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Coordinator: V Scarlato

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Giacomo Vezzani (34, Frigimelica / Scarlato):
Use of B-cell platform to characterize antigens of interest in Gram-negative bacteria

Human antibody response to infections or vaccinations is achieved through an enormous number of sequence diversity that often target the same antigens or epitopes although with variable affinity and efficacy. Therefore, sequencing antibodies in the course of an immune response can reveal individual diverse response to the same antigenic challenge and provide crucial information to design optimized vaccine or therapeutics. Single B cell screening strategies have emerged as important technologies for efficiently sampling the natural antibody repertoire of infected or vaccinated subjects. Having access to a method to interrogate different B cell subsets provides an attractive option to produce large and diverse panels of high-quality antibody, with the possibility to find rare B cell clones producing IgG with unique and desirable characteristics.
We have developed in-house a method enabling contextual VH and VL cloning and expression, while keeping their endogenous VH:VL pairing. This approach consists in the cloning, expression and functional evaluation of antibodies derived from single cell sorted B-cells derived from human peripheral blood. We are exploiting our B-cell platform to isolate and characterize human monoclonal antibodies (HumAbs) derived from subjects immunized with Meningococcus B vaccine Bexsero.
Bexsero is a multicomponent vaccine against one of the major etiological agents of sepsis and invasive meningococcal disease: *Neisseria meningitidis* serogroup B (MenB). Detergent-extracted outer membrane vesicles (dOMVs) are one of the components of the vaccine, whose broad protection could be due to several antigens present on dOMV surface. To unravel the relative contribution of the different antigens in eliciting protective antibody responses, we isolated human monoclonal antibodies (HumAbs) from single-cell sorted Plasmablasts (PBs) of adult vaccinees peripheral blood. Recombinant HumAbs have been screened for binding to dOMVs and Bexsero recombinant proteins by Luminex bead-based assay. dOMVs positive mAbs are tested for functionality by serum bactericidal assay (SBA) and characterized in a tailor-made protein-microarray containing a panel of prioritized dOMV-specific proteins, with the aim to identify meningococcal antigens/epitopes that contribute to the protection induced by dOMVs. The bactericidal mAbs identified so far recognize the outer membrane protein PorB binding, confirming the high contribution of this protein in eliciting protective antibody response. Surface exposed PorB loops have been expressed and purified in a scaffold protein to be test with bactericidal mAbs to investigate on PorB immunoreactivity. Additional analyses are ongoing to further elucidate the contribution to Bexsero protection of different MenB antigens.

Bexsero is a trademark owned by or licensed to the GSK group of companies. G Vezzani participated in a post graduate studentship program at GSK

Federico Antoniciello (36, Scarlato / Roncarati):
Inhibition of the essential transcriptional regulator HP1043 as a potential therapeutic approach against *Helicobacter pylori* infection

The Gram-negative bacterium *Helicobacter pylori* is one of the most widespread human pathogens and class 1 carcinogen, believed to have colonized half of the world's population. By infecting the gastric epithelium, *H. pylori* is responsible for the development of chronic gastritis that could result in peptic

ulceration and gastric cancer. The current guidelines recommend the use of a triple therapy, consisting of a proton-pump inhibitor and two antibiotics, or a quadruple therapy, with three antibiotics when persistent infections occur. The rapid development of antibiotic resistance drastically reduces the eradication rates of the standard therapies, imposing the development of new approaches. We focused on the HP1043 protein, a dimeric orphan response regulator, and its mRNA as alternative molecular targets. Due to its crucial role in regulating essential cellular processes, it has been impossible to modulate HP1043 transcriptional levels, hampering a deep understanding of its function and, at the same time, making it a good candidate for novel pharmaceutical approaches. In the present study, 18300 FDA-approved molecules were tested in silico by a molecular docking-based high throughput virtual screening (HTVS) and the highest-scoring ones were further investigated through MM-PBSA molecular dynamics (MD) simulations. The HTVS and the following MD simulations revealed fourteen promising compounds which highlighted binding patterns that could result in a destabilization of the protein-DNA complex or the dimer, therefore blocking HP1043 activity. In parallel, five top-ranked molecules were tested through in vitro protein-DNA binding assays. Furthermore, Peptide Nucleic Acid (PNA)-peptide conjugates were designed to target hp1043 mRNA nearby the ribosome binding site and tested in vivo. By virtue of the promising antisense antimicrobial activity shown recently, the use of PNAs to silence HP1043 translation could allow us to elucidate its regulatory mechanism and exploit it as a therapeutic strategy to stop *H. pylori* infection.

Luigia Cappelli (35, Cozzi / Maione / Scarlato):

Exploring different self-assembling nanoparticles in displaying structurally defined epitopes

To cope the growing need of medical interventions for many infectious diseases, novel vaccines containing isolated and highly purified antigenic protein have been developed. Compared to early vaccines, they are safer but unable to induce high level of protective immunity. To overcome this latter, the design of antigen nanoparticles results to be a promising strategy. (J. López-Sagaseta et al., 2016). In fact, virus-like particles (VLPs) and protein nano-particles (NPs), thanks to their repetitive and highly ordered structure, might present multiple copies of the desired antigens. So, the supra-molecular assemblages can potentially mimic the size and the shape of the natural host-pathogen surface interactions with potential to induce potent B- and T-cell responses (Y Hsia et al. Nature 1–3 (2016) doi:10.1038/nature18010).
The primary aim of this project is to explore the potential of self-assembling NPs as platform for the display of antigen in the vaccine research evaluating their heterologous expression with different systems, purification and characterization in terms of solubility, stability and immunogenicity.
Antigens from different pathogenic bacteria have been genetically fused to several NPs of different size and geometry. All the fusion proteins have been successfully cloned, expressed and purified. The correct assembly of chimeras has been verified with size exclusion chromatography and TEM analysis.
The approach used for rational design of new chimera-NPs and the preliminary results obtained regarding the recombinant production of those NPs and their biochemical characterization will be presented.

Annapaola Petrosino (35, Danielli):

A potent orthogonal phage vector platform for targeted photodynamic therapy of Gram-negative bacterial pathogens

The increase in bacterial antibiotic resistance has encouraged the revival of phage-inspired antimicrobial approaches. Photodynamic therapy (PDT) is considered a very promising research area for the protection against infectious diseases. Yet, very few efforts have been made to combine the advantages of both approaches in a modular, retargetable platform. Here, we propose the M13 bacteriophage as a multifunctional scaffold enabling selective photodynamic targeting of bacterial cells. We took advantage of the well-defined molecular biology of the phage to functionalize its capsid with hundreds of photo-activable chemical sensitizers (Rose Bengal) and contemporarily target this suicide vector to a specific bacterial species via pIII-phage-display of bacteria-binding peptides or nanobodies. By this method, we managed to concentrate the number of PS per binding event, thereby increasing PDT efficacy.

Chimeric phages were engineered to attach specifically Gram (-) bacteria including the human pathogens *Escherichia coli*, *Pseudomonas aeruginosa* and *Acinetobacter baumannii*. Specificity was investigated with flow cytometry and further validated in PDT assays, in which photoactive phages conjugated with LPS binding peptide killed *P. aereuginosa* and *A. baumannii* cells while causing minimal damage to *Staphylococcus aureus*, used as Gram (+) control.
Together, these results anticipated to impact positively on the development of new antimicrobial solutions, tailored to target selectively different pathogens with the same scaffolding platform.

Jacopo Rossi (34, Trost / Zaffagnini):

S-nitrosoglutathione homeostasis in plants: the central role of GSNOR and a new enzyme class on the horizon

Among post-translational modifications (PTMs), S-nitrosylation has been recently established to play a major role in the regulation of various plant metabolic pathways, especially responses to diverse type of biotic and abiotic stresses. During these events, plants face an overproduction of reactive nitrogen species (RNS), among which nitric oxide (NO) is the most common. NO derivatives are highly reactive and can react with reactive thiols of critical Cys residues of proteins leading to the formation of nitrosothiols (i.e. S-nitrosylation). The presence of nitrosylated cysteine can influence both protein activity and structural stability with a consequent effect on plant metabolism. The main S-nitrosylating agent relies on nitrosoglutathione (GSNO), which derives from the spontaneous reaction between NO-derivatives and reduced glutathione (GSH). This molecule act as NO reservoir and the control of its intracellular concentration directly reflects on the amount of protein S-nitrosothiols allowing the cell to modulate protein activities and consequently stress responses. As part of my PhD work, I attempted a biochemical and structural characterization of plant S-nitrosoglutathione reductases (GSNORs), the main enzyme involved in the control of GSNO homeostasis. Furtherly, I combined this study with the analysis of GSNO-degrading activities using protein extracts from Arabidopsis plants expressing or lacking the *GSNOR* gene. Finally, I carried out in collaboration with the laboratory of prof. E. Vierling (University of Massachusetts Amherst), proteomic studies on wild-type and mutant plants allowing the identification of a novel class of enzymes that could be significantly involved in GSNO homeostasis.

Francesca De Chirico (35, Monti):

Understanding the role of microglial extracellular vesicles in neuroinflammation spreading: an in vitro study

Neuroinflammation is a crucial and common mechanism for the initiation and progression of neurodegeneration in the majority of central nervous system (CNS) disorders, including Alzheimer's, Parkinson's, Huntington's disease and Amyotrophic Lateral Sclerosis. Microglia, the immune cells of the brain, are tasked as the first responders of the inflammatory response following the onset of a neuropathology. In fact, in pathological conditions, microglia can acquire two different activated phenotypes: i) in early phases, an anti-inflammatory phenotype (M2) with the release of neurotrophic and neuroprotective factors, while later ii) a pro-inflammatory phenotype (M1) associated with neurotoxic properties, the secretion of pro-inflammatory cytokines and of nitric oxide (NO). The shift of microglial phenotype from the neuroprotective M2 to the neurotoxic M1 could be related to a change in the protein and/or miRNA content in extracellular vesicles involved in intercellular communication. This suggests that the neuroinflammation spreading could be mediated by the release of vesicles in the extracellular environment and, therefore, by the effect that these vesicles have on the non-activated/surveying microglia.
First, to evaluate whether activation could be transmitted among microglial cells indirectly through vesicles, activation has been pharmacologically induced in a microglial murine cell line (N9) by using LPS towards a M1 activation or ATP towards M2. Then, the media conditioned by differentially activated microglia or the vesicles extracted from these media has been given to non-activated cells to evaluate their activation though the analysis of specific commonly used markers in Western blot.
Therefore, we focused our attention on miRNAs, already identified as involved in microglial activation, trying to understand their role in the spreading of neuroinflammation. We identified through qRT-PCR analy-

sis miRNA-155, miRNA-124, miRNA-34a miRNA-125b as the most up-regulated ones upon LPS and ATP stimulation in both activated cells and in their secreted EVs. To evaluate the role of miRNA-34a and to try to block microglial activation spreading through vesicles, DNA nanostructures for delivery of DNAzymes were produced. DNAzymes were designed starting from known cleaving sequences, with a preference for Mg2+-dependent DNAzymes. Tetrahedral DNA nanostructures of different architectures and sizes were designed and characterized as to include the anti-mir34a DNAzyme.
Given that evidence, the role of EVs miRNAs released by microglia deserves to be deeply investigated both as potential therapeutic targets and as biomarkers for neurodegenerative diseases.

Giorgia Babini (36, Monti / Massenzio):

Metabolic alterations of fatty acids synthesis in OPCs and NSCs models of AGC1 deficiency

AGC1 deficiency is an ultra-rare demyelinating disease caused by mutations in the *SLC25A12* gene, encoding for the mitochondrial aspartate-glutamate carrier isoform 1 (AGC1). Main pathological features are secondary hypomyelination, altered myelin formation in central nervous system, together with brain cells proliferation deficit. Likely, the abnormal myelin production is due to a reduced N-acetyl-aspartate (NAA) synthesis, from which acetyl groups are mainly derived. This, in turn, leads to epigenetic alterations in brain precursor cells and consequent transcriptional dysregulation, causing proliferation and differentiation defects, as proved by previous data on our in vitro AGC1 deficiency models (mouse oligodendrocyte precursor cells -OPCs- where SLC25A12 is silenced by a shRNA and neurospheres from mouse model of AGC1 deficiency) from our laboratory.
Together with epigenetic alterations, lower NAA production specifically leads to reduced acetyl-CoA levels, involved in a high number of biological activities, including fatty acids synthesis. These biomolecules in CNS are the main components of myelin sheath, thus an alteration of their production brings to hypomyelination. Also, the RNA-seq analysis on OPCs confirms this metabolic impairment, showing altered expression of transcriptional factors and enzymes involved in the fatty acids synthesis pathway, such as SREBP (Sterol Regulatory Element Binding Protein) and FASN (Fatty Acids Synthase N).
Thus, we would like to verify the in silico data from RNA-seq analysis, to assess the potential of this bioinformatic tool and prove the metabolic alteration also in our in vitro models. In addition, to try to compensate the lack of acetyl-CoA, and the consequent epigenetic and metabolic impairments, experiments with amino acids and vitamins supplementations directly involved in NAA synthesis are performed on OPCs. Specifically, starting from preliminary experiments on cells viability and mitochondrial activity, we will want to move towards morphological and biochemical analysis to identify the main compounds responsible for a potential recovery of differentiation/proliferation defects in our in vitro AGC1 models.

Maria Meloni (36, Zaffagnini / Crozet / Trost):

Uncovering the functional properties of ribulose-5-phosphate epimerase (RPE) from the microalga *Chlamydomonas reinhardtii*

Photosynthetic carbon fixation is realized by eleven enzymes in the so-called Calvin-Benson cycle (CBC), a metabolic pathway highly conserved in oxygenic phototrophs. Essential epimerization of xylulose-5-phosphate into ribulose-5-phosphate is catalyzed by ribulose-5-phosphate-3-epimerases (RPEs) during the regeneration phase of the cycle.
Here, we set a structural and functional study of RPE from the model single-cell alga *Chlamydomonas reinhardtii*, with a particular interest on its regulation. In particular, after the setting of the coupled assay, the kinetic parameters were estimated.
Finally, a regulation mediated by redox agents does not seem to be predominant. Intriguingly, a regulation based on the modulation of the quaternary structure could be physiologically relevant, where the hexameric form seems to be the active state and the dimeric form the inactive state.
This work aims at a thorough understanding of CBC regulation that lays the basis for future improvements in carbon fixation

Luigi D'Angelo (36, Porcelli / Gasparre):

Mitochondrial metabolism in ovarian cancer

Epithelial ovarian cancer (EOC) standard treatment includes debulking surgery followed by chemotherapy, but the outcomes are poor since the disease is often diagnosed at late stages and chemoresistance occurs frequently [1]. Therefore, the understanding of the molecular mechanism driving chemoresistance is vital to develop novel therapeutic strategies. Metabolic reprogramming is a hallmark of cancer with the mutual regulation of glycolysis and oxidative phosphorylation (OXPHOS) being crucial in the process [2]. EOC was recently classified into two bioenergetic subgroups with a preference for either aerobic glycolysis or OXPHOS metabolism, low- and high-OXPHOS respectively, on the base of the expression levels of some OXPHOS complexes subunits [3]. The former subgroup is characterised by higher stabilization of Hypoxia Inducible Factor 1-alpha (HIF1α), the transcription factor orchestrating the adaptation to low oxygen conditions, whereas the latter subgroup is associated with higher expression of Peroxisome proliferator-activated receptor Gamma Coactivator 1-alpha (PGC1α), the master regulator of mitochondrial biogenesis. The focus of this project is to understand how EOC metabolic rewiring drives chemoresistance, thus we aim to dissect the metabolic status of EOC tumour masses and ascites in a cohort of chemo-sensitive and -resistant patients. However, before exploring the metabolism in tumour biopsies from patients, we sought to investigate the bioenergetic signature in five ovarian cell lines (OVSAHO, SKOV3, CAOV3, OC314, and OV90) previously reported to belong to different metabolic subgroups [3]. Firstly, we measured oxygen consumption rate (OCR) and protein levels of five subunits representing each OXPHOS complexes to link protein expression to bioenergetic function. Our analysis reveals that respiration correlates only with complex III (CIII) subunit UQCRC2 and complex IV (CIV) subunit MT-CO2 levels rather than correlating with the whole OXPHOS protein expression, likely because these complexes are bottleneck enzymes in the respiratory chain. Considering this, just UQCRC2 and MT-CO2 levels can be sufficient to define a high- or low-OXPHOS status. Secondly, we show that HIF1α is generally less stabilized in those cell lines with higher OXPHOS protein expression. Overall, our preliminary results in ovarian cell lines depict a heterogenous metabolic scenario with SKOV3 clearly showing a low-OXPHOS phenotype when compared to the other cell lines. We also assessed OXPHOS protein and HIF1α levels both in malignant ascites and tissue biopsies from up-front patients. Interestingly, this preliminary experiment suggests a dependence of the metabolic status on tumour stage and anatomic location, besides it confirms the inverse correlation between OXPHOS protein abundance and HIF1α stabilization. Indeed, high levels of MT-CO2 is mirrored by HIF1α absence in ascites, whereas high levels of HIF1α is accompanied by low levels of MT-CO2 in metastatic tissue from omentum. Further analyses on patient pre- and post-chemotherapy matched samples, as well as the assessment of HIF1α downstream targets and the analysis of PGC1α expression, are needed to get a deeper comprehension of EOC metabolic heterogeneity and chemoresistance to develop a new therapeutic opportunity.

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Renée Concetta Duardo (34, Capranico):

Replication stress and under-replication as main mechanisms of Topoisomerase I poison-induced micronuclei

The success of classical antitumor chemotherapy has recently been shown to be due to the stimulation of innate and adaptive immunity by anticancer drugs. However, how this occurs has not been fully clarified yet.

Topoisomerase I (Top1) poisons are among the clinically-effective drugs used in standard chemotherapeutic regimen for colon, ovary and lung cancers. Top1 is a key enzyme that resolves topological stress accumulated during transcription, replication and chromatin remodelling by allowing strand rotation through a transient single-strand break introduced by the enzyme into the DNA duplex substrate.

Anticancer Top1 poisons trap DNA-enzyme intermediates that can lead to irreversible DNA double-strand breaks (DSB), cell death and genome instability.
In agreement with previous data from our and other laboratories, I now show that structurally different Top1 poisons, Camptothecin (CPT) and LMP776, induce the formation of micronuclei in human cancer cells. Micronuclei can be a source of cytoplasmic DNAs, which can activate STING-dependent pathways leading to an innate immune genes activation and production of type I interferons and other cytokines in cancer cells. As mechanisms of micronuclei formation have not been fully established, the main aim of my PhD project is to establish the mechanism of micronuclei induction by Top1 poisons in human cancer cells.
Using immunofluorescence assays and EdU labelling of nascent DNAs, my results show that, after 24 hours of recovery, short treatment with sub-cytotoxic doses of Top1 poisons induces the formation of micronuclei that do not contain nascent (EdU-labelled) DNA in human cancer HeLa and U2OS cells. Under similar experimental conditions, the drugs were able to produce significant levels of γH2AX, a marker of DSBs in replicating cells but much lower levels in non-replicating cells. Further results demonstrate that EdU-negative micronuclei formation is not dependent on transcription. As we have also data showing that micronuclei formation is dependent on R-loops, I used DRIP-seq techniques to map genome-wide CPT-induced R-loop distributions in HCT116 cells. Bioinformatic analyses show that R loop levels are both decreased and increased by CPT depending on the specific genomic regions. In particular, increased R loops are found mainly at active genes and always overlapped with Top1cc sites as defined by Baranello et al (2016) in the same cell line. Thus, our data are consistent with Top1ccs causing an increase of both R-loop levels and then irreversible DNA cleavage at selected genomic regions. The data altogether are consistent with the formation of micronuclei due to R-loop increase and under-replication at specific regions caused by Top1 poisons.
In addition, I have data showing that CPT/LMP776 treatments lead to an asymmetric distribution of newly synthesized DNA and micronuclei at mitosis, which appears to depend on ATR activity. Next, I will thus determine whether micronuclei formation is related to a non-random chromatid segregation at mitosis, which would mean that they are formed through an active process to preserve genome integrity at least in one daughter cell.
My results will eventually help in developing new strategies for effective personalized interventions using Top1-targeted compounds as immuno-modulators in cancer patients.

Andrea Arleo (34, Capranico / Marinello):

Topoisomerase 1 poisons as modulators of innate immune gene activation in human cancer cells

DNA topoisomerase I (Top1) regulates DNA supercoiling by catalyzing a controlled rotation of a cut strand around the intact one of a DNA duplex. During the catalytic activity, Top1 forms a complex intermediate known as DNA cleavage complex, which is recognized and bound by Top1 poisons, preventing Top1 enzyme from re-ligating DNA. In our previous studies, we observed that Top1 poisons trigger the formation of unscheduled R-loops, which eventually lead to irreversible DNA double-strand breaks (DSB), cell apoptosis and genome instability. This project aims to investigate the role played by non-cytotoxic doses of Top1 poisons, such as Campthotecin (CPT) and LMP776, in inducing the formation of micronuclei, DNA damage, and immune gene response.
In the first year of my PhD, I observed that Top1 poisons induce micronuclei formation in several cancer cell lines, including HeLa, U2OS and three different small cell lung cancer cell lines, with small differences among them. Next, I selected a U2OS cell line bearing an inducible RNAseH1 gene to investigate the role of R loop in Top1 poison effects, showing that the overexpression of RNAseH1 reduced the number of micronuclei as compared with non-induced cells. The data thus support a main role of R loop in micronuclei formation by Top1 poisons. Moreover, I demonstrated that in the analyzed lung cancer cell lines an innate immune response correlates to the basal expression level of cGAS/STING pathway proteins and varies across cell lines.
During the second year of my PhD course, I focused the attention on defining the mechanism that links Top1 poisons with the activation of innate immune genes and, in order to do that, I selected a model (HeLa cells) in which the cGAS/STING pathway is fully active. Thus, I performed immunofluorescence microscopy

analyses of the activation of cGAS and STING upon treatment with poisons. The number of cGAS positive micronuclei observed in Top1 poison-treated cells resulted 2-3 times higher than untreated cells. To further validate these findings, I conducted an ELISA test to measure cGAMP levels in time course experiments with CPT and LMP776, since cGAMP is known to be involved as a second messenger in the cGAS-STING pathway. cGAMP levels exhibited a significant increase in treated cells as compared to untreated cells. Next, I tested whether Top1 poisons are able to trigger immune gene expression as a result of cGAS/STING pathway activation by measuring mRNA levels of 5 different cytokines in human HeLa and murine B16 cells. For the latter cell model, I studied both the wt line and a CRISPR STING-KO cell line. The results show that Top1 poisons can stimulate the transcription of the studied cytokines in both HeLa and B16 WT cells, but not in STING-KO B16 cells, supporting that cytokine gene activation by Top1 poisons is mainly dependent on the STING pathway. Taken together, the findings show that non-cytotoxic doses of Top1 poisons activate the cGAS/STING pathway mediated by micronuclei formation.

Martina Gatto (36, Gardini / Perini):

PP2A Methylation status during PP2A-Integrator interaction

Phosphoprotein Phosphatase 2A (PP2A) is one of the most abundant phosphatases that plays many roles in cells both in nucleus and cytoplasm. It is composed of three subunits: A subunit, also known as Scaffolding subunit, B regulatory subunit which determines the role of PP2A, and the C catalytic subunit. PP2A activity is influenced by methylation status occuring on C subunit (Leucin-methyltransferase 1 LCMT1 is responsible for PP2A methylation, whereas Protein Methylesterase1 PME1 for demethylation).

The phosphatase PP2A along with the kinases CDK9, is responsible of the RNA Polymerase II (PolII) transition from pause-release to the active elongation. Recently, it has been shown that PP2A is recruited to the chromatin during transcription by the interaction with Integrator (S. Vervoot, Sarah A. Welsh et al., 2021), a 14-subunit complex involved in many nuclear activities. Immunoprecipitation experiments demonstrated that two of the fourteen Integrator subunits are responsible of the binding of PP2A: INTS6 (subunit 6) and INTS8 (subunit 8). It is thought that PP2A, recruited to the chromatin via INTS6, dephosphorylates RNA PolII CTD and allows its pausing before the transition to productive transcriptional elongation. Although today we know how PP2A and Integrator interact each other (Zheng et al., Science 2020), we do not know yet when this interaction occurs; hence, the main question to be addressed is: is the methylation status of PP2CA influencing its interaction with Integrator?

My experimental work includes:

- Genesis of recombinant proteins from bacteria and insect cells
- In vitro methylation assays using recombinant proteins
- Mass spec data from LCMT1 KO, LCMT1 KD and PME1 KD cells

Simona Lombardi (36, Zhang / Perini):

Targeting cancer stemness in High Grade Serous Ovarian Cancer

High Grade Serous Ovarian Cancer (HGSOC) is one of the most frequent histological subtypes of ovarian cancer with poor outcome [1]. The HGSOC initially responds well to the platinum-based chemotherapy (e.g., cisplatin) but due to cancer cells heterogeneity, a subpopulation of cells is chemoresistance causing relapse [2]. These cells are named cancer stem cells or stemness cancer cells and contribute to chemoresistance [3]. To achieve complete cancer regression, targeting cancer stemness is a new approach that has been developing to therapeutically suppress cancer relapse [4].

ARID4B is a member of the *ARID* (AT-rich interacting domain) gene family and encodes the Arid4b epigenetic modulator. It has been identified as a member of the mSin3a chromatin repressive complex, but recently its function as co-activator has been suggested [5-6]. Very little is known about its role in cancer, and it has not yet been studied in ovarian cancer. Its involvement in cancer stemness is not yet explored. Based on three different public TGCA datasets, ARID4B is overexpressed in around 20% of high grade serous ovarian cancer samples [7-9]. Preliminary results of this project supported the hypothesis that ARID4B may be involved in HGSOC cancer stemness. Therefore, this phenotype has been investigated.

ALDH is a common stemness marker in ovarian cancer [10] and ARID4B KO cells, from two different HGSOC cell lines, show decreased ALDH activity supported by a decrease in ALDH mRNA expression compared to wt. Moreover, ARID4B depletion sensitizes cells to cisplatin, reducing relapse and cell proliferation. That is especially relevant in clinic application since cancer stem cells are resistant to conventional chemotherapy.

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Davide Maestri (36, Tempera / Perini):

The role of YY1 in the 3D organization of the EBV genome in EBV-positive tumors

Epstein-Barr Virus (EBV) is a ubiquitous gamma-herpesvirus highly prevalent in the world's population (approximately 95%). EBV is the most common etiological agent of infectious mononucleosis (1) and is the first oncogenic virus that has been identified. It is estimated that 1-2% of human cancers are attributable to EBV transformation, including nasopharyngeal carcinomas (NPC) and EBV positive gastric cancers (GC) (2). Immunosuppressed and EBV-positive individuals have a higher incidence of lymphomas including Burkitt's Lymphoma (BL), Hodgkin's lymphoma and diffuse large B cell lymphoma (3). In most healthy individuals, however, the viral genome undergoes a process of circularization, forming an episome assuming a specific three-dimensional (3D) structure and establishing a chronic latent infection in which it replicates together with the genome of the infected B cells. The virus is able to hijack host processes through the production of specific viral proteins in order to maintain the latency program. The presence of numerous copies of EBV genomes and latency proteins, such as EBNA1, can also affect the host genome in its 3D structure. Understanding the mechanism and the players by which the host-virus interplay is able to modify both genomes 3D structures is of pivotal importance in order to identify novel and specific therapeutic strategies to counteract the growth and metastasis of EBV-positive tumors. The host proteins involved in this reorganization process include the transcription factor CTCF, implicated in long-distance DNA looping interactions along with Cohesin, and Poly (ADP) Polimerase 1 (PARP1). These two proteins cooperate in determining the 3D structure of the EBV genome (4) and consequently in regulating the different virus latency type. Recent experimental evidence obtained by the Dr. Tempera group have shown how differences in the 3D structure of the EBV genome are attributable to the activity of PARP1 which is able to stabilize the binding of CTCF to the virus genome and to CTCF itself. However, the binding of the latter does not change between the different latency types (Type I and III) as demonstrated by Chromatin Immunoprecipitation followed by Next Generation sequencing (ChIP-seq) experiments performed on isogenic cells both for human genome and for EBV strain (Mutu I/III). Therefore, since the different 3D structure of the EBV genome is not entirely attributable to the binding of CTCF, it is interesting to identify other host TFs that can alone or in cooperation with other proteins determine these differences between different types of latency. Results of bioinformatic analysis conducted on ChIP-seq data from the ENCODE project on EBV-positive lymphoblastoid cell lines (LCLs) revealed how other transcription factors bind to the EBV genome including Ying Yang 1 (YY1) (5). YY1 is a transcription factor that belongs to the Gli-Kruppel TFs family and derives its name from its dual activity as activator and repressor of transcription depending on its interactors including PRC2 and INO80. Recent studies have identified Coesin and Condensin among the numerous interactors of YY1, which are proteins involved in regulating the 3D structure of the genome (6). ChIP experiments

conducted on both LCLs and EBV-positive epithelial cancer cell lines allowed to identify how YY1 is differently bound to the EBV genome at the level of some EBV promoters between the two distinct latency types. YY1 knock-down experiments on LCL as well as EBV-positive Gastric Cancer cell lines followed by ChIP experiments revealed differential binding of both CTCF and Cohesin on the same promoters where YY1 binds between the different cell lines. It is tempting to speculate whether these proteins cooperate with each other in order to determine the 3D structure of EBV and the mechanism by which this phenomenon occurs.

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Eugenia Lorenzini (34, Ciarrocchi / Ambrosetti):

TRIM28 keeps the time of mitosis sustaining proliferation and progression of Malignant Pleural Mesothelioma (MPM)

Malignant Pleural Mesothelioma (MPM) is a rare but aggressive cancer arising from pleural mesothelial cells. It is characterized by high mortality and dismal prognosis (1). Molecular bases of this disease remain unknown, representing a relevant limitation to the development of effective targeting strategies. We performed a CRISPR-Cas9 genome-wide screening in the MPM cell line MSTO-211H. We identified 233 genes essential for MPM progression, most of which are involved in epigenetic regulation and chromatin organization. Among these, TRIM28 showed a dramatic effect on MPM cells' survival and a striking correlation with patients' survival probability. Functionally, TRIM28 KD resulted in a delay of cell cycle re-entering upon MPM cells' synchronization, together with an increase of apoptotic cells. RNA-sequencing performed on TRIM28 KO cells identified many genes involved in cell cycle regulation, and particularly in mitosis, as strongly downregulated. Coherently, TRIM28 KD cells showed several defects in mitotic spindle organization and chromosome segregation. The expression of cell cycle genes is highly coordinated to ensure proper cell division. G2/M genes share a common sequence in their promoter, called CHR element, and are transcribed by the MYBL2/FOXM1-MuvB complex (2). We found that, TRIM28 binds the promoter of many G2/M genes and form a transcriptional complex with RNA Polymerase II (RNAPII), CDK9, FOXM1 and RBBP4, subunit of the MuvB complex. Silencing of TRIM28 caused displacement of RNAPII from the promoter of G2/M genes and reduction of Ser2-phophorylated RNAPII along the gene body of AURKB, suggesting elongation impairment. Confirming these observations, silencing of FOXM1 or MYBL2 as well as pharmacological inhibition of CDK9 recapitulated TRIM28 KD phenotype. Finally, gene expression analysis by Nanostring Technology in a retrospective cohort of 86 MPMs confirmed in vivo the association of TRIM28 with G2/M genes and the correlation of this gene signature with adverse clinical outcome. Overall, through an integrated approach we discovered TRIM28 as a new dependency for MPM, showing that its centrality in this setting is linked to a previously unknown function of this factor in setting the timing of G2/M gene expression.

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Piergiuseppe De Rosa (35, Perini):

Characterization of Eight-Twenty-One (ETO) protein complex in neuroblastoma

Neuroblastoma (NB) is one of the most frequent solid tumor in childhood and infancy, deriving from neural crest. NBs carrying MYCN gene amplification are predicted to have poor prognosis and a bad response to therapy. Therefore, understanding the mechanisms driving its oncogenesis may help develop new therapeutic protocols. The most widely used animal model for NB research is the Th-MYCN transgenic mouse, expressing high levels of MYCN in immature neuroblasts inducing tumor formation after few weeks from the birth. Preliminary data obtained by random mutagenesis of Th-MYCN mice revealed that a single missense mutation in the sequence encoding the NHR4 domain of ETO gene strongly reduces the severity of tumor phenotype, thus, suggesting a crucial role of this gene in MYCN-driven NB insurgence.

To understand the molecular mechanisms in which ETO is involved in NB, we analyzed its interactome in the MYCN-amplified NB cell line SK-N-BE(2)C. We found some of the well-known ETO interactors as well as four new partners: the histone demethylase LSD1 and the corepressor RCOR3, which are part of the Co-REST repressor complex, and the transcription factors HAND2 and TWIST1, involved in the neural crest differentiation. ChIP-seq data revealed a significant overlap between ETO peaks and LSD1, RCOR3 and HAND2 peaks, suggesting that these factors could be part of a unique protein complex involved in the regulation of gene expression.

To shed light on the role of the ETO missense mutation, we tested the interaction of ETOmut with some of the protein partners emerged from the proteomic analysis. Notably, we found that in the context of the tested proteins, RCOR3 is the only factor that totally loses the interaction with the mutant. Moreover, ETO silencing abolishes RCOR3 association with chromatin in NB cell line Kelly, demonstrating that ETO is required for recruitment of RCOR3 to ETO binding sites.

To further characterize the interaction between ETO and the proteins of this putative chromatin remodeling complex, we dissected ETO by deleting its highly conserved Nervy Homology Region (NHR) domains. Next, we tested the interaction between the deleted forms of ETO with LSD1, RCOR3, HAND2, HDAC1 and HDAC2. As expected, NHR4 domain was necessary for the interaction with RCOR3 and, interestingly, the homodimerization domain NHR2 was required for the interaction with both RCOR3 and LSD1. Furthermore, we identified a region of RCOR3 required for the interaction with ETO.

In conclusion, our work demonstrated that ETO-RCOR3 interaction is required for MYCN-driven NB, and that these two proteins play role in a chromatin remodeling complex involved in neural crest gene expression regulation. Moreover, we also characterized the essential domains of ETO involved in this complex, providing information for further structural studies.

Marta Viggiano (34, Maestrini):

Contribution of compound heterozygous CACNA1H mutations in Autism Spectrum Disorder susceptibility

Autism Spectrum Disorder (ASD) is a complex neuropsychiatric disorder with a strong genetic component. So far, more than one hundred high-confidence susceptibility genes have been identified and recent efforts have led to an ever-growing list of ASD candidate genes. Among these, low-voltage activated T-type calcium channels (Cav3) genes (*CACNA1G*, *CACNA1H*, *CACNA1I*) have been consistently implicated, which nicely correlates with the role of calcium signaling in neuronal function.

We performed whole genome sequencing analysis in a cohort of 105 families, consisting of 125 ASD individuals, 210 parents and 57 unaffected siblings, to explore the presence of rare damaging variants in Cav3 genes.

We have identified inherited damaging variants in Cav3 genes in 19 ASD families. Interestingly, compound heterozygous rare damaging missense variants were detected in the *CACNA1H* gene in 4 ASD subjects (2 MZ twins and 2 independent cases), belonging to 3 different families. The identified biallelic damaging variants could affect the CACNA1H protein activity with a recessive model and contribute to the disease development in the context of a high-risk genetic background. Thus, we are performing functional analysis, using whole-cell patch clamp technique, to clarify the role of the *CACNA1H* variants on the calcium channel activity.

The identification of biallelic mutations in different ASD families provides further support for a role of *CACNA1H* in ASD susceptibility, and for the first time highlights that this ASD candidate gene may act through a recessive mode of inheritance.

Nicola Balboni (36, Giorgi / Monti):

A transcriptome-wide analysis of the rare genetic disease AGC1 deficiency

AGC1 deficiency is a rare genetic disease caused by the impaired activity of the mitochondrial aspartate-glutamate carrier isoform 1, also known as SLC25A12. Patients affected by the disease show arrested psychomotor development, seizures, cerebral atrophy and global hypomyelination. The cause for these symptoms can be explained by the reduced levels of N-acetyl-aspartate, a key metabolite for myelin formation.
This study focuses on the bioinformatic analysis of a bulk RNAseq dataset obtained by silencing Agc1 in mice oligodendrocyte precursor cells (OPCs).
By analysing the data, we found a significant downregulation in genes involved in the fatty-acid synthesis pathway, a key pathway for membrane building, including myelin. Also, the downregulation of a large region of mouse chromosome 11 and data on Agc1 localization that prove it can move inside the nucleus, suggest a sort of epigenetic involvement of Agc1, which will be further investigated by using ATAC-seq. We also tested a possible method to integrate transcriptomics data with metabolomics data using an algorithm for gene network analysis to infer correlations between metabolites levels and transcripts levels, in order to subsequently use them as a predictor of metabolite levels. Splicing analysis was also carried out, showing a significant alternative exon usage in a neuron-specific RNA-binding protein.
All these results suggest how Agc1 can impact the nervous system development not only from a metabolic point of view, but in other different ways which need further investigation.

Friday, September 10, 2021

Serena Jasmine Aleo (34, Ghelli):

Dissecting the role of NQO1 in the efficacy of idebenone as a treatment for Leber's hereditary optic neuropathy

Leber's Hereditary Optic Neuropathy (LHON) is a disease caused by point mutations on genes mainly encoding subunits of NADH dehydrogenase (Complex I; CI) of the respiratory chain [1]. In most patients, vision loss is devastating in terms of the impact on their quality of life, and often remains permanent [2]. Although LHON is a well characterized optic neuropathy, no effective treatment is available to date, and currently, idebenone, a ubiquinone analogous, is the only approved drug, by the European Medicine Agency, as treatment for LHON [3]. However, the real effect of idebenone is still controversial, because only a limited subset of patients may recover to some extent visual acuity, depending on the age of onset and the mutation type [4, 5]. It has been demonstrated that the potent antioxidant effect of idebenone and its ability to maintain high levels of ATP in cells, in which CI was pharmacologically inhibited, depend on its reduced form that is able to transport electrons directly to complex III (CIII), partially restoring the functionality of the respiratory chain bypassing the compromised CI [6]. In cells, the enzyme identified as the key player in idebenone reduction is NAD(P)H-quinone dehydrogenase (NQO1), a well-known inducible cytosolic enzyme involved in the antioxidant response through the reduction of quinones and their derivates that in turn act as antioxidants [7]. However, the demonstration of the role of NQO1 in promoting the therapeutical effect of idebenone in cells with genetic defect of complex I is still lacking.
The aim of this study was to investigate the role of NQO1 expression and activity on the efficacy of idebenone treatment, analyzing cellular respiration and ATP synthesis as well as ROS production in cells carrying LHON mutations. We observed that, in a cellular model overexpressing NQO1, idebenone was able to maintain mitochondrial respiration after treatment with a CI inhibitor (rotenone) and ameliorated respiration in cells with defective CI due to one of the LHON mutations. Furthermore, we showed that in the same cells, mitochondrial ATP synthesis could also occur adding external NADH/NADPH in presence of idebenone. Tacking together, these results demonstrate that NQO1 can support oxidative phosphorylation bypassing CI and directly transferring electrons to the CIII via the NQO1-idebenone-CIII pathway. In

addition, we also assessed the possibility that the reduction of idebenone by NQO1 could enhance its antioxidant activity. We observed that in the same cellular model, overexpressing or not NQO1, ROS production induced by the LHON mutation was reduced in cells treated with idebenone and overexpressing NQO1, indicating that reduced idebenone also acts as antioxidant.
To translate our analysis in a cellular model closer to physiological conditions, the next step was to study the effect of idebenone related to NQO1 expression directly in fibroblasts derived from healthy controls and LHON patients. Therefore, we evaluated the expression levels of NQO1 and measured mitochondrial respiration in presence or absence of idebenone, demonstrating that only cells expressing NQO1 were able to maintain respiration in presence of rotenone or increased oxygen consumption when LHON mutations were present. Finally, we treated fibroblasts that poorly expressed NQO1 with dimethyl-fumarate (an inducer of NQO1 expression) and we observed that mitochondrial respiration in presence of idebenone was partially rescued. In conclusion, these results strongly indicate that NQO1 expression levels correlates with the efficacy of idebenone, providing the possibility to design strategies to enhance NQO1 activity to maximize idebenone therapeutic potential in LHON.

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Ottavia Tartagni (34, Zuccheri):

Microstructured device fabrication for 3D cell cultures: drug assays and advanced microscopy techniques

In vitro models are constantly being improved to better imitate tissue structure and function, as well as reflect organism response to treatments. Three-dimensional (3D) cell culture systems, as multicellular tumor spheroids (MTS), provide numerous advantages over traditional two-dimensional (2D) cell culture. MTS better simulate the complex in vivo milieu and produce cellular behavior which is more similar to natural settings. Tumor spheroids are still the extensively studied 3D model and various techniques have been developed for their production. However, spheroid sizes are prone to heterogeneity, rendering them unsuitable for high-throughput drug testing.
We focused our research on manufacturing defined size microwell arrays using a nonadhesive silicon elastomer technology to overcome this difficulty. The prototype device developed contains more than 400 microwells in order to provide a robust and effective platform for in vitro drug testing. After assessing seeding technique and device cell culture application, we demonstrated the use of this platform to generate size-controlled microtumors from a variety of human cancer cell lines originating from ovarian, breast, colon, renal, thyroid and mouse brain cell lines.
Specifically, to test our device prototype for anticancer drug screening application, we investigated the drug response of MTS cultivated in our device using a human colorectal carcinoma cell line (HCT116). We already treated spheroids with various drug concentrations of Staurosporine and Paclitaxel – now, we tested Doxorubicin and Vorinostat (HDAC inhibitor shown to have anticancer activity in solid tumors). Growth and morphological aspects before and after drug exposure have been monitored. Then, as an analytical end-point for drug testing, we modified the standard MTT assay to assess cell viability. Devices were also included with low-melting-point agarose for in-situ chemotherapeutic effects analysis using fluorescein diacetate and propidium iodide staining. With this method we were able to observe morphological effects and the distri-

bution of live and dead cells in microtumor structures without any recovery. Fluorescence image analysis with Matlab allowed us to analyze the fluorescence intensity of a large number of spheroids at the same time, thus providing a fast-automated analysis.
Finally, one of the biggest drawbacks of 3D cellular model analysis is the inability to observe sample deeper areas with standard research microscopes; in the majority of applications, cellular resolution can only be achieved in areas not more than 50-100um away from the surface. Thus, with the collaboration of Prof.Hofkens from KU Leuven University, we used light-sheet and 2-photon microscopy on spheroids grown in our device and compared with the confocal microscope analysis application. We were able to deepen the imaging advanced techniques that provide a better characterization of the generated MTS. To conclude, I contributed to a review paper on technological approaches over microglia activation mechanisms and I am currently working on a manuscript on my PhD project.

Suleman Khan Zadran (36, Perini / Danielli):

Engineering of an orthogonal phage-vector platform for photosonodynamic of Neuroblastoma

Gangliosides are modified sphingolipids that are extensively expressed in cancer cells and relatively low expressed in healthy tissues. Disialoganglioside GD2 is a tumor-associated antigen expressed on the surface of neuroblastoma (NB) cells that is well-suited target for immunotherapy. Previously, GD2 was targeted by dinutuximab, a chimeric monoclonal antibody (mouse-human) based on the structure of the murine monoclonal antibody 14G2a FDA approved for NB immunotherapy. With recent advances, a modified chimeric antigen receptor (CAR) T cells generated with 14G2a-based Single Chain Variable fragment scFv (VL-VH) against GD2 to induced cytotoxicity and improve stability and affinity. However, there are still substantial hurdles to efficacy, lack of T cell retention and potency, difficulties identifying targets, and an immunosuppressive tumor microenvironment are among them. Keeping all these challenges in view, we hypothesized a novel therapeutic approach to target GD2 by manipulating M13 filamentous phage particles against NB. Single Chain Variable Fragments of 14G2a and 5F11 monoclonal antibody were cloned in the pComb3XSS phagemid as a fusion protein with the minor capsid protein pIII, allowing scFv expression and display on the tip of the vector phages using a helper phage construct. Furthermore, we tested 6 neuroblastoma cell lines (IMR-32, CHP-134, Kelly, SH-SY5Y, LAN-5 and SK-N-SH) for GD2 expression, all cell lines were positive for GD2 with different level of expression except SK-N-SH tested negative for GD2 expression. Next, the VIII protein of the phagemid will be conjugated with photosensitizers and sonosensitizers that can be activated with light and ultrasound to induce the production of reactive oxygen species. The selectivity and cytotoxicity of these vectors will then be tested on the cell lines expressing different levels of GD2. In conclusion, based on our hypothesis this novel potential biotechnological therapeutic tool could be more effective, potent, non-invasive, and selective compared to the previous reported strategies.

Key Words: Neuroblastoma. GD2. Phage display. Cytotoxicity

Ylenia Beniamino (34, Zambelli):

Insights into the structure and dynamics of the NDRG1 intrinsically disordered region, combining NMR spectroscopy with in vivo and in vitro SDSL- EPR approaches

N-myc downstream regulated gene 1 is a human protein of 43 kDa, working as regulator of tumor progression. Recently proposed as new target in lung cancer, its up-regulation is associated to higher cancer aggressiveness and worse prognosis in this tumor form [1][2]. Although the mechanism of action of NDRG1 is not clear, an important role in the regulation of its biological functions, is assigned to the intrinsically disordered C-terminus of the protein itself. This region is 83 residues long [3], shows nickel binding activity and undergoes functional serine and threonine phosphorylation by different kinases [4].
In this work, the structure and the dynamics of the NDRG1 intrinsically disordered region were investigated combining NMR spectroscopy and site-directed spin labeling (SDSL) approach coupled to electron paramagnetic resonance (EPR), an efficient tool to elucidate the protein structure and dynamics [5]. This technique, introduced in my research project during my period as visiting PhD student at the laboratory of

"Bioénergétique et Ingénierie des Protéines" of the CNRS of Marseille, under the supervision of Dr. Elisabetta Mileo, contemplates the covalent incorporation of a nitroxide label, stable and sensible to the local environment, onto a specific site of the protein, generally a cysteine residue [6]. The targeted cysteines for the labeling reactions were introduced by mutagenesis and expressed and purified from E.coli following the protocol previously developed and optimized by myself for the native protein. The protein variants were labeled with the nitroxide label maleimido-proxyl (M-Proxyl) and used for in vitro EPR experiments in the presence of several osmolytes and crowding agents. The protein structural dynamics was also investigated by in cell-EPR spectroscopy, delivering the protein inside E. coli cells by electroporation and following the protein internalization and localization by confocal fluorescence microscopy. The NMR spectroscopy confirmed the disordered structure of this region and the assignment of the peaks of the HSQC spectrum was completed.

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Luca Ulfo (36, Danielli / Calvaresi):

Molecular engineering of the M13 phage into a nanobiotechnological platform for photodynamic, sonodynamic and photothermal cancer therapy

Photodynamic therapy (PDT) represents a promising modality for cancer treatment. We are developing and optimizing a PDT platform using engineered M13 bacteriophages as vector for photodynamic killing of cancer cells by using an orthogonal approach (genetic/chemical). Phages were refactored to display several targeting moieties (peptides or Nanobodies) that bind specifically various exposed tumor markers. Primarily we optimized the engineered phage production to have a homogeneous population of phages (in size and expression of the retargeting factor). Engineered phages were then chemically conjugated with hundreds of photosensitizers molecules (as Rose Bengal). The retargeting rate and internalization was assessed in vitro on cell lines and three-dimensional structures (spheroids); then the killing efficiency was assessed after a 30' irradiation with a low intensity white light or after few seconds of laser irradiation, on both 2D and 3D cells structures. Promisingly the killing activity for cancer cell lines and the disaggregation of spheroids is observed at picomolar concentration of engineered phage vectors.

Benedetta Manca (34, Giuliani / Scarlato):

Functional characterization of the gonococcal polyphosphate pseudo-capsule

Neisseria gonorrhoeae is an exclusively human pathogen able to evade the host immune system through multiple mechanisms. Gonococci accumulate a large portion of phosphate moieties as polyphosphate (polyP) on the exterior of their cells, however its function has not been yet clarified. Although its polyanionic nature has suggested that it may form a protective shield on the cell surface its role has remained controversial.

Taking advantage of a recombinant His-tagged polyP-binding protein, the presence of a polyP pseudo-capsule in gonococcus was demonstrated. Interestingly, the polyP shield was found to be strain specific. To investigate its functional role in host immune evasion, such as resistance to serum bactericidal activity, phagocytosis and antimicrobial peptides, enzymes involved in polyP metabolism were genetically deleted, generating mutants with altered polyP external production. To this regard, mutants not expressing polyP on their surface, became sensitive to complement mediated killing in presence of normal human serum. Conversely, naturally serum sensitive strains didn't display polyP pseudo-capsule and could reverse their phenotype in the presence of exogenous polyP. Resistance to phagocytic killing was assessed by using differentiated HL60, a validated cell model for gonococcus-neutrophils interaction. Results exhibited a significant

increase in viability of strains shielded with polyP in comparison to those lacking it on their surface. Interestingly, the addition of exogenous polyP restored bacterial survival. Finally, the presence of polyP pseudo-capsule has been revealed critical also in the protection from antibacterial activity of cationic antimicrobial peptide, like the cathelicidin LL-37. Indeed, results evidenced that the minimum bactericidal concentration was significantly lower in strains lacking polyP than in those harboring the pseudo-capsule, a condition that overturned after the complementation with exogenous polyP.
Taken together, data presented suggested an essential role of polyP pseudo-capsule in the gonococcal pathogenesis, paving the way to a new perspective on gonococcal biology and new treatments.

This work was sponsored by GlaxoSmithKline Biologicals SA. BM is a student of University of Bologna and participates in a post graduate studentship program at GSK.

Federico D'Agostino (35, Scarlato / Roncarati):
Identification of *Helicobacter pylori cncR1* targets through MAPS technology

The importance of *Helicobacter pylori* as a human pathogen is underlined by the plethora of diseases it is responsible for. The peculiar small genome size combined with its paucity of transcriptional regulators, highlights the relevance of post-transcriptional regulatory mechanisms as small non-coding RNAs (sRNAs). Among them, the *cag*- non-coding RNA1 (*CncR1*) is a *trans*-acting sRNA that appears to play a pivotal role in the programmed expression of genes involved in *H. pylori* infection and persistence in the gastric niche. Although *CncR1* sequence is well conserved among all *cag*-pathogenicity island positive (*cag*-PAI+) strains, its targets have not been deeply characterized and its regulatory circuit remains poorly understood.
Here we aim to uncover the *CncR1* full targetome through an MS2-Affinity Purification coupled with RNA Sequencing (MAPS) technology. This method takes advantage of the strong affinity between MS2 phage capsid protein and MS2 RNA aptamer used to tag in vivo the *CncR1* sRNA.
To this end, a set of *H. pylori* G27 mutants harbouring the 5′ MS2-tagged or untagged gene in a C*ncR1* null background were generated. We show that the complementation of the 5′-MS2-tagged *CncR1* sequence in an ectopic locus restore the wild type sRNA expression levels. MS2-based in batch chromatography experiments reveal a peculiar recovery of the MS2-tagged *CncR1*. Finally, we prove that *CncR1* enrichment in RT-qPCR experiments increase in a degradosome deficient mutant, thus possibly increasing CncR1 recovery in the MS2-based chromatography.
We are planning to improve the MS2-based chromatography to increase the recovery of putative targets obtained from transcriptome analysis of *CncR1* deleted mutant. Furthermore, in addition to the previous bioinformatic prediction models of sRNA-target interaction, a deep *in vitro* analysis will be performed to better characterize these complexes.

Sonia Nicchi (34, Brettoni / Maione / Scarlato):
Investigating the mechanisms of *Moraxella catarrhali*s resistance to oxidative stress

Moraxella catarrhalis (Mcat) is the second most prevalent bacterium associated with exacerbation of Chronic Obstructive Pulmonary Disease (COPD), a multifactorial pathological condition. Among the aspects that characterize COPD pathogenesis, oxidative stress is a hallmark. The main biologically relevant sources of reactive oxygen species (ROS) include innate immune cells, alteration of the microbiota composition and bacterial load. Most pathogens survive the action of ROS by employing intrinsic mechanisms such as detoxification of radical species while few bacterial pathogens exploit extrinsic resistance mechanisms to actively suppress ROS production by eukaryotic cells.
Unlike non-typeable *Haemophilus influenzae* (NTHi), we showed for the first time that Mcat is able to actively interfere with ROS-related responses in activated neutrophil-like cells. It survives intracellularly, suggesting a clear capability to subvert the host innate immune responses supporting its replicative life cycle. Interestingly, in co-infection, its presence actually provides a safer niche for NTHi which is otherwise highly susceptible to the host antimicrobial arsenal.
Mcat shows a higher level of resistance to exogenous oxidative stress compared to the co-infecting pathogens pneumococcus and NTHi but the underlying intrinsic mechanisms are currently not well defined. Even if less obvious than H_2O_2, copper represents a key component of innate immune bactericidal defenses acting

as a catalyst for the production of hydroxyl radicals via the Fenton reaction. To date, few transcriptional studies related to this response are present despite the fact that copper efflux genes are frequently identified as crucial virulence factors for successful intracellular pathogens. Therefore, to better characterize the global transcriptional response to oxidative stress, RNA-Seq of exponentially growing Mcat exposed to sublethal amounts of H_2O_2 or $CuSO_4$ was performed. We found 225 and 140 genes as differentially expressed (DE) compared to untreated bacteria, respectively. All DE genes were classified according to 13 major functional categories and KEGG pathways enrichment analyses were carried out. A good portion of these genes falls into intrinsic resistance mechanisms and interestingly the glyoxylate metabolism emerged among the top significantly enriched pathways upon both treatments. Comparing the two transcriptomes of Mcat treated with H_2O_2 or $CuSO_4$, we found 61 commonly regulated genes. Ten genes were subsequently selected because of their relevance in DE analysis and functionally characterized through the generation of knock-out (KO) mutants. Among them, deletion mutants for *badM*, *katA* and *MCR_0349* showed a different phenotype compared to the wild-type strain in terms of sensitivity to H_2O_2 and intracellular survival in ROS-induced neutrophil-like cells. Altogether, this work represents the first comparative global transcription analysis of the two stimuli and unravel a previously unknown overlap of the two transcriptional responses.

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Paolo Cinelli (35, Cozzi / Maione / Scarlato):

Computational design, generation and periplasmic expression of new protein carriers for bioconjugation technology

Glycans presented on the surface of bacteria mediate some of the early interactions between microbes and mammalian host. These polysaccharides are involved in a multitude of processes, including resistance to serum-mediated killing, bacterium-host interactions, and regulation of the host immune response and for this reason, surface polysaccharides have been used as target antigens for the development of vaccines. It is well known that most polysaccharides are not strong antigens because they act as T cell-independent antigens, therefore they're unable to induce immunoglobulin class switching and long-term B cell memory in children below the age of 2 years. For this reason, starting in the early 1980s, glycoconjugate vaccines were developed in order to obtain more effective and long-lasting immunogenic vaccine products. Traditionally, glycoconjugate vaccines are obtained through a semi- or a fully-synthetic approach. Although these types of vaccines have seen immense commercial success, their manufacturing processes are not free from drawbacks, including batch to batch variation, heterogenous product formation and high manufacturing costs.

Recently, bacterial glycosylation systems have been developed to produce innovative vaccines substantially simplifying the production of glycoconjugates. In these systems, heterologous expression of polysaccharides can be associated with the glycosylation of selected protein targets by expressing an oligosaccharyltransferase (i.e. C. jejuni PglB) (bioconjugation) in an engineered E. coli strain. These systems have been shown to be efficient and promising tools to produce glyco-conjugated vaccines. An optimum carrier for bioconjugation should be highly expressed and correctly folded in the periplasmic space of *E. coli* and should allow the insertion of the glycosite in positions accessible to the pglb enzyme. To enrich the molecular repertoire of protein scaffolds that can be used in bioconjugation, we selected 15 proteins from the PDB, with different size, structure and chemical-physical characteristics to be used as carriers for the polysaccharide moiety. In this context, *in silico* and rational designed glycoengineering was applied to analyze the 3D structure of the different selected proteins. From the computational analysis, the most promising sites to insert the glycosylation sequence were identified. For protein expression, different leader sequences were screened in order to explore different secretion pathways for the export in the periplasm. To date, it was found that 2 out of 7 leader sequences tested dramatically increase the export of most of the carriers tested in this subcellular space. The next step will be to test the new protein scaffolds in the glycol-competent E. coli strains to test the best carriers for bioconjugation. This approach can broaden the repertoire of glycoconjugates vaccines and deepen the knowledge on the mechanisms underlying the bioconjugation mechanism.