyl groups of successive residues in the 19 beta-turn classes recently defined by Shapovalov and coworkers [2]. Our dataset was based on high-resolution X-ray structural data for a total of 36949 analyzed beta turns.
We also performed a statistical analysis on the presence of n → π* interactions and their geometrical features in turn mimetic compounds as cyclic peptides, cyclic depsipeptides and cyclic peptoids. A total of 232 macrocyclic compounds (whose structure were solved by X-ray crystallography) were considered.
The results obtained show that the n → π * interactions allow to discriminate among beta turn classes. We therefore highlight how weak chemical interactions may be used to classify beta turns.

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21) Effect of manganese on Antarctic esterase

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Organisms able to survive and thrive in cold environments are widespread and have developed several molecular adaptations to survive in these extreme conditions, including the production of cold-active enzymes. These enzymes are characterized by high activity at low temperatures and thermolability, making them suitable for bioprocesses involving thermolabile substrates or products [1]. Esterases are widespread enzymes that hydrolyze ester bonds present in different molecules, and they are important for several biotechnological applications such as food processing and detergency [2]. Here, we report the functional and structural characterization of an esterase identified in the genome of the Antarctic bacterium *Marinomonas ef1* (M-Est). M-Est is a strictly cold-active enzyme with an optimum temperature of catalysis at 5°C and high thermolability. Interestingly, the addition of Mn^{2+} ion improves the thermal stability of this enzyme. The effects of Mn^{2+} ion on functional and structural properties of M-Est are investigated combining site directed mutagenesis and biophysical analysis.

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22) Deepening into the biochemical properties of human D-3-phosphoglycerate dehydrogenase

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L-Serine plays a pivotal role in the metabolism of eukaryotic cells and is involved in several physiological functions [1]. Moreover, L-serine is the precursor of glycine and D-serine, two neuroactive signaling molecules that modulate the activation of NMDA receptors [2, 3]. Human D-3-phosphoglycerate dehydrogenase (hPHGDH, EC 1.1.1.95) is responsible for the first, reversible and rate-determining step in the "phosphorylated pathway", which regulates the *de novo* biosynthesis of L-serine [1]: this protein catalyzes the transformation of D-3-phosphoglycerate (3PG, generated by glycolysis) into 3-phosphohydroxypyruvate (PHP) with NAD^+ reduction. Here, we biochemically characterized hPHGDH by investigating the effect of pH, temperature, ligands, and ions on the enzymatic activity. We established the kinetic parameters of the forward reaction for 3PG, and alternative carboxylic acids and we clarified that 3PG is the best substrate. We found that PHP and α-ketoglutarate are efficiently reduced by hPHGDH (in the reverse direction), indicating that *in vivo* α-ketoglutarate could compete with PHP. Notably, PHP, L-serine, glycine, and D-serine were not able to inhibit hPHGDH activity. Moreover, we suggest that hPHGDH is present in solution in different conformations and/or oligomeric states.
Taken together, the comprehension of the biochemical properties of hPHGDH will allow a better understanding of the molecular mechanisms underlying brain L-serine biosynthesis, and of the effects of mutations related to pathological states. This work is also aimed at the identification of new approaches to modulate L-serine levels in order to reduce cancer progression and treat neurological disorders.
This project was founded by "PRIN-2017 - Dissecting serine metabolism in the brain".

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23) Omics strategies unveil a novel DJ1 proteoform accounting for metabolic epigenetic misregulation

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In cancer cells, the high glycolytic flux induces carbonyl stress, a damaging condition that increases reactive carbonyl species making DNA, proteins and lipids more susceptible to glycation. Together with glucose, methylglyoxal (MGO), a by-product of glycolysis, is considered the main glycating agent. MGO is highly diffusible, enters the nucleus and reacts with lysine- arginine-rich tails of histones.
Glycation adducts on histones undergo oxidization and rearrange to form stable species known as advanced glycation end-products (AGEs). This modification alters nucleosomes stability and chromatin architecture deconstructing the histone code. Formation of AGEs has been associated with cancer and several age-related diseases. DJ1, a cancer-associated protein that protects cells from oxidative stress, has been described as a deglycase enzyme. In several human tumours, its expression, localization, oxidation, and phosphorylation were found altered. This work aims to explore the molecular mechanism that triggers the peculiar cellular compartmentalization and the specific post translational modifications (PTM) that influences the DJ1 dual role, in breast cancer cells. Using a proteomic approach, we identify on DJ1 a novel threonine phosphorylation, part of a putative Akt consensus.
Interestingly we found that pharmacological modulation of Akt pathway induces a functional tuning of DJ1 proteoforms revealing that the pathway is critical for DJ1 pro-tumorigenic abilities.
In breast cancer cells, the overactivation of Akt signaling enhances DJ1-phosphorylation. Phosphorylated DJ1 increases its glyoxalase activity therefore preventing glycation-induced histones misregulation. In this work we report the characterization of a novel proteoform of DJ1 accounting for the ability of cancer cells to counteract carbonyl stress escaping the metabolic induced epigenetic misregulation that otherwise could impair their malignant proliferative potential.

24) Structure-function correlation of Phox2B in the congenital central hypoventilation syndrome (CCHS)

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About 90% of congenital central hypoventilation syndrome (CCHS) patients show polyalanine triplet expansions in the coding region of transcription factor PHOX2B, which renders this protein an intriguing target to understand the insurgence of this syndrome and for the design of a novel therapeutical approach (1). Consistently with the role of PHOX2B as a transcriptional regulator, it is reasonable that a general transcriptional dysregulation caused by the polyalanine expansion might represent an important mechanism underlying CCHS pathogenesis. Comparison of variants displaying different polyalanine expansions by a multidisciplinary approach based on different methodologies (including circular dichroism, spectrofluorimetry, light scattering, and Atomic Force Microscopy studies) highlighted the propensity to aggregate and to form fibrils for the PHOX2B variant containing the polyalanine expansion (+7-alanines) suggesting a plausible role of such fibrils in the insurgence of CCHS (2). More recently a biochemical characterization of the paralogue Phox2A, lacking the alanine stretch, was performed and thermophoresis data revealed a direct interaction between the two forms of Phox2B and Phox2A *in vitro* showing a higher affinity between Phox2A and the pathological variant of Phox2B rather than with Phox2B wild-type. This prompts to speculate Phox2A could stabilize pathological forms making them functional and limiting the aggregation process. In parallel, high resolution structural characterization of the wild-type Phox2B and of its DNA binding domain was performed via NMR spectroscopy. At last, through NMR CSP analysis, binding experiments between Phox2B wild-type and its DNA target were undertaken aimed at the exploration of a powerful approach for the screening of molecules with therapeutic purposes.

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25) Evolutionary, structural and functional differences between alpha amylases

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The possibility of gathering and transferring information from prokaryotes and simple eukaryotes to more complex systems is a relevant tool which offers structural, methodological and economic advantages. Enzyme template transfer often relies on comparable amino acid sequences, that frequently result in similar protein structures and functions. This may also be the case of α-amylases from *Homo sapiens* saliva (HSA) and from *Aspergillus oryzae* (AAO), which share a good degree of aminoacid sequences homology. However, the relationship between structure similarity and function similarity is influenced by multiple factors, such as divergence and convergence evolution. This project aims at comparing HSA and AAO by *in silico* and *in vitro* approaches to evaluate the possible use of the simpler and less expensive fungal protein as a substitute of the human protein in future pharmaceutical studies.
As expected, in silico investigation highlighted a significant overlap of the aminoacids of the active site as well as of those involved in calcium binding. The only relevant difference is the presence of a chloride ion in HSA, which is absent in the fungal enzyme. In addition, despite broad structural similarities, the fungal enzyme was functionally more efficient than the human enzyme and oppositely regulated by the presence of ions (calcium, chloride), which inhibit the fungal protein and activate the human one.
In conclusion, these functional differences are to be taken into account when using the fungal enzyme as a model of the human one, with a particular attention in the setup of the experimental conditions.

26) p53-MDM2/MDM4 complex and chemokine receptor CXCR4 activation in Glioblastoma cells and Glioblastoma stem-like cells

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The protein-protein interaction is one of the main mechanisms regulating cancer progression. Particularly, the p53 protein is regulated by the interaction with numerous proteins, among which murine double minute 2 (MDM2) and 4 (MDM4) proteins have a pivotal role: they negatively regulate p53, inhibiting its transcriptional activity and favouring its degradation. Thus, the inhibition of p53-MDM2/MDM4 complex is one of the most promising strategies to block tumour progression. Moreover, tumour resistance/invasiveness has been recently attributed to an overexpression of the C-X-C chemokine receptor type 4 (CXCR4) that further cooperates with MDM proteins in promoting tumour invasion/progression, especially deregulating the p53-MDM2 axis. However, the cooperation of CXCR4 with p53-MDM2/MDM4 complex has never been investigated in Glioblastoma (GBM). Thus, the current study aimed to assess the effect of CXCR4 signal blockade in enhancing the effect of p53-MDM2/4 complex inhibition, using new synthetic compounds, leading to a complete functional reactivation of p53 in GBM cells (U87MG) and GBM stem-like cells (neurospheres), which are critical for tumour recurrence and chemotherapy resistance. The obtained data have demonstrated that the inhibition of MDM2/4 and the selective CXCR4 antagonism reduced GBM cell invasiveness/migration. It is noteworthy that the simultaneous MDM2/4 inhibition and CXCR4 antagonism exerts a synergic effect on cancer stem components: indeed, the MDM2/4 block inhibited the growth and formation of neurospheres, the CXCR4 antagonism induced differentiation of neurospheres and enhanced the MDM2/4 block effectiveness preventing their proliferation/clonogenicity.
These results confirm that blocking CXCR4/MDM2/4 represents a valuable strategy to reduce tumour proliferation and invasiveness, acting on the stem cell component too.

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- Zhang et al. Cancers. 2018
- Presti et al. Oncol Lett. 2018

27) Characterization of the two main isoforms of human D-aspartate oxidase by cellular studies

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Human D-aspartate oxidase (hDASPO) is a peroxisomal flavoenzyme that selectively degrades acidic D-amino acids, in particular D-aspartate (D-Asp). In mammalian central nervous system D-Asp acts as a signaling molecule and is involved in neural development, brain morphology and behavior [1]. Studies performed in animal models demonstrated that a persistent upregulation of D-Asp levels causes severe deficits in synaptic plasticity, spatial learning and memory [2]: by strictly regulating the brain levels of D-Asp, DASPO exerts a protective role and prevents these precocious deterioration processes.
Apparently, the human DASPO gene produces alternative transcripts encoding for three putative hDASPO isoforms constituted by 341, 369 and 282 amino acids. Unfortunately, the hDASPO_369 isoform was produced in *E. coli* as inclusion bodies, this hampering its biochemical characterization. However, the functional properties, the degradation kinetics and the mechanisms involved in cellular turnover of hDASPO_341 and hDASPO_369 were investigated by ectopically expressing these isoforms in the U87 human glioblastoma cell line. Both protein isoforms are active, localize to the peroxisomes, are stable and are primarily degraded through the ubiquitin-proteasome system [3]. Notably, the additional N-terminal peptide of the hDASPO_369 isoform was identified only in the hippocampus of female Alzheimer's disease (AD) patients, while peptides common to hDASPO_369 and hDASPO_341 isoforms were present in both male and female healthy controls and AD patients. The hDASPO_369 isoform seems to be selectively expressed in the hippocampus of AD female patients.

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28) Fibrosis in human cancers: a BAG3 perspective

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Bcl-2-associated athanogene 3 (BAG3) belongs to the family of co-chaperone proteins that interact with the heat shock protein 70 (Hsp70). Due to the presence of several domains involved in protein-protein interactions, BAG3 binds also to other partners, modulating a number of cellular processes including proliferation, apoptosis and autophagy (Marzullo et al., BBA Gen Subj. 2020). BAG3 can be also released, through the unconventional secretion pathway, in the extracellular medium by some neoplastic cells and in particular pancreatic cancer cells, and can activate macrophages through binding to a specific receptor, InterFeron- Induced TransMembrane (IFITM)-2 protein. This induces PI3K and p38 MAPK activation, expression of COX2 and iNOS genes and the release of cytokines, including IL-6 and IL-10 (Rosati et al., Nat Commun. 2015). More recently, new pieces of evidence revealed a pivotal role of BAG3 in pro-tumor cell signaling in tumor microenvironment, as well as its involvement in the development of fibrosis in tumor tissues (Iorio et al., Gut 2018, Clin Cancer Res. 2019). Here we report further data showing the presence of the BAG3 receptor (IFITM-2) on the plasma membrane of normal dermal fibroblasts and the activity of BAG3 as a factor able to induce the expression of alpha-SMA and the phosphorylation of AKT and FAK kinases, that sustain CAF functions in tumor microenvironment. The results obtained provide encouraging data that identify BAG3 as a promising therapeutic target to counteract fibrosis in tumors.

29) Post-translational modifications of human D-amino acid oxidase: new regulation mechanisms of an old flavoenzyme

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A relevant role has been highlighted for the FAD-dependent human flavoenzyme D-amino acid oxidase (hDAAO) in the central nervous system: it is responsible for the degradation of a key signaling molecule, D-serine (D-Ser), the main endogenous coagonist of synaptic N-methyl-D-aspartate receptors. hDAAO is therefore indirectly involved in modulating the activation state of these receptors and plays a role in NMDA receptor signaling pathway impairments, which are known to occur in acute and chronic neurological diseases and are often due to a dysfunctional D-Ser metabolism (Ploux et al., 2020).
Due the proposed biological role, hDAAO structural/functional relationships have been extensively investigated and several strategies aimed at controlling the enzymatic activity have been identified (Murtas et al., 2017). Here, we focus on post-translational modifications: by using a combination of structural analyses, biochemical methods, and cellular studies, we investigate whether hDAAO is subjected to nitrosylation, sulfhydration, and phosphorylation.
Our results indicate that hDAAO is S-nitrosylated and this negatively affects its activity. In contrast, the hydrogen sulfide donor NaHS seems to alter the enzyme conformation, stabilizing a species with higher affinity for the FAD cofactor, thus positively affecting the enzymatic activity. Moreover, for the first time, we show that hDAAO is phosphorylated in cerebellum; however, the protein kinase involved is still unknown. Taken together, these findings indicate that D-Ser levels can be also modulated by post-translational modifications, which intriguingly are common to serine racemase, the D-Ser biosynthetic enzyme.

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30) Signal transduction and C-di-GMP second messenger: novel tools to investigate the mechanism of nutrient sensing

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Nutrients may dictate the fate of bacterial cell including biofilm formation by tuning the intracellular levels of c-di-GMP [1]. Many proteins involved in controlling c-di-GMP levels through diguanylate cyclase or phosphodiesterase domain (or both) are linked to periplasmic domains devoted to nutrient perceiving. Among nutrients, L-Arginine represents a key metabolite required as nitrogen, carbon and ATP source, associated to chronic infections, biofilm/virulence and antibiotic resistance [2]. Understanding the mechanism of L-Arginine-dependent signal transduction is crucial to identify possible strategies to tune biofilm through nutrients. Nevertheless, the architecture of such transducers is often too complex to be suitable for full-length studies, including many regulatory domains downstream the transmembrane helices joining the catalytic moiety (as in the case of RmcA from P. aeruginosa [3, 4]).
To by-pass this limitation, we identify a possible prototype protein able to change c-di-GMP levels in response to L-Arginine, showing a minimal architecture i.e., periplasmic L-Arginine binding domain, transmembrane helices, catalytic domain(s). The purification as soluble protein arranged into nanodisc and its preliminary characterization is shown, in comparison with protein engineering studies aimed at producing a water-soluble version of the protein.

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31) SS-nitrosylation and glycine control the activity of human serine racemase through an allosteric interplay

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D-serine is a neuromodulator that co-activates NMDA glutamatergic receptors, produced in several tissues and mainly in cerebral regions. D-serine is synthesized by serine racemase (SR), a pyridoxal 5'-phosphate (PLP)-dependent homodimeric enzyme that catalyzes the reversible racemization of L-serine to D-serine and the irreversible deamination of L- and D-serine to pyruvate and ammonia. D-serine has been demonstrated to be involved in amyotrophic lateral sclerosis (ALS) pathogenesis. Indeed, the deletion of the gene encoding SR slowed down the disease progression in ALS mouse models and protected against cerebral ischemia and excitotoxicity. Therefore, SR is an interesting target for the development of innovative ALS treatments. It was found that SR is regulated by S-nitrosylation at Cys113, with a 7-fold reduction of the enzyme activity. To get insights into the mechanism of regulation, we investigated the correlation between S-nitrosylation and SR conformations stabilized by ATP, a positive allosteric effector of SR, and glycine, a physiological inhibitor of SR, by integrated experimental and theoretical approaches. We demonstrated that S-nitrosylation-mediated negative regulation occurs through the stabilization of an open, less-active conformation of the enzyme. The reaction of SR with either NO or nitroso donors is conformation-dependent and occurs only in the conformation stabilized by ATP, in which the - amino group of Lys114 acts as a base towards the thiol group of Cys113. In the closed conformation stabilized by glycine, the side chain of Lys114 moves away from that of Cys113, while the carboxyl side-chain group of Asp318 moves significantly closer, increasing the thiol pKa and preventing the reaction. We concluded that ATP binding, glycine binding, and S-nitrosylation constitute a three-way regulation mechanism for the tight control of SR activity [1].

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32) Cytosolic localization and in vitro assembly of human de novo thymidylate synthesis complex

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Cancer cells reprogramme onecarbon metabolism (OCM) to support enhanced growth and proliferation, in this contest Serine hydroxymethyltransferase (SHMT) is a pivotal enzyme.
SHMT mainly exists in three isoforms; two localized in the cytosol (SHMT1/SHMT2 α) and one (SHMT2) in the mitochondria. SHMT1 undergoes to a nuclear localization during the Sphase of the cell cycle to sustain de novo dTMP synthesis [1]. The de novo thymidylate synthesis is a crucial pathway for normal and cancer cells. Deoxythymidine monophosphate (dTMP) is synthesized by the combined action of three enzymes: serine hydroxymethyltransferase (SHMT), dihydrofolate reductase (DHFR) and thymidylate synthase (TYMS), the latter two targets of widely used chemotherapeutics such as antifolates and 5fluorouracil. It had been suggested that these three proteins assemble in the nucleus into the thymidylate synthesis complex (dTMPSC) [1].
situ proximity ligation assay, showing that it is also detected in the cytoplasm. This result strongly indicates that the role of the dTMPSC assembly may go beyond dTMP synthesis.
We have also successfully assembled the dTMP synthesis complex in vitro, employing tetrameric SHMT1 and a bifunctional chimeric enzyme comprising human TYMS and DHFR by using a different array of techniques.
Moreover, we have demonstrated that the SHMT1 tetrameric state is required for efficient complex assembly, indicating that this aggregation state is evolutionary selected in eukaryotes to optimize protein protein interactions. Lastly, we have setup an activity assay of the complete thymidylate cycle in vitro, which may provide a useful tool to develop drugs targeting the entire complex instead of the individual components.

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33) Structure, Function and dysfunction of human riboflavin transporter 2

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Riboflavin (Rf) is an essential dietary component, representing the precursor of FMN and FAD, the redox cofactors of several flavoenzymes involved in bioenergetics, ROS balance, as well as protein folding and epigenetics [1].
Rf cellular uptake in humans is mediated by three transporters belonging to the SLC52 family. SLC52A1-3 exhibit no significant similarity to bacterial or yeast Rf transporters and they show different tissue expression and functional properties [1]. RFVT2 is ubiquitously expressed, but it is relevant especially for the brain [2, 3]. Alterations of its activity/expression have been correlated with a rare inherited neurodegenerative disorder, the Rf Transporter Deficiency 2 (previously known as Brown Vialetto-van-Laere syndrome), sometimes treatable with high Rf doses [3]. To study RFVT2 structure-function relationships, we over-expressed RFVT2 in *E. coli* and assayed the transport in proteoliposomes [4]. Kinetics of mutants mimicking the patient mutations were compared to the WT RFVT2 and in most cases a reduction of catalytic efficiency was found.
We addressed the morphological and biochemical consequences of alteration of flavin cofactor availability in RTD2 patients. RFVT1 and RFVT2 expression was investigated in fibroblasts from a compound heterozygote affected child, in her parents and in age-matched controls. In the patient, RFVT2 protein amount was significantly reduced, while RFVT1 was expressed as in controls, but at a higher level than in parents. We hypothesize that the presence of RFVT1 in children could compensate the malfunctioning of RFVT2 in patients. This correlates with an unaltered level of flavins in cells. Further investigation is needed to explain the observed mitochondrial derangements in patients.

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34) Insights into the molecular consequences of phosphorylation of androgen receptor: implications for the pathogenesis of spinal and bulbar muscular atrophy

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Androgen receptor is a transcription factor implicated in the etiopathogenesis of prostate cancer, androgen insensitivity syndrome and spinal bulbar muscular atrophy. The latter is a rare neurodegenerative disease affecting male adults with a rate of 1 on 40000. The genetic hallmark of the disease is the expansion of CAG triplet in the exon 1 of androgen receptor gene, yielding a protein with extended poly-glutamine tract in the N-terminal region of the protein. So far, the causes of the disease have not been identified, yet transcriptional dysregulation is known to play a role. The aim of the project is to investigate the molecular details leading to disease. In particular, I am analysing the role of phosphorylation of the N-terminal domain of androgen receptor, focusing on the structural and functional consequences of phosphorylation on the biology of the protein, to understand the molecular details for transcription dysregulation. Results indicate minor changes to structural propensity due to phosphorylation at a specific Serine, yet the combined contribution of several phosphorylation sites is able to increase transcriptional activity of androgen receptor.

35) Abstraction of the topology of protein structures enabling automated analyses

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The structure of a protein determines its function. Therefore, the search for similar structures, comparison and classification of structures are important tasks in identifying the function of a protein. The analysis can be conducted on different levels of abstraction. As the Protein Data Bank daily increases in size, automatic methods are required. We represent protein structures as graphs. The graph computation is fully automated. The graphs enable the application of graph-theoretic methods, e.g., the computation of graph measures or the search for similar graphs. The Protein Topology Graph Library (PTGL) [1,2,3] is a database for topologies of protein structures. It comprises graph-based descriptions of the protein structure at secondary structure element (SSE) level and chain level. The database utilizes PDB entries and the corresponding DSSP annotation of SSEs. We define a Protein Graph (PG) to describe the topology at secondary structure level and a Complex Graph (CG) to describe the topology at chain level. In PGs, vertices are either helices, strands or ligands. In CGs, vertices are either protein or RNA chains. Edges denote spatial neighborhoods. In PGs, edges are labeled according to the orientation of the SSEs as parallel, antiparallel or mixed. In CGs, edges are weighted by the number of residue-residue contacts. PTGL is an online database and webserver. PTGL currently holds graphs of 151,837 PDB entries, including 921 large structures. The results are pre-computed and therefore available in real time. The webserver allows searches for pre-defined motifs and arbitrary topologies at SSE level. Concluding, PTGL computes graphs that represent topologies of protein structures at different levels of abstraction. Graphs provide a clear visualization and the application of graph-theoretic methods.

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36) Impact of pathogenic amino acid substitutions on the activity, structure and stability of human phosphoserine phosphatase

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Phosphoserine phosphatase (PSP) catalyzes the Mg^{2+}-dependent hydrolysis of L-phosphoserine to L-serine. This reaction is the last and irreversible step in the "phosphorylated pathway", the main contributor of endogenous L-serine in the brain(1). In this study, we investigated the impact of the D32N, M52T and A35T substitutions on the structure, stability and function of human PSP. The corresponding mutations have been identified in a patient with Williams' Syndrome (D32N and M52T) (2) and in a family in which individuals suffered from intellectual disability and seizures (A35T) (3). While M52 is located in a dynamic loop that folds in the presence of L-phosphoserine, D32 and A35 residues lie in the active site and are not directly involved in catalysis. Circular dichroism spectra in the far-UV of D32N and M52T revealed no major effects on the secondary structure, along with an overall retention of the thermal stability, whereas A35T shows a small, but significant, 3°C decrease in Tm with respect to wt PSP. However, substitutions show a great impact on the kinetic parameters of the reaction. Specifically, the comparison between the wild-type enzyme (*kcat* 44.8 ± 0.9 s^{-1}, *K*M 40.3 ± 2.8 mM) and its variants shows that: i) D32N exhibits a reduction of both *kcat* and *K*M (14.4 ± 0.3 s^{-1} and 14 ± 1 mM, respectively), suggesting that the substitution might favor an unproductive substrate binding; ii) A35T and M52T exhibit similarly strong drops of *kcat* (1.4 ± 0.1 s^{-1} and 1.7 ± 0.1 s^{-1}, respectively) and small but opposite changes of *K*M (76 ± 8 mM and 33 ± 2 mM, respectively). Interestingly, the D32N substitution is the only one that does not affect the overall catalytic efficiency but is expected to limit the flux through the metabolic pathway when L-phosphoserine concentration is above the Km.
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