7th Experience in biomedical research: young minds at work

Desenzano del Garda (BS) • Park Hotel

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SOCIETA' ITALIANA DI VIROLOGIA
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Dear Friends,

We are very pleased to host you here in Desenzano del Garda at the 7th Annual Experience in Biomedical Research: Young Minds at Work.

During the conference, you will be in touch with peers (PhD students, postgraduates, Post-Doctoral Scientists, etc.) from across all biomedical disciplines encompassing the latest and hottest frontiers of research and their innovative translational applications. We want you to take this opportunity and to make this conference very special. Be participative! Be curious! Ask! Be ready to share your knowledge! Even more importantly: Think! Think of possible new scenarios for your research!
Think of how fascinating and intriguing is the work we are all involved in!

Your peers are not here to judge you, but to give you a fantastic opportunity to share your data in an extremely informal environment.

Your active participation is important to us and for the real effectiveness of the conference. We also hope it is important to you!

Kind regards,

Francesca Caccuri
Claudia Del Vecchio
Nicasio Mancini

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Index

DELIVERY SYSTEMS AND ANTI-CANCER THERAPY

EXPERIMENTAL ONCOLOGY

VIROLOGY AND CLINICAL VIROLOGY

VIRAL PATHOGENICITY

ANTIVIRAL MECHANISMS

BACTERIA PATHOGENICITY AND ANTIBACTERIAL DRUGS

NEW TRENDS IN MICROBIOLOGICAL DIAGNOSIS

STRUCTURAL ANALYSIS OF ALPHA-1 ANTITRYPSIN POLYMERS EXTRACTED FROM AN EXPLANTED MZ HETEROZYGOTE LIVER

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Background: The most common variant associated with alpha-1-antitrypsin (AAT) deficiency (AATD) is Z AAT (E342K), which forms ordered polymeric chains in the endoplasmic reticulum of hepatocytes that aggregate into inclusion bodies. Polymerisation contributes to AAT plasma deficiency, emphysema and liver cirrhosis in ZZ patients. Around 2% of European and US populations are heterozygotes for the wild-type M and the Z alleles. While MZ subjects generally do not exhibit the predisposition to COPD and liver disease that ZZ homozygotes do, these individuals have a higher risk of liver disorders when compared to MM homozygotes.
We have previously demonstrated that M and Z can co-polymerise in the ER of AATD cellular models. Here, we extend the analysis to *ex-vivo* AAT polymers purified from inclusion bodies of an MZ heterozygote liver explant.
Methods: To this end, we developed a conformational antibody that showed specificity for the M over the Z variant in ELISA and surface plasmon resonance assays, and we determined its epitope by X-ray crystallography.
Results: We used Fab fragments of our M-specific antibody to label any M molecules present in MZ liver-derived polymers and visualised them by negative-stain electron microscopy. Our data show these polymers were morphologically indistinguishable from those extracted from a ZZ explant and we found that around 6% of the subunits were M AAT.
Conclusions: These data provide evidence that a detectable fraction of the M variant becomes trapped in Z polymers and confirm that the MZ co-polymerisation observed in cellular models occurs *in vivo*.

ACID CERAMIDASE GENE DELIVERY PROTECTS AGAINST DOXORUBICIN-INDUCED CARDIOTOXICITY

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Introduction: Doxorubicin is a cytotoxic agent utilized for the standard therapy of various tumours, but causes severe and untreatable cardiotoxicity. The cytotoxic action of doxorubicin is mostly mediated by the overproduction of ceramides, bioactive lipids that trigger apoptosis. Often, cancer cells resist to doxorubicin by upregulating the acid ceramidase (AC), a lysosomal enzyme that converts pro-apoptotic ceramides into sphingosine. While this cellular response prevents an efficacious tumour treatment, it might prove beneficial for non-cancerous tissues.
Methods: Experiments were performed in 2 groups of C57/Bl6 mice (14/group), one transduced with empty AAV9 and one with AAV9-AC, all administered by peri-ocular injection. Each group was divided in two sub-groups (7/group), Control and Doxorubicin (25 mg/kg).
Results: At 14 days after doxorubicin, mice transduced with AAV-AC displayed a better survival rate, 6/7 against 3/7 of mice with empty AAV9 ($p<0.001$) and their heart function was preserved, with no significant changes in ejection fraction (EF) and LV end-diastolic volume (LVEDV), whereas mice transduced with empty AAV9 developed a severe cardiomyopathy, with a fall in EF from 53.8±2.3% to 43.3±1.7% and an increase in LVEDV from 61.2±0.7 µl to 108.6± 4.7 (all $p<0.01$). Moreover, AC gene delivery completely prevented myocardial ceramide accumulation after doxorubicin. Finally, in a separate group, the co-administration of the AC inhibitor ARN14975 and doxorubicin caused severe cardiotoxicity and death (10/10), which supports a key protective role for AC. Mechanisms were further tested, in vitro, by transducing neonatal cardiomyocytes with AC and exposing them to doxorubicin. AC increased autophagy, a process notoriously involved in doxorubicin elimination, by 5 folds ($p<0.001$) and enhanced survival rate after doxorubicin (90% vitality vs 56% of controls).
Conclusions: Our results provide novel insights in the protective effects of AC against doxorubicin-induced toxicity suggesting a possible strategy for the cardio-selective prevention of this major side effect of chemotherapy.

EPIRETINAL MEMBRANE: GLIAL-TO-MESENCHYMAL TRANSITION IN MIO-M1 MÜLLER GLIAL CELLS

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Background: Formation of the epiretinal membrane (ERM), a thin layer of scar tissue at the vitroretinal interface on the macular/perimacular region of the eye, may cause severe visual defects in the elderly population. In the retina, Müller glial cells (MGCs) may undergo glial-to-mesenchymal transition (GMT) in response to different growth factors and cytokines, thus contributing to ERM formation, Here, we investigated the capacity of vitreous fluid obtained from patients affected by different ocular pathologies to modulate GMT in MIO-M1 cells, a prototypic MGC line.
Methods and Results: MIO-M1 cells were treated with TNFα, TGFβ, NGF, CTGF, insulin, VEGF, or FGF2 and expression levels of *GFAP, αSMA, CRALB,* and *CYCLIN-D1* were assessed by RT-qPCR. The results demonstrate that these cytokines exert a different impact on MIO-M1 cells. In particular, TGFβ, TNFα and FGF2 induce a pro-GMT expression profile characterized by *αSMA* upregulation and/or *CRALB* down-modulation. Next, MIO-M1 cells were treated with vitreous samples obtained from patients affected by macular hole, macular puker or retinal detachment. All vitreous samples caused the downregulation of *CRALB* and *GFAP* expression that was paralleled by a limited upregulation of *αSMA*. Notably, macular hole and macular puker samples were also able to induce *CYCLIN-D1* upregulation, an index of MIO-M1 cell proliferation.
Conclusions: The results demonstrate that the vitreous fluid of patients affected by eye pathologies associated to ERM development modulates GMT in MIO-M1 cells. This can be due to the activity of various cytokines usually present in this biological fluid. Based on our observations, vitreal TNFα, TGFβ and FGF2 may provide an important contribution to ERM formation.

THE SIMPLE WAY TO LOAD THE SMALL MOLECULE COMPOUNDS INTO MESENCHYMAL STEM CELLS AS THE DRUG CARRIER

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The potential use of stem cells as therapeutics in disease has gained momentum over the last few years and recently phase-1 clinical trials showed favourable results in treatment of a small cohort of acute stroke patients. Similarly, they have been used in pre-clinical models-drug-loaded for the effective treatment of solid tumours.

Firstly, we characterised uptake and release of a novel p5-CDK5 inhibitory peptide by human adipose-derived mesenchymal stem cells (hAD-MSCs) and showed release levels capable of blocking aberrant CDK5 signalling pathways, through phosphorylation of CDK5 and p53. These pathways represent the major acute mechanism stimulating apoptosis after stroke and hence modulation of this could benefit patient recovery. In vivo administration of hAD-MSCs loaded with this therapeutic peptide to post-stroke rats created conditions that supported survival of drug-loaded hAD-MSCs after cerebral ischemia, suggesting its therapeutic potential in patients with stroke.

Secondly, we investigated whether hAD-MSCs can be used as potent and safe tumour tropic vehicles for conventional drug delivery to tumours. The results showed that paclitaxel and carboplatin, the first line chemotherapeutic drugs for lung cancer treatment, produced a strong anti-proliferative effect on three lung cancer cells (HCC827, A549 and H520) whereas hAD-MSCs were strongly resistant to paclitaxel and relatively resistant to carboplatin, respectively. The conditioned media from paclitaxel or carboplatin primed hAD-MSCs produced a dose dependent growth inhibition of lung cancer cells, whereas conditioned media from untreated hAD-MSCs were not effective. LC-MS/MS analysis further confirmed the time dependent release of drugs by primed hAD-MSCs. The kinetics of doxorubicin internalization into hAD-MSCs revealed the appreciable internalization of doxorubicin by hAD-MSCs after 1 hour of priming and the intense staining in cytoplasm at the end of priming (24 hours). The distribution of doxorubicin in cytoplasm decreased after 24 hours, suggesting a possible excretion. Furthermore, transwell assays showed that conditioned media from lung carcinomas induced the migration of hAD-MSCs.

Our work indicates a potential use for drug-loaded stem cells as delivery vehicles for stroke therapeutics and in addition as anti-cancer receptacles particularly, if a targeting and/or holding mechanism can be defined.

AVIAN REOVIRUS P17 INHIBITS MOTILITY, MIGRATION AND ANGIOGENESIS OF HUMAN ENDOTHELIAL CELLS

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Background: Avian Reovirus p17 (ARVp17) is a 17-kDa non-structural protein encoded by the second open reading frame of S1 gene and contains 146 amino acids. ARVp17 has been observed to induce cell growth retardation, cell cycle arrest and host cellular translation shutoff in different epithelial and fibroblast cell lines by suppression of CDK1 and PLK1 like signaling pathways, and regulation of p53/PTEN/mTORC1 like pathways. ARVp17 is also known to induce autophagy and activate protein kinase RNA-activated signaling, thereby activating the innate immune system, which may induce the immune response against tumors.
Methods: Human Vascular Endothelial Cells (HUVECs) were used to perform wound healing assay, 2-D and 3-D Matrigel assay, spheroids invasion, cell motility and co-cultivation assay. Diprotin A, a DPP4 inhibitor, was used to modulate the activity of ARVp17. The same set of experiments were also performed with the recombinant GST tagged ARVp17 purified protein. The irrelevant protein GST or empty vector was used as a control.
Results: ARVp17 is able to induce loss of the ability of endothelial cells to form a network of capillary-like structures when seeded with extracellular matrix (BME) and suppresses the sprouts formation by spheroids embedded in collagen. Moreover, migratory capacity of HUVECs treated with ARVp17 was inhibited, as demonstrated by wound sealing and cell migration assays. The anti-angiogenic activity of the viral protein was confirmed both by co-cultivation and testing culture supernatant. However, the same was found to be suppressed, at least partially, in the presence of Diprotin A.
Conclusion: The ability of ARVp17 to suppress endothelial cells motility, impeding angiogenic processes and new vessels formation, may lead to the inhibition of tumor nutrition, growth and spreading.

IMMUNO-ONCOLYTIC HSV-1-BASED VIRUSES FOR THE TREATMENT OF SOLID TUMORS RESISTANT TO CURRENT IMMUNOTHERAPIES

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Immunotherapy is achieving impressive results in the treatment of solid tumours for which curative medical therapy is still not available. Oncolytic viruses (OVs) are antitumoral therapeutics that can serve as both cancer cell killers and gene therapy vectors. OVs are also a form of immunotherapy, by means of which a replicating virus elicits an antitumoral immune response. A herpes simplex type 1 (HSV-1) based OV (talimogene laherparepvec) has been approved for the treatment of metastatic melanoma following successful clinical trials. However, solid tumors surrounded by an immunosuppressive tumor microenvironment (TME), including triple negative breast cancer (TNBC) and others, are usually resistant to immunotherapy and OVs, requiring an improved approach.

We therefore engineered an attenuated HSV-1 genome to express multiple therapeutic genes that disrupt the immunosuppressive features of the TME. A strain 17+ HSV-1 genome including a double deletion of the "neurovirulence" γ34.5 gene, embedded in a bacterial artificial chromosome (BAC), was further modified by BAC mutagenesis in a strain of Escherichia coli expressing heat-inducible recombinases. Viral replication and therapeutic gene expression are being evaluated by plaque titration assay, Western blotting and reverse transcriptase PCR (RT-PCR) in vitro.

The Us12 gene was deleted to obtain a starting backbone analogous to the clinically approved virus. Therapeutic genes were selected for insertion in the UL55-UL56 intergenic region, including 1)enhanced green fluorescent protein, 2)human IL-12, a cytokine with anti-tumoral activity 3)murine IL-12, 4)FMS-like tyrosine kinase ligand 3 (FLT3L), a chemokine active on dendritic cells, 5) soluble programmed cell death 1 (sPD-1), 6)a single chain antibody against the CCR4 receptor on regulatory T cells, 7)a synthetic peptide which blocks the IL4 receptor, important for the activation of myeloid-derived suppressor cells (MDSCs).

Viruses 1, 2, 3, 5 and 6 were generated and efficiently replicated and expressed therapeutic genes in human and murine breast cancer cell lines *in vitro*. They also showed selectivity for murine breast cancer organoids compared to non-tumoral murine breast organoids.

Our OVs are also being tested on *in vitro* or primary cell counterparts of non-tumoral cells present in the TME, such as macrophages and fibroblasts. Little is known about the replication and therapeutic genes expression pattern of OVs in these cells, that indeed are important players in the biology of tumors. OVs will be subsequently tested in an immunocompetent TNBC mouse model (4T1 cells in Balb/c mice), to allow evaluation of their efficacy in the presence of an intact immune system, while also providing an important opportunity to study the relative importance of different immunosuppressive mechanisms in the TME *in vivo*.

INDUCTION OF APOPTOSIS AND INHIBITION OF DNA REPAIR MECHANISMS ON TRIPLE NEGATIVE BREAST CANCER CELLS BY U94 OF HUMAN HERPES VIRUS 6

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Introduction: Triple-negative breast cancer (TNBC) prognosis is still poor with no effective specific targeted therapy available. U94, the latency gene of human herpes virus 6 (HHV-6), is able to interfere with proliferation and crucial steps of the metastatic cascade in TNBC models. U94 exerts its activity upon its arrival to the nucleus. Based on these evidences, we characterized the molecular targets of the viral protein.
Materials and Methods: TNBCs (MDA-MB 231, MDA-MB 468 and BT-549) were transduced for twenty-four hours with amplicon vectors expressing U94 or enhanced green fluorescent protein (EGFP). The Affimetrix array was performed and its results were confirmed by real-time PCR. Apoptosis was assessed by flow cytometry, western blot and TUNNEL assay. Chemotherapeutic drugs cytotoxic rate was evaluated by counting cells using the COULTER COUNTER® Analyzer.
Results: The Affimetrix array analysis demonstrated that U94 is able to inhibit TNBCs proliferation and DNA repair mechanisms. Flow cytometric and TUNNEL analysis revealed that following U94 expression, TNBCs undergo apoptosis as demonstrated by morphological changes in cell membranes and by DNA fragmentation. Western blot analysis showed that U94 induces apoptosis through down-modulation of the anti-apoptotic protein Bcl-2 and activation of the pro-apoptotic proteins Bax and BAD. Moreover, U94 increased the expression of cleaved poly (ADP-ribose) polymerase and caspase-3. Here we show that, U94 induces apoptosis via the intrinsic pathway as attested by the expression of active caspase-9. Based on this evidence, we tested whether U94 could act as chemo-sensitizer in TNBCs. Interestingly, U94 transduction of TNBCs enhanced cisplatin and doxorubicin cytotoxicity whereas no differences were detected after taxol treatment.
Discussion and Conclusions: U94 can be considered as a potential selective chemotherapy sensitizer for the killing of cancer cells which lack DNA repair. In this scenario, U94 is a hopeful therapeutic TNBC treatment as a single agent or in combination.

DEVELOPMENT OF FGF TRAP MOLECULES FOR THE TREATMENT OF FGF-DEPENDENT CANCERS

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Background: The FGF/FGFR system is involved in tumor/stroma compartments of different tumors, representing a major target for novel anti-tumor drugs. PTX3 is a natural FGF antagonist able to inhibit the growth and vascularization of FGF-dependent cancers. Molecular characterization of interaction between FGF2 and PTX3 allowed the identification of the first orally active low molecular weight FGF trap (NSC12) with a significant anti-tumor activity in various FGF-dependent tumor models.
NSC12 structure consists of a steroid backbone linked to a hexafluoroisopropanol group. To better characterize the structure/function relationship of NSC12 and to improve its potency and physicochemical properties, chemical derivatives were generated and tested on the FGFR3-overexpressing human multiple myeloma cell line KMS-11.
Method and Results: Cell proliferation and FGFR phosphorylation assays identified four active derivatives out of the 27 molecules tested, modification of the hexafluoroisopropanol group causing the loss of the antitumor activity. Like NSC12, active derivatives blocked the proliferation of KMS-11 cells with an $IC_{50} \cong 3$ μM, whereas they appeared to be more effective in inhibiting FGFR phosphorylation. Notably, only one of them was better than NSC12 to prevent the formation of the bioactive HSPG/FGF2/FGFR ternary complex in a FGF2-dependent "cell-cell adhesion assay". In vivo this new lead compound. appeared to be more effective, compared to NSC12, in reducing the KMS-11 xenografts growth in immunodeficient mice.
Conclusions: These results define the importance of the hexafluoroisopropanol group in the NSC12 FGF trap activity and identify a new FGF trap compound with improved efficacy in vivo.

A NOVEL RECURRENT ACTIVATING MUTATION OF VEGFR2 DRIVES MELANOMA PROGRESSION THROUGH ENERGY METABOLISM REWIRING

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Background: Melanoma is an aggressive cancer which partially responds to existing therapies. The tyrosine kinase receptor (RTK) VEGFR2 is the fourth most mutated tyrosine kinase protein in cancer, following BRAF, EGFR and ERBB2. VEGFR2 is expressed in melanoma cells where it regulates cell proliferation. Here, we identified a new mutation of VEGFR2 which recurs in position 258 of the aligned tyrosine kinase domain of other RTKs and which correlates with reduced overall survival, suggesting that mutation of this residue in RTKs may promote tumor progression.

Methods and Results: Functional characterization demonstrated that $VEGFR^{2258}$ is active in the absence of VEGF ligand and, as a consequence, it has a reduced membrane lateral diffusion when compared to $VEGFR2^{WT}$, similarly to VEGF-activated wild-type receptor.

When expressed in human melanoma SK-MEL-31 cells, mutated $VEGFR^{2258}$ increases their proliferative/metastatic capacity. The expression of $VEGFR^{2258}$ upregulates the phosphorylation of several proteins of the Akt/mTOR pathway, a master regulator of cell metabolism. In keeping, SK-MEL-31 cells expressing $VEGFR^{2258}$ show increased energy metabolism, ATP production and glutamine dependency when compared to cells expressing $VEGFR2^{WT}$. Accordingly, glutamine deprivation normalizes mitochondrial respiration and growth of melanoma cells expressing mutated VEGFR2. Of note, expression of $VEGFR^{2258}$ renders melanoma cells highly sensitive to Linifanib TK inhibitor.

Conclusion: mutated VEGFR2 increases the oncogenic capacity of melanoma cells *in vitro* an *in vivo* by inducing a metabolic rewiring. Screening for somatic VEGFR2 mutations could help to predict inter-individual differences in terms of tumor growth and response to therapy.

ORTHOTOPIC VACCINATION OF CIITA-DRIVEN MHC CLASS II-POSITIVE GL261 GLIOMA CELLS CONFERS PROTECTION AGAINST GL261 PARENTAL TUMOR

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Background: The recognition of tumor antigens by the immune system remains a critical step for optimal induction of adaptive anti-tumor immunity. The latter's terminal effectors are MHC-I restricted cytolytic T cells, whose activity and persistence is dependent on the action of CD4+ T helper cells that, instead, are MHC-II restricted. Previously, we showed that tumor cells genetically modified with CIITA, master regulator of MHC-II expression, were rejected or their growth strongly retarded. Importantly, CIITA-tumor vaccinated mice were protected against challenge with the parental tumor cells. We aimed to apply this strategy to glioblastoma, one of the most malignant and yet untreatable tumor.
Methods: Murine GL261 glioblastoma cells were transfected with CIITA. MHC-II-positive cells were intracranially injected in the left hemisphere of syngeneic C57BL/6 mice. At day 21 after tumor injection, half the mice were sacrificed, their brains harvested and checked for the presence or absence of tumors. The remaining mice were challenged with parental tumor cells in the right hemisphere and after 21 days were sacrificed to check their brain for tumor take and growth. Splenocytes from the sacrificed mice were used for adoptive cell transfer.
Results: Our results showed that after genetic transfer of CIITA, GL261 cells were rejected or their growth strongly reduced in-vivo. Importantly, CIITA-tumor vaccinated mice resisted the challenge in the opposite hemisphere with parental GL261 tumor cells. Moreover, preliminary results suggest that CD4+ T cells isolated from vaccinated mice can transfer protection from parental GL261 tumor growth when injected into naïve mice.
Conclusion: Our results demonstrate the feasibility of CIITA-modified tumor cell approach in glioblastoma, one the most malignant tumor of the central nervous system, opening the possibility for the development of immunotherapeutic strategies for its cure. Further experiments are needed to asses the molecular correlation of this protection and define the potential of this approach for brain tumors.

THE CIRCRNAS LANDSCAPE OF T-CELL ACUTE LYMPHOBLASTIC LEUKEMIA

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Background: T-cell Acute Lymphoblastic Leukemia (T-ALL) is an aggressive hematological malignancy of precursor T-cells. T-ALL subgroups are classified according to driver genetic events, aberrant activation of specific transcription factor oncogenes, and stages of T-cell development. Previous studies of gene, miRNA and lncRNA expression contributed to understand the molecular network driving T-ALL transformation in distinct T-ALL subgroups. Emerging data on circular RNA (circRNAs) functions and oncogenic potential and variable expression in normal haematopoiesis motivated the study of circRNAs in T-ALL.
Methods: CirComPara pipeline was used to characterize the expression landscape of circRNAs by analysis of ribosomal RNA-depleted RNA-seq data of 25 T-ALL patients representing 5 cytogenetic subgroups (HOXA, TLX1, TLX3, TAL1/LMO2 rearranged T-ALLs and an immature/LYL1+ subgroup) and of 5 sorted populations of developing thymocytes from two healthy donors.
Results: Our data disclosed that the malignant cell transcriptome harbors numerous functional entities not observed with traditional strategies. The 3,447 circRNAs that we identified in our data set revealed deregulation of the circRNAome in T-ALL compared with normal thymocytes, notably with an exceeding number of circRNAs upregulated in malignant cells and only few downregulated. CircRNAs deregulated in T-ALL samples included circRNAs with well known oncogenic potential or previously reported associations to specific molecular functions, whereas most of them are not yet characterized. Interestingly, novel circRNAs from genes linked to leukemogenesis were deregulated and highly expressed as well. Comparing genetic subtypes of human T-ALL we found highly group-specific expression patterns of circRNAs, a promise for a better understanding of the disease mechanisms.
Conclusions: Our study provides a comprehensive overview of circRNAs expression in T-ALL, setting the stage for functional evaluation of specific circRNAs in tumor biology.

DIRECT INHIBITION OF THE VEGF/VEGFR2 SYSTEM BY THE EPH/EPHRIN ANTAGONIST UNIPR1331: A NEW DUAL-ACTING ANTIANGIOGENIC COMPOUNDS

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Background: Angiogenesis, the formation of new blood vessel from pre-exisiting ones, is crucial for tumor growth and metastasization, being thus considered a promising therapeutic target. Unfortunately, drugs specifically directed against a specific pro-angiogenic growth factor or receptor turned out to be of limited benefit for oncological patients, likely due to the high biochemical redundancy of the neovascularization process. In this scenario, multitarget compounds able to tackle different pro-angiogenic simultaneously are eagerly awaited. UniPR1331 is a 3β-hydroxy-Δ^5-cholenic acid already known to act as a potent pan-Eph receptor inhibitor.
Methods: We adopted a pipeline of analysis made up by surface plasmon resonance (SPR) spectrometry, biochemical assays and endothelial cell models to demonstrate that UniPR1331 also interacts directly with the vascular endothelial growth factor receptor 2 (VEGFR2).
Results: By using SPR, we observed that UniPR1331 interact with VEGFR2 in a specific and dose-dependent way with a Kd equal to 62.2 μM (UniPR1331 binding to immobilized-EphA2 resulted equal to 3.3 μM). Similarity, in ELISA UniPR1331 prevents the binding of VEGF to VEGFR2 with an IC 50 equal to 46 μM, while the IC 50 for eprhin A1/EphA2 interaction is equal to 4 μM, mirroring the higher affinity for the eprhin A1/EphA2 interaction in respect to the VEGF/VEGFR2 one, as measured by SPR. UniPR1331 retains its capacity to bind VEGFR2 at the cell surface, preventing its binding to VEGF and the formation of the productive VEGF/VEGFR2/HSPGs. The compound effectively inhibits the phosphorylation and internalization of VEGFR2 and consequent ERK 1/2 activation in different EC lines and in HUVECs. Finally, UniPR1331 inhibits VEGF-dependent proliferation, motogenic activity in wounded HUVEC monolayers and sprouting formation.
Conclusion: Taken together, these data point to cholenic acid as a promising scaffold for the development of multitarget anti-angiogenic compounds.

HHV-6A INFECTION AND AUTOIMMUNE SYSTEMIC SCLEROSIS: CLUES OF A POSSIBLE ASSOCIATION

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Background: Systemic sclerosis (SSc) is a very severe autoimmune multisystem disease characterized by vasculopathy and fibrosis of the connective tissues, which is often fatal. Its etiology remains unknown, and several infectious agents, including human herpesvirus 6 (HHV-6), have been suggested as possible triggering factors, although direct association between infection and SSc is still missing. Since HHV-6 infection/reactivation has been associated to many autoimmune conditions, including thyroid disease, that is often present in SSc, the aim of our study was to investigate such possible association.
Methods: Twenty SSc patients were analyzed for the presence of HHV-6 in tissues and blood by a specifically designed digital droplet PCR allowing the distinction between 6A and 6B species. Anti-HHV6 antibody response was also analyzed. Furthermore, as endothelial cells (EC) are a target for HHV-6 infection and have a main role in the disease pathogenesis, the expression of fibrosis-associated factors was analyzed in HHV-6 infected EC.
Results: HHV-6A was detected at the skin level in all SSc available biopsies, and higher virus loads correlated with higher disease severity. HHV-6B was instead detected only at the blood level. SSc patients also displayed a higher frequency and titer of antibodies directed against the virus U94 product, compared to controls, similar to what already observed in other autoimmune pathologies that have been associated with HHV- 6 infection. Furthermore, EC infection with HHV-6A induced the expression of several pro-fibrosis factors.
Conclusion: Our data show that HHV-6A is present in skin of SSc patients, and can induce pro-fibrotic factors expression in EC, suggesting that HHV-6A infection/reactivation might be associated with the disease development and could be correlated to the progression of the disease at the tissue level. These data open the way to future studies aimed to understand the mechanisms by which HHV-6 might favor fibrotic transition in infected cells.

HHV-6A INFECTION OF ENDOMETRIAL EPITHELIAL CELLS AFFECTS IMMUNE PROFILE AND TROPHOBLAST INVASION

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Background: We first reported Human herpesvirus (HHV)-6A DNA presence in 43% of endometrial cells from women with idiopathic infertility, whereas no fertile control women harbored the virus. We investigated the effect of HHV-6A infection on the immunological status of the endometrium.
Method of study: Endometrial biopsies, uterine flushing, and whole blood samples were collected from 67 idiopathic infertile women (mid-secretory phase).
Methods: We analyzed the endometrial immunological status evaluating: i) the effect of HHV-6A infection on endometrial immune profile analyzing the ratio of Interleukin (IL)-15/ Fibroblast growth factor-inducible 14 (Fn-14) and IL-18/ TNF-related weak inducer of apoptosis (TWEAK) mRNA as a biomarker of endometrial (e)Natural killer activation/maturation, angiogenesis, and Th1/Th2 balance; ii) endometrial receptivity to trophoblasts in endometrial 3D in vitro model; iii) Natural killer (NK) cells and T cells percentage and subpopulations by flow cytometry.
Results: We confirmed the presence of HHV-6A infection in a 40% of idiopathic infertile women, characterized by an immune profile reflecting eNK cell cytotoxic activation and a decrease in CD4+CD25+CD127dim/- regulatory T cells. The co-culture of endometrial epithelial cells with spheroids generated from the extravillous trophoblast-derived cell line JEG3 showed a 2-fold expansion of spheroids on endometrial epithelial-stromal cells (ESC) culture surface from HHV-6A negative women while no expansion was observed on the surface of ESC from HHV-6A positive women.
Conclusions: The identification of an effect of HHV-6A infection on endometrial immune status opens new perspectives in idiopathic infertile women care management. In addition, it would be possible to select antiviral therapies as novel, non-hormonal therapeutic approaches to those idiopathic infertile women characterized by the presence of endometrial HHV-6A infection, to increase their pregnancy rate.
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HERPES SIMPLEX VIRUS-MEDIATED INHIBITION OF ADENO-ASSOCIATED VIRUS GENOME CIRCULARIZATION

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Background: Adeno-associated virus (AAV) is a small helper-virus-dependent parvovirus, which is mainly known for its application as a gene therapy vector. Transduction with recombinant AAV (rAAV) vectors *in vivo* has shown astonishing durability of transgene expression up to several years post-injection. The AAV genome consists of a single-stranded DNA (ssDNA) molecule encoding two major ORFs flanked by inverted terminal repeats (ITRs). The genome is known to convert into different forms within the host cell e.g., double-stranded DNA (dsDNA), circular DNA, as well as being integrated into the host genome. Although the AAV genome in its episomal, circular dsDNA state is suggested to be the genome structure which persists over a long period of time in wtAAV as well as rAAV infected tissues, the mechanisms behind circularization and persistence are not clear yet. In the present study we make use of the properties of HSV-1 to further elucidate the underlying mechanisms of AAV genome circularization.
Methods: We used a circularization-dependent recombinant (self-complementary) AAV vector (scAAVGFP_CD), which enables transcription of functional GFP only when the genome is circularized. As additional circularization readout we quantified the AAV DNA using qPCR in the presence or absence of exonuclease V, which specifically degrades linear DNA but preserves circular DNA.
Results: In the presence of HSV-1, significantly less GFP+ cells were observed in scAAVGFP_CD infected cells. This inhibitory effect of HSV-1 on AAV genome circularization was absent when HSV-1 underwent UV treatment prior to infection. Co-infection with AdV did not lead to a reduction of the GFP+ cell population in scAAVGFP_CD infected cells. HSV-1 was also found to inhibit genome circularization of wtAAV.
Conclusions: We conclude that HSV-1, but not AdV, inhibits the circularization of the AAV genome. We are currently investigating which HSV-1 gene products are responsible for the observed inhibition and which cellular mechanism they employ to achieve this.

PREVALENCE OF NON B HIV-1 SUBTYPES IN NORTH ITALY AND ANALYSIS OF TRANSMISSION CLUSTERS BASED ON SEQUENCE DATA ANALYSIS

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Background: HIV-1 diversity is increasing in European countries due to immigration flows and this may have important implications for public health. We characterized HIV-1 diversity and transmission clusters to identify the extent to which non B-strains are transmitted locally.
Methods: We analyzed 732 HIV-1 sequences comprising protease and reverse transcriptase (PR/RT) coding regions, sampled from 2012 to 2017 from naive patients in Spedali Civili Brescia. The sequences were subjected to an initial typing using a consensus of online tools. The identification of the recombination site and the assignment of the subtype was performed using a local bio-informatic pipeline; phylogenetic analysis with a structured coalescence model and MCMC was used on the datasets to determine clusters and evolution.
Results: We detected 279 (38.1%) patients infected with HIV-1 non B variants: 3 pure subtypes and more than four recombinant forms; subtype F (17.9%) and CRF02_AG (53.7%) prevalently. Baseline clinical, demographic and laboratory characteristics, recorded as percentages, were compared between non B HIV-1 subtypes. They were mostly men, aged between 37 and 46 years, heterosexuals and from Italy. 28 transmission clusters were identified, three of which included >10 patients; two of subtype CRF02_AG and one from subtype F. Most cases of alleged transmission were between heterosexual couples. Probably due to strong migratory flows we have identified individual subtypes with particular patterns of recombination or, as in the case of the subtype G 17/279 (6.1%), to a complete lack of relationship between the sequenced strains revealing that they are all singletons.
Conclusions: The methods of phylogenetic analysis are advancing rapidly with very accurate analysis, providing consistent data in phylogenetic inference. In our analysis we have determined that, if used correctly, these tools can provide transmission and evolution data with greater precision and resolution than conventional analyzes of the HIV non B and recombinant forms.

HEPARIN AND HEPARAN SULFATE PROTEOGLYCANS PROMOTES HIV-1 P17 MATRIX PROTEIN OLIGOMERIZATION: COMPUTATIONAL, BIOCHEMICAL AND BIOLOGICAL IMPLICATIONS

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Background: p17 matrix protein released by HIV+ cells interacts with leukocytes heparan sulfate proteoglycans (HSPGs), CXCR1 and CXCR2 exerting different cytokine-like activities that contribute to AIDS pathogenesis. Since the bioactive form of several cytokines is represented by dimers/oligomers and oligomerization is promoted by binding to heparin or HSPGs, here we evaluated if heparin/HSPGs also promote p17 oligomerization.
Methods and results: Heparin favours p17 dimer, trimer and tetramer assembly, in a time- and biphasic dose-dependent way. Heparin-induced p17 oligomerization is of electrostatic nature, being it prevented by NaCl, by removing negative sulfated groups of heparin and by neutralizing positive lysine residues in the p17 N-terminus.
A new computational protocol has been implemented to study heparin chains up to 24-mer accommodating a p17 dimer. Molecular dynamics show that, in the presence of heparin, two p17 molecules undergo conformational modifications creating a continuous "electropositive channel" in which heparin sulfated groups interact with p17 basic amino acids, promoting its dimerization. At the cell surface, HSPGs induce p17 oligomerization, as demonstrated by using B-lymphoblastoid Namalwa cells overexpressing the HSPG Syndecan-1. Also, HSPGs on the surface of BJAB and Raji human B-lymphoblastoid cells are required to p17 to induce $ERK_{1/2}$ activation.
Conclusion: the results obtained indicate that HS-induced oligomerization plays a role in p17-induced lymphoid dysregulation during AIDS.

DETECTION OF HPV16/18 E6 ONCOPROTEINS IN CARCINOMA OF THE OROPHARYNX OR METASTATIC CARCINOMA OF UNKNOWN ORIGIN IN THE CERVICAL LYMPH NODES

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Background: Accurate identification and diagnosis of clinically relevant human papillomavirus (HPV) infections in patients with oropharyngeal squamous cell carcinoma (OPSCC) and neck lymph node metastasis of SCC from unknown primary tumor (NSCCUP) represents a critical topic in clinical research. We determined the feasibility and accuracy of HPV 16/18 E6 oncoprotein detection in cytological specimens from primary tumors and neck metastases.
Methods: Cytological specimens from primary tumor and neck metastases were collected from 34 patients with OPSCC with lymph node involvement or NSCCUP by fine needle aspiration (FNAC) and tested with a commercial lateral flow test (OncoE6, Arbovita) that detects the HPV16 or 18 E6 oncoproteins. Results were compared to presence of HPV-DNA together with $p16^{INK4a}$ overexpression or HPV-DNA together with HPV E6 seropositivity as reference method.
Results: Eighteen of 29 OPSCC (62%) and 3 of 5 NSCCUP (60%) were HPV-driven according to the reference method. The HPV 16/18 E6 oncoprotein test had a sensitivity of 94% (95% CI: 70%-100%) and a specificity of 100% (95% CI: 66%-100%) on OPSCC, and a sensitivity of 88% (95% CI: 64%-99%) and a specificity of 100% (95% CI: 74%-100%) in the neck metastases analyzed. Test agreement between the E6 lateral flow test and the clinical reference method was excellent both for primary lesion and neck metastases (Cohen's kappa=0.92 and 0.88 respectively).
Conclusions: In this study, we found the detection of HPV 16/18 E6 oncoproteins to be feasible on cytological samples from OPSCC and NSCCUP, allowing clinicians to get reliable HPV status information with a less invasive procedure.

HHV-6A INFECTION INDUCES AMYLOID-BETA EXPRESSION AND ACTIVATION OF MICROGLIAL CELLS

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Background: The control of viral infections in the brain involves the activation of microglial cells, the macrophages of the brain that are constantly surveying the central nervous system, and the production of amyloid-beta (Aβ) as an antimicrobial molecule. Recent findings suggest a possible implication of HHV-6A in AD. We evaluated the effect of HHV-6A infection on microglial cells expression Aβ and the activation status, determined by TREM2, ApoE, cytokines and tau expression.
Methods: We have infected microglial cells (HMC3, ATCC®CRL-3304), in monolayer and human peripheral blood monocyte-derived microglia (PBM-microglia) spheroid 3D model, with HHV-6A (strain U1102) cell-free virus inocula with 100 genome equivalents per 1 cell. We collected the cells 1, 3, 7 and 14 days post infection (d.p.i.) and analyzed them for viral DNA and RNA, ApoE, Aβ (1-40, 1-42), tau and phospho-tau (Threonine 181) by Real Time, immunofluorescence and cytokines by immunoenzymatic assay.
Results: We observed a productive infection by HHV-6A. The expression of Aβ 1-42 expression increased from 3 d.p.i., while no significant induction was observed for Aβ 1-40 expression. The HHV-6A infection induced the activation (TREM2, IL-1beta, ApoE) and migration of microglial cells. The secretion of Tau started from 7 d.p.i., with an increasing percentage of the phosphorylated form.
Conclusions: In conclusion, microglial cells are permissive to HHV-6A infection, that induces the expression of Aβ and an activation status. Meanwhile, we hypothesize a paracrine effect of HHV-6A infection, that activates and induces microglia migration to the site of infection.

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THE HIV-1 MATRIX PROTEIN P17 INFILTRATES THE CENTRAL NERVOUS SYSTEM CROSSING THE BLOOD BRAIN BARRIER

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Background: Human immunodeficiency virus-1 (HIV-1) infiltrates the central nervous system (CNS) where it is relatively protected from anti-viral drugs. The HIV-1 has also neurotoxic effects that result in HIV-1-associated cognitive neurological disorders termed as HIV-1 encephalopathy and AIDS dementia. The degree of neurologic dysfunction was found to be strongly related to chronic inflammation, with the neurovascular unit as the most critical target. Many studies showed a positive correlation between the amount of HIV-1 and/or viral proteins in the brain and the extent of histopathological changes. The HIV-1 matrix protein p17 was found to be expressed in autoptic brain tissues of HIV+ patients and it was found to be prone to oligomerize and form toxic amiloidogenic assemblies capable of inducing neurocognitive disorders in mice. Herein we have assessed the ability of p17 to cross the blood brain barrier (BBB) in a BBB in vitro model of translocation.
Methods: BEnd.3 cells were grown in tissue culture inserts and starved or not for 16 h. After starvation, atto488-p17 was added to the apical chamber for 6 h. The apical and base volume were collected after incubation and the fluorescence was measured.
Results: We demonstrated that p17 was able to transmigrate the BBB in normal condition of growth, with up to 26.1 ± 9.8% of the viral protein found in the base after 6 h. In addition, serum starvation of bEnd.3 cells increases p17 transmigration of BBB with up to 40.2 ± 10.3%. P17 receptor, CXCR2, plays a key role in p17 transmigration of BBB and the activity of the viral protein was neutralized by a specific p17 neutralizing mAb. .
Conclusion: In conclusion, p17 can cross the BBB and rapidly arrive to the CNS and it can contribute to HIV-1-associated cognitive neurological disorders.

HTLV-1 TAX AND HBZ ONCOPROTEINS EXPRESSION AND SUBCELLULAR LOCALIZATION IN INFECTION AND DISEASE PROGRESSION: IMPLICATIONS FOR IMMUNE-BASED THERAPEUTIC APPROACHES

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Background: HTLV-1, the first discovered human oncogenic retrovirus, is associated to the onset of the two major diseases: acute T cell leukemia-lymphoma (ATL) and tropical spastic paraparesis (HAM/TSP). Two viral products, Tax-1 and HBZ, are involved in the pathogenesis of both diseases. However, many cell biology aspects of Tax-1 and particularly of HBZ remain elusive concerning the subcellular localization of the proteins at the onset of infection and during the progression of the distinct diseases
Materials and Methods: Subcellular distribution of endogenous HBZ and Tax-1 proteins was assessed by confocal microscopy with the 4D4-F3 anti-HBZ and the A51-2 anti-Tax-1 mAbs in a large panel of PBMC samples derived from HTLV-1 infected asymptomatic carriers (AC), ATL and HAM/TSP patients.
Results: In ATL samples, HBZ was demonstrated to be preferentially expressed and localized in the nucleus, while Tax-1 was rarely detected in these leukemic cells. On the contrary, HBZ was confined in the cytoplasmic compartment in HAM/TSP samples while Tax-1 was observed to be localized in both nucleus and cytoplasm. Interestingly, in AC samples, both HBZ and Tax-1 were expressed in the cytoplasm. Only few cases were found co-expressing the two oncoproteins in a very limited number of PBMC cells. Cells expressing HBZ and Tax-1 were exclusively found in CD4+ T cell compartment and very rarely in CD8+ cells.
Conclusion: Strict HBZ cytoplasmic localization in AC indicates that HBZ is originally expressed in this compartment and retained in the cytoplasm during the chronic inflammatory process leading to HAM/TSP. The predominant nuclear localization of HBZ in ATL strongly suggests that the oncogenic transformation is accompanied with the newly acquired function of nuclear HBZ.

EXTRACELLULAR VESICLES AS AN ALTERNATIVE MODEL FOR HCV GLYCOPROTEINS STRUCTURAL STUDY

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Background: Structural studies of hepatitis C virus (HCV) internal proteins represented a powerful tool to establish a structure-function relationship that ultimately led to the development of the direct-acting antivirals currently in use.
Conversely, HCV outer structures are poorly characterized. The virus envelope glycoproteins E1 and E2 play key roles at different stages of HCV life cycle. They are highly glycosylated, single-pass, type I transmembrane proteins, organized in covalent heterodimers and heterohexamers on the viral envelope. They both present an N-terminal ectodomain directly involved in viral entry and a conserved C-terminal transmembrane domain implicated in oligomerization.
E1 and E2 flexibility, high glycan content and complex disulfide connectivity have limited their structure analysis to short synthetic constructs.
We propose the use of membrane protein-enriched extracellular vesicles (MPEEVs), already successfully applied to other fusion proteins, as a robust model for the structural study of native HCV glycoproteins.
Methods: E1 and E2 genes were cloned from several HCV isolates in a eukaryotic expression vector and subsequently overexpressed in human cell lines. Different cell lines and purification methods were tested to improve MPEEVs yield and purity.
MPEEVs morphology was assessed in electron microscopy, biochemical characterization of HCV glycoproteins was performed and entry in human hepatoma cell line tested.
Results: MPEEVs represent an ideal system for structural study as they provide a platform in which the native conformation of entire membrane proteins is conserved.
We demonstrated E1-E2 MPEEVs release in culture medium and glycoproteins exposure in their native conformation as shown by their oligomerization state and interaction with monoclonal antibodies and soluble host receptor. Furthermore, MPEEVs seem also to recapitulate viral entry in human cells.
Conclusions: The presented data support MPEEVs application to HCV glycoproteins structural definition in their native, complexed form.

THE EFFECT OF HUMAN DDX3 HELICASE INHIBITORS ON VIRUS REPLICATION

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Introduction: DDX3 is a human RNA helicase playing a key role in cell gene expression and cycle regulation. Since DDX3 has been shown to be involved in replication of HIV, HCV, Dengue, and other viruses, it is considered a potential antiviral target. Indeed, some DDX3 inhibitors have proved broad-spectrum antivirals, particularly against HCV and drug-resistant HIV. From this ground, and as part of PANVIR, we demonstrated that some DDX3 inhibitors are also effective against virus with a positive single-stranded RNA (ssRNA+) genome such as Coxsackie B (CV-B), Chikungunya virus (CHIKV) and against virus with a DNA double stranded (dsDNA) genome such as herpes virus (HSV-2) while they are not effective against virus a negative single-stranded RNA (ssRNA-) genome such as measles virus (MeV) and vesicular stomatitis virus (VSV).
Materials and Methods: DDX3 inhibitors (UVRs) were designed by in silico modelling of the human DDX3 RNA helicase, to target specifically the RNA binding domain leaving the ATP-binding domain unchanged. The percentage of inhibition (I%) of RNA helicase activity was measured *in vitro* by Helicase Assay Based on FRET. Antiviral activities of UVRs against CHIKV, CV-B and HSV-2, VSV and MEA were determined *in vitro* by plaque assay and virus yeald reduction assay and the inhibition concentration 50% (IC50) and selective Index (SI) was measured. Western blot and molecular analyses were used to investigate viral protein and genome production. Time of addiction assay (TOA) was performed to further investigate the mechanism of action.
Results: Almost all compounds showed antiviral activities against ssRNA+ virus with IC50 values ranging from 0.35 to 158 microM and SI up to 35, while they were not effective against ssRNA-. DDX3 inhibitors efficiently stop viral protein production and were able to act up to 12 h post infection. Some of the UVRs showed antiviral activities against HSV-2.
Conclusion: DDX3 proved to be an excellent target to develop antivirals against clinically relevant ssRNA+ viral species. However, DDX3 inhibitors were un-effective against ssRNA- viruses, such as VSV, and MeV. We hypothesize that DDX3 is important to unwind RNA during translation of specific mRNAs that have secondary structures in their 5' ends. This possibly allows viral protein expression after shutoff, whereas viruses that have a traditional cap-de-

pendent translation, are less dependent on the function of this cellular protein, if at all. This suggests that DDX3 can be a target to develop broadly acting compounds against viruses that rely on IRES-dependent translation, such as most ssRNA+ viruses, including HIV.

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IFI16 IS A RESTRICTION FACTOR OF AAV2 INFECTION

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Background: Adeno-associated virus (AAV) is a small, non-pathogenic, helper virus-dependent parvovirus with a single-stranded (ss) DNA genome. In absence of a helper virus, AAV establishes a latent infection either by integrating its genome into the host cell genome or by persisting as an episome. We recently assessed the global gene expression profile of AAV2 infected normal human fibroblasts (NHFs). IFI16, a cytosolic and nuclear sensor of ds- and ssDNA, was among the top 50 differentially expressed (DE) genes of the gene ontology (GO) term "innate immune response". IFI16 has been shown to be a restriction factor of many different viruses through various mechanisms, including interferon response and epigenetic modifications. In this study we addressed the question whether IFI16 is a restriction factor for AAV2 infection.
Methods: We recently performed a global gene expression analysis of AAV2 infected NHF cells, including GOterm analysis and visualizations of enrichment maps. To assess the role of DE genes, post-transcriptional knockdown experiments were performed. Multicolor immunofluorescence analysis combined with fluorescence in situ hybridization (FISH) provided information about the subcellular localization of capsids, genomes and proteins of interest.
Results: The screening revealed 1`929 genes as DE ($p < 0.01$, number of reads ≥ 40) in AAV2 infected cells. Among the top 50 DEs in the GOterm "innate immune response" was IFI16. Indeed, the post-transcriptional knockdown of IFI16 resulted in a significant increase in transduction efficiency of AAV2 vectors in NHF and 2fTGH Jak1-/- cells. Moreover, AAV2 transduction efficiency was enhanced also upon knockdown of both IFI16 and STING. Besides, multicolor immunofluorescence analysis of AAV2 infected NHF cells showed a nucleolar accumulation of IFI16 together with AAV2 capsids and genomes.
Conclusions: The data implies an IFI16 mediated inhibition of AAV2 transduction independent of interferon or STING signaling, regardless of the vector genome structure, single-stranded or self-complementary. We hypothesize that the inhibitory effect of IFI16 on AAV2 infection is linked to its sub-nucleolar localization.

A NEW THERAPEUTIC APPROACH IN HSV-1 INFECTION: SYNERGIC EFFECT OF YY1I AND HDACI

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Background: Yin Yang 1 (YY1) is a multifunctional mediator of several signaling processes, such as cell growth, tumor development and apoptosis. The name derives from its dual role: it can either activate or inhibit gene expression, depending on the cofactors that it recruits. YY1 recognizes a consensus sequence in promoter gene region rich in CArG. Different studies showed that YY1 regulates histone acetylation, deacetylation and methylation through the interaction with p300, HDACs, Ezh2 and PRMT1. Herpes Simplex Virus-1 (HSV-1) is a common human pathogen, which can establish productive lytic infection in epithelial cells and latent infection in the nervous system. YY1 plays a role in HSV-1 gene regulation through the transactivation of different viral genes.
Methods: Vero cells were pre-treated with YY1 inhibitor, called NPI-0052, in combination with several HDAC and Ezh2 inhibitors (valproic acid, trichostatin A, entinostat, vorinostat and GSK126). Subsequently, cell monolayer was infected with HSV-1 carrying a EGFP gene fused to the HSV-1 tegument protein VP22. The infection was monitored via plaque assay and fluorescence microscopy. It was also evaluated the putative apoptosis effect through Annexin V assay. Finally, a Chromatin immunoprecipitation (ChIP) followed by a Real-Time PCR was performed using viral gene primers.
Results: The data indicate a synergic regulation of HSV-1 infection driven by YY1 inhibitor together with HDAC and Ezh2 inhibitors. Indeed, HSV-1 infection was significantly decreased after the treatment of NPI-0052 in combination with several epigenetic drugs. No apoptosis was revealed after the treatment, supporting that the reduction in HSV-1 infection was referred only to the antiviral activity of the different combinations. The results were supported by ChIP analysis, highlighting that NPI-0052, together with the epigenetic drugs, prevent the recognition and the modulation of HSV-1 genome.
Conclusion: Acyclovir, penciclovir, and their prodrugs have been widely used for the treatment of herpesvirus infections. Antiviral resistance of HSV has emerged very rapidly and the research of new antiviral drugs is mandatory for the scientific community. The synergism between YY1 inhibitor NPI-0052 and several epigenetic drugs could represent a new and efficient therapeutic approach in the prevention and healing from HSV infection.

DISRUPTION OF PROTEIN-PROTEIN INTERACTIONS AS A NEW ANTIVIRAL STRATEGY AGAINST INFLUENZA VIRUS AND HUMAN PAPILLOMAVIRUS

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Background: Influenza viruses cause a highly contagious respiratory disease in humans. Currently available drugs have several drawbacks, including development of drug resistance and toxic side effects. On the other hand, vaccines provide good protection, but must be reformulated every year according to the circulating strains. Recently, the viral RNA polymerase complex emerged as an interesting target for antiviral discovery.
Human papillomaviruses are small DNA viruses with epithelial tropism. Low-risk genotypes mainly cause cutaneous warts, while high-risk HPVs are responsible for several types of cancers. E7 oncoprotein of high-risk HPVs is able to interact with many cellular proteins and subvert their physiological pathways. Among these interactions, E7 interacts with and leads to the proteasomal degradation of the cellular phosphatase PTPN14, which is an inhibitor of the co-transcriptional activator YAP.
Methods: The disruption of the interaction between the PA and PB1 subunits of the influenza virus RNA polymerase was assessed through ELISA-based assays. Antiviral activity was assessed by means of plaque reduction assays. Compounds cytotoxicity was assessed through MTT assay.
Protein levels were visualized by means of Western Blotting. Intracellular localization of proteins was visualized by immunofluorescence.
Results: Some of the anti-PA-PB1 compounds were able to inhibit influenza virus replication at low concentrations, and some of them showed also activity in the ELISA assay. Unfortunately, some compounds resulted to be toxic.
Preliminary data confirmed the importance of few critical residues implied in E7-PTPN14 interaction, the role of E7 in PTPN14 degradation, and the consequent effects on YAP shuttling and localization.
Conclusions: SAR analysis will allow the determination of chemical groups responsible for the disruption of PA-PB1 interaction and the development of more potent compounds.
Taking advantage of our preliminary data, we will proceed with an *in silico* screening of small-molecule compounds, to search for inhibitors of the E7-PTPN14 interaction.

INFLUENZA VIRUS MODULATES G6PD ENZYME TO CONTROL ITS REPLICATION AND HOST RESPONSE

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Background: Glucose-6-phosphate dehydrogenase (G6PD), the first and the rate-limiting enzyme of pentose phosphate pathway, is responsible for the production of reducing equivalents of NADPH, used for regenerating the reduced form of GSH. It has been reported that G6PD-deficiency could increase the susceptibility to viral infections even if the modulation mediated by influenza virus (IV) remains to be elucidated. Moreover, G6PD activity is regulated by the deacetylase SIRT2, but G6PD acetylation processes during viral infection are still unknown.

The aim of this study was to deep inside the regulation of G6PD in both epithelial and innate immune cells infected with IV.

Methods: Epithelial cell line (A549) and monocytes-derived macrophages cell line (U937) were silenced for G6PD expression and infected with influenza A PR8/H1N1 virus. After 24h of infection the viral titer by TCID50 assay and GSH production by using a colorimetric assay kit were evaluated. Cytokines production was evaluated by ELISA.

Results: We found that the expression level of G6PD and activity decreased in infected cells compared to uninfected ones in both cell lines at 24h post infection. Epithelial and macrophages cells silenced for G6PD expression and infected with IV showed an increased expression of influenza viral proteins and a further decrease of GSH relative to control-infected cells. Furthermore, both cells silenced for G6PD showed a reduction in pro-inflammatory cytokines production. Finally, IV infected-A549 cells showed a reduction of the deacetylase SIRT2 expression and its chemical inhibition further reduced G6PD expression while its induction restored G6PD expression and decreased the viral titer.

Conclusion: The results showed a central role of G6PD in contributing to virus-induced redox imbalance and in IV replication in both cell lines. The decrease of SIRT2 expression suggests a possible mechanism by which IV modulates G6PD activity and indicates a possible target for restoring redox equilibrium during IV.

INHIBITION OF HCMV REPLICATION BY SMALL MOLECULES INTERFERING WITH THE DIMERIZATION OF DNA POLYMERASE PROCESSIVITY FACTOR UL44

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Human cytomegalovirus (HCMV) is a leading cause of severe disease in immunocompromised individuals, and in congenitally infected newborns. Despite the availability of several drugs, pharmacological treatment is associated with toxicity and the emergence of resistant strains. Therefore, it is essential to identify new potential targets of therapeutic intervention. One of those is represented by the dimerization of HCMV DNA polymerase processivity factor UL44. Indeed, UL44 plays an essential role in viral replication by tethering the DNA polymerase holoenzyme to the DNA and its dimerization is absolutely required for DNA binding and Ori*Lyt*-dependent DNA replication since point mutations disrupting protein self-interaction also prevent DNA binding and abolish viral replication. The aim of this study is therefore to identify small molecules (SMs) that hinder viral replication by interfering with UL44 homodimerization. To this end, we first validated UL44 crystal structure by a variety of *in vitro* (GST-Pull Down and Thermal Shift) and in cells (Fluorescence and Bioluminescence Resonance Energy Transfer) assays and used it to perform a virtual screening to identify SMs potentially interfering with UL44 homodimerization. Based on cluster analyses and commercial availability, 18 out of 140 identified SMs were tested for their ability to impair replication of the TB4-UL83-EYFP recombinant HCMV by means of Fluorescent Reduction Assay (FRA). Four SMs reproducibly inhibited viral replication in the absence of evident cytotoxicity. Subsequently, MTT and FRA assays allowed to calculate the 50% cellular toxicity (CC50) and effective dose (ED50) relative to each hit. The 3 compounds with the highest selectivity index (ranging from ~5 to ~20) were further tested for their ability to inhibit the replication of an AD169-GFP recombinant virus and a GCV-resistant derivative, resulting in similar ED50. The most active compound inhibited AD169 replication by Plaque Reduction Assays with an ED50 of ~ 15 μM, and specifically impaired expression of late genes, as accessed by Western Blotting assays. Overall, our data suggest that SM-mediated impairment of UL44 dimerization and viral replication could be employed as a valuable therapeutic approach for the treatment of infections caused by drug resistant HCMVs, and the SMs identified here could represent a starting point for the development of new, highly-needed antiviral compounds.

GIVING DRUGS A SECOND CHANCE: REPURPOSING HEPARIN TO PREVENT ZIKA VIRUS-INDUCED CELL DEATH IN HUMAN THREE-DIMENSIONAL (3D) NEUROSPHERES (NS) DERIVED FROM HUMAN INDUCED-PLURIPOTENT STEM CELLS (HIPSC)

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Background: The severe consequences of fetal ZIKA virus (ZIKV) infection have highlighted the need of antiviral agents for the treatment of infected pregnant women. One attractive approach for combating emerging and rapidly spreading infectious diseases is drug repurposing. In this regard, heparin, a safely used anticoagulant in pregnant women, has antiviral activities against attachment and entry of several enveloped viruses. Aim of our study was to explore the effects of heparin on ZIKV replication and cytopathic effects (CPE) in human 3D NS derived from hiPSc.
Methods: NS were incubated for 1 h with heparin and then infected with recent ZIKV strains. Testing the NS supernatant in a Vero cell-based plaque assay assessed viral replication. CPE was determined by measuring the diameter of the spheres and by quantifying the levels of adenylate kinase (AK) activity released in the culture supernatant. As vacuole formation is a hallmark of ZIKV-induced cell death, their presence and number were evaluated by transmission electron microscopy in heparin-treated vs. untreated infected cells.
Results: ZIKV infection significantly reduced the NS diameter as a consequence of ZIKV-induced CPE, whereas heparin restored it to the control value. A significant increase of AK activity was determined in infected cells whereas heparin decreased it to the levels observed in uninfected cells. Moreover, heparin prevented the formation of intracellular vacuoles, a typical feature of paraptosis. Heparin significantly decreased the release of infectious virus by 5-10 fold. Whether this unexpected effect is consequent to interference with virus-induced CPE or whether it is dependent upon a true antiviral effect will need further investigations. Ultimately we are still investigating the role of heparin on ER stress induced by Zika virus infection and which pathway of the unfolded protein response is involved.
Conclusion: Heparin could be potentially exploited as lead compound to discover novel agents for preventing ZIKV replication and virus-induced cell death.

UNRAVELLING THE ROLE OF IL-17 RECEPTORS DURING CHRONIC RESPIRATORY INFECTION BY *PSEUDOMONAS AERUGINOSA*

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Background: The IL-17 pathway, through interleukin(IL)-17 receptor A (IL-17RA), contributes to exaggerated inflammation and tissue damage during chronic lung diseases associated to persistent infections, such as those by *Pseudomonas aeruginosa*. In this context, we aim at dissecting the pleiotropic activities of IL-17RA mediated by the interaction with other IL-17Rs (IL-17RC, IL-17RB, IL-17RE) during chronic lung infection by *P. aeruginosa* in murine models.
Methods: C57BL/6N mice were infected with *P. aeruginosa* embedded in agar beads. The levels of IL-17Rs and IL-17 cytokines (IL-17A, IL-17F, IL-17E, IL-17B and IL-17C) were evaluated by flow cytometry and ELISA respectively. To directly address the contribution of each IL-17R during chronic respiratory infections, three new knockout (KO) murine models for IL-17Rs were generated by CRISPR/Cas9 technology, validated through bioinformatic analysis and western blot, and infected with *P. aeruginosa*.
Results: Long term chronic infection by *P. aeruginosa* is characterized by chronic neutrophilic inflammation (CD11+, GR-1hi) and T cell (CD3+, CD4+) recruitment. We found that overall IL-17 cytokines increased and IL-17Rs are differently expressed among stromal and immune cells in the lung during the development of chronic respiratory infection (2 days and 28 days post-infection). Moreover, we generated new KO murine models for Il17rc, Il17rb, Il17re genes in order to address the mutual contribution of each IL-17R to the host defense. Among Il17rc, Il17rb, Il17re KO mice, preliminary data show that the lack of IL-17RC modulates host resistance in term of bacterial burden and incidence of chronic infection, while the evaluation of immunopathology, is still in progress.
Conclusions: The interaction of IL-17RA with other IL-17Rs may selectively modulate host defense and immunopathology in the lung. Further mechanistic studies on IL-17RC, IL-17RB, IL-17RE in our new KO mouse models might help the design of novel immunotherapeutic strategies limiting exaggerated inflammation and tissue damage during chronic respiratory diseases.

LONGITUDINAL GENOMIC ANALYSIS OF *PSEUDOMONAS AERUGINOSA* AS A TOOL FOR THE DEFINITION OF PERSISTENCE/REINFECTION IN THE AIRWAYS OF CYSTIC FIBROSIS PATIENTS

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Introduction: In cystic fibrosis (CF) patients chronic *Pseudomonas aeruginosa* (Pa) infection is associated with lung damage, a more rapid decline in lung function, and is an important prognostic factor of morbidity and mortality. *Pa* earlier acquisition shortens life expectancy, therefore, attempts to eradicate initial *Pa* acquisition and delay chronic airway infection is crucial for patient care. Aim of this study was to analyse the whole genome sequences (WGS) of *Pa* isolates obtained from a child over a 4 years period in order to define if she was subjected to uncommonly frequent reinfections or if she has acquired an early chronic *Pa* infection.
Materials and Methods: *Pa* isolates (n = 32) were subjected to genomic DNA extraction. Nucleic acid was quantified by Quantus fluorimetric system (Promega) and evaluation of its purity was carried out by Nanodrop (Thermo Scientific). Genomic libraries were prepared using the Nextera XT Flex DNA kit and were run using the Miseq system (Illumina) for the generation of paired-end 2x250bp reads. Raw reads were evaluated on the basis of sequence quality criteria using FastQC. Filtered reads (Trimmomatic software) have been *de novo* assembled through the SPAdes v3.9.0 software. Assembled products were used as input for genetic characterization by comparison with specific databases such as virulence gene databases (Virulent Factors Database, VFDB) and antibiotic resistance (Resfinder). Phylogenetic relationship of the isolates was evaluated using a SNP-based approaches (CFSAN, FDA or kSNP3). SNPs matrix was used as input for phylogenetic analyses performed with the RaxML software that uses Maximum Likelihood (ML) algorithms to determine the relationships between genomic sequences of *Pa* isolates obtained from this study and the ones that are present in databases.
Results: WGS analysis carried out with CFSAN pipeline using PAO1 as reference genome of *Pa* highlighted the presence of two clusters whose isolates differ in about 1000 pairwise SNPs. Within the same cluster, *Pa* isolates had a maxi-

mum of 6 SNPs difference confirming the clonality of different isolates. The main cluster comprises all the *Pa* strains isolated in the period 2015-2017, when the child had two >6-months period of *Pa*-free cultures and some strains isolated in 2018/2019 (cluster I), whereas the cluster II contains only recent strains (years 2018-2019).

Conclusions: Results have shown that, starting the first *Pa* isolation, the child suffered from a chronic infection and that a superinfection occurred some years later. Evaluation of *Pa* clonality by WGS may support studies aimed to determine efficacy of eradication therapies and may help to manage patients for obtaining a better clinical outcomes.

TEMPORAL DYNAMICS OF AIRWAY MICROBIOME IN A CYSTIC FIBROSIS PATIENT CHRONICALLY INFECTED BY *NOCARDIA OTITIDISCAVIARUM*

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Background: In cystic fibrosis (CF) mortality is mostly caused by respiratory impairment due to mucus retention and chronic infections. A wide range of bacteria are described as a cause of CF infections, nevertheless many pulmonary exacerbations occur due to atypical and fastidious bacteria. Microbiome analysis by NGS technologies overcomes culture-based limits and allows to study airways microbiome in detail. Here we used such technology as follow-up tool to evaluate respiratory samples of a CF patient chronically infected by *Nocardia otitidiscaviarum*.
Methods: The bacterial DNA was extracted with the Qiagen kit. The libraries were prepared with the Ion 16S™ Metagenomics Kit and loaded onto the Ion S5. The reads obtained were analyzed by the Ion Reporter™ Software to identify the microorganisms present, using the Greengenes and the MicroSEQ ID 16S rRNA reference database.
Results: *N. otitidiscaviarum* loads and percentages of NGS reads reflected the aggressive therapeutic approach in the earlier samples, while longitudinal evaluation revealed a loss of microbial community diversity throughout the time of observation with an elevated load of *N. otitidiscaviarum* emerging after the settlement of the chronic infection despite the use of antibiotics to control symptoms.
Conclusions: Combination of culture-based methodology and NGS technology allowed to draw an accurate portrait of microbial changes occurred in the airways during the transition from acute to a chronic nocardial infection. An enhanced comprehension of the role of all pathogens in the polymicrobial airway microenvironment will help to design new strategies for CF patients' treatment.

TRACKING DOWN THE PRO-INFLAMMATORY POTENTIAL OF NONTYPEABLE *HAEMOPHILUS INFLUENZAE* IN CHRONIC RESPIRATORY INFECTIONS

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Background: Nontypeable *Haemophilus influenzae* (NTHi) is a bacterium commonly isolated in chronic respiratory diseases, such as chronic obstructive pulmonary disease and cystic fibrosis (CF). However, how far NTHi persistence contributes to the lung inflammatory burden during chronic airway infections remains unclear.
Here, we aim at determining the pathogenic potential of NTHi persistence in a small cohort of CF patients and in a newly generated mouse model.
Methods: The pathogenic potential of NTHi persistence in CF patients in term of cytokines production and lung functionality has been assessed, respectively, by ELISA on respiratory samples and through a retrospective study in CF patients with NTHi colonization or uncolonized by any pathogen. Using C57BL/6N mice, we evaluated NTHi persistence and the associated pro-inflammatory profile (lung cellular infiltrates and cytokines production) during the development of chronic infection at 2 and 14 days post-infection.
Results: In our study cohort, we found that CF patients chronically colonized by NTHi had significantly higher levels of IL-8 and CXCL1 than those uninfected. The reduction of lung function (%FEV_1) after two year was greater among NTHi colonized patients in comparison to those uncolonized.
In mice, we successfully induced long term NTHi infection with sustained bacterial load up to 14 days post challenge. NTHi persistence was associated with lung chronic inflammation, characterized by recruitment of neutrophils and cytokine release (KC, MIP-2 and IL-6) at 2 and 14 days post infection. Moreover, we observed an increased burden of T cell mediated response ($CD4^+$, $CD8^+$ and γδ cells) associated with tissue remodeling marker (pro-MMP9) at 14 days post-infection.
Conclusions: Our results demonstrate the pathogenicity of NTHi persistence in the human and murine lung. Thus, we propose NTHi as an active player of lung immunopathogenesis in chronic respiratory diseases.

CHARACTERIZATION OF THE ACTIVATION PROCESS THAT INDUCES AN INCREASE IN THE EXPRESSION OF THE SIGMA FACTOR σE IN *MYCOBACTERIUM SMEGMATIS* UNDER SURFACE STRESS CONDITION

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Background: σE is one of the 13 sigma factors encoded by the *Mycobacterium tuberculosis* chromosome, it is engaged in a very complex regulatory network involving other regulators as the two component system MprAB and the pleiotropic regulator ClgR, and its role is of prime importance for physiology and virulence.
Methods: A series of *Mycobacterium smegmatis* mutants were constructed and characterized in order to understand the SigE regulatory network. we used mutant strains missing *mprAB* and *clgR* to understand the effective role of the two component system MprAB and the transcriptional regulator ClgR in SigE-mediated surface stress response. Through real-time PCR analysis the dynamic of transcription of several *SigE* dependent genes was studied. Specifically, we evaluated the transcriptional level at different time points of *sigE* itself, *sigB* that is induced by *sigE* both at physiological and stressed condition, *rseA*, the σ^E-specific anti sigma factor, and *clpP2*, coding a protease responsible for the degradation of the SigE-RseA complex. We simulated surface stress conditions by the exposure to sodium dodecyl sulfate (SDS) 0.05% that is an anionic detergent able to modify bacterial surface inducing the denaturation of membrane proteins.
Results: The data strongly support the fundamental role of both MrpAB and ClgR to act out an efficient stress response indeed their presence is necessary to induce the expression of *sigE*. The effect of SigE activity could be seen in the transcriptional dynamic of *sigB*, that requires the two component system to be induced and ClgR to be substained during time. The absence of MprAB led to the absence of stress response, while the lack of ClgR resulted in a ready but partial activation of the *sigE* network.
Conclusion: In this work, we started the characterization of the activation process that induces an increase in the expression of the sigma factor $\boldsymbol{\sigma E}$ in *M. smegmatis* under surface stress condition. This regulatory network is assumed to be very similar to that of *M. tuberculosis* so this data will be early corroborate in pathogenic strain.

THE NUCLEOTIDE EXCISION REPAIR (NER) IN *MYCOBACTERIUM TUBERCULOSIS*: BIOCHEMICAL AND STRUCTURAL STUDIES

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***Summary**:* Mycobacterium tuberculosis *(MTB) is a species of pathogenic bacteria and the causative agent of tuberculosis. During the infectious process MTB is exposed to many types of DNA-damaging agents. MTB has efficient DNA repair systems (like the NER system), that are believed to play essential roles in pathogenesis.*

***Key words**:* Mycobaterium tuberculosis*, DNA repair, NER*

Introduction: *Mycobacterium tuberculosis* (MTB) is a human pathogen that during its complex life cycle undergoes to different types of DNA-damaging insults posed by host immune system and antibiotic treatments. During its infection, MTB resides in the host macrophages and is continuously exposed to a variety of DNA damaging stresses by endogenous and esogenous factors. Genome integrity and stability are essential for the survival and proliferation of all organisms. In addition to its biological significance, it has now been realized that DNA repair has a role in genome diversification and consequently the development of drug resistance in mycobacteria [1, 2].
During the evolution, mycobacteria have evolved different DNA repair mechanisms to contrast the different nature of stress that the bacteria counteract. In particular, the Nucleotide Excision Repair (NER) toolbox is a multi-enzyme system, highly conserved, which is composed of four core proteins UvrA, UvrB, UvrC and UvrD, that operate in a concerted manner [3, 5]. The first step of the reaction are carried out by the coordinated action of the UvrA, UvrB and UvrC proteins which represent the core of the complex. To better understand the features of the core complex we performed biochemical and structural analysis at a single-protein and in-complex level.
Experimental: We purified the proteins related to the sensor NER complex, MTB UvrA and UvrB (*Mt*UvrA, *Mt*UvrB); we analyzed the behavior in solution and the features of MtUvrB and the sensor complex by SAXS (Single Angle X-ray Scattering) analysis[6]. Moreover we performed an analysis of the sensor complex *Mt*UvrA-UvrB with a SEC (Size Exclusion Cromatography) based approach. Furthermore to better understand the biochemical properties of the complex archetiture, we performed a Surface plasmon resonance analysis to obtain the cinetic constant of the complex.
Results: We analyzed the hydrodynamic properties and the oligomeric state of *Mt*UvrB, by SAXS analysis, showing that the protein assumes a dimeric form in solution, which are characterized by an elongated shape. Furthermore, using

a SEC-based approach, we analyzed the *Mt*UvrA-UvrB sensor complex, observing that the two proteins interact in solution, in absence of ligands, with an A2B2 stoichiometry. Surface plasmon resonance analysis showed that the dissociation constant of the complex falls in the low micromolar range that could represent the basis for a fine modulation of the complex architecture.
Conclusions: Deciphering the molecular aspects of the NER system of MTB is important to better understand the mechanism that underlies one of the most important DNA repair system that mycobacteria use during the infection. Considering that the interdiction of different MTB responses to oxidative stresses, which are constantly present in its life cycle, the inhibition of NER system could be as target for drug discovery for potential antitubercular treatments.

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INHIBITION OF TRANSGLUTAMINASE 2 AS A POTENTIAL HOST-DIRECTED THERAPY AGAINST *MYCOBACTERIUM TUBERCULOSIS*

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Introduction: Host-directed therapies (HDTs) are emerging as a potential valid support in the treatment of drug-resistant tuberculosis (TB). Following our recent report indicating that genetic and pharmacological inhibition of transglutaminase 2 (TG2) restricts *Mycobacterium tuberculosis* (*Mtb*) replication in macrophages, we aimed to investigate the potentials of the TG2-inhibitors cystamine and cysteamine as HDTs against TB.
Materials and Methods: To investigate whether cysteamine and cystamine had an anti-microbial activity against *Mtb* in macrophages, THP-1 monocyte-derived macrophages and in primary human monocyte-derived macrophages (hMDM), were infected with *Mtb* H37Rv and then treated with cystamine and cysteamine at concentrations compatible to those that achieved *in vivo*. We previously showed that genetic inactivation of TG2 in murine macrophages results in the impairment of the LC3/autophagy homeostasis, which nevertheless correlates with the restriction of *Mtb* intracellular replication. To further investigate the impact of the two TG2 inhibitors cystamine and cysteamine on autophagy, we quantitatively evaluated the autophagic flux by confocal pH-imaging of the autophagic intermediates on THP-1 cells transfected with mRFP-GFP-LC3B. To analyze the activity of these drugs as HDTs against TB, we infected human peripheral blood monocyte cells (PBMCs) with *Mtb*, evaluating the effect on granuloma-like structures (GLS).
Results: We showed that both cysteamine and cystamine restricted *Mtb* replication in infected macrophages when provided at equimolar concentrations and did not exert any antibacterial activity when administered directly on *Mtb* cultures. Interestingly, infection of monocyte-derived THP-1 mRFP-GFP-LC3B

cells followed by the determination of the autophagic intermediates pH distribution (AIPD) showed that cystamine inhibited the autophagic flux while restricting *Mtb* replication. Moreover, both cystamine and cysteamine had a similar antimicrobial activity in primary macrophages infected with a panel of *Mtb* clinical strains belonging to different phylogeographic lineages. Evaluation of cysteamine and cystamine activity in the human ex vivo model of granuloma-like structures (GLS) further confirmed the ability of these drugs to restrict *Mtb* replication and to reduce the size of GLS.
Discussion and Conclusions: The antimicrobial activity of the TG2-inhibitors synergized with a second-line anti-TB drug as amikacin in human monocyte-derived macrophages and in the GLSs model. Overall, the results of this study support the potential usefulness of the TG2-inhibitors cysteamine and cystamine as HDTs against TB.

SYNTHESIS AND MICROBIOLOGICAL EVALUATION OF NOVEL ASCORBIC ACID CONJUGATES AS BIOFILM INHIBITORS ACTIVE AGAINST *PSEUDOMONAS AERUGINOSA*

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Background: *Pseudomonas aeruginosa* is a Gram-negative ubiquitous bacterium, that mainly colonizes lungs of patients with cystic fibrosis. This event leads to chronic infections due to the excessive accumulation of pulmonary mucus and also to the ability of this type of bacteria to be organized in biofilm. Biofilm is represented by a huge aggregation of bacteria colonies, settled down by a polysaccharide matrix. Biofilm formation is regulated by the "Quorum Sensing" (QS), a communication system between bacteria, self-induced by 3O-C12-HLS (Fig. 1) and its receptor LasR. For these reasons the QS inhibition could represent a good target to avoid the biofilm formation.

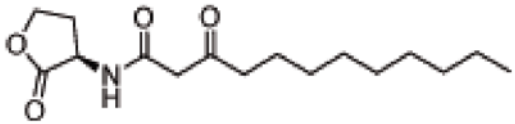

Figure 1. 3O-C12-HSL structure

Methods: Taking as reference compounds two derivatives already reported as inhibitors of the QS pathway through LasR binding, we decided to synthesize a series of analogues to increase water solubility and to improve the chemical stability. In particular, we decided to investigate how the cyclic and the linear portion contributes to the activity of LasR inhibition.
Results: We have modified the lactone moiety with a cyclopentylaminic one (to avoid the catabolism induced by lactonases) and the linear carbon chain with a polyoxyethylenic one (to improve the hydrophilicity). We also decided to conjugate our compounds to the ascorbic acid, to further increase the water solubility.
Based on these considerations, we have obtained PF28 (Fig. 2), a compound that is able to reduce up to 50% biofilm formation at 0.5 μM concentration (as demonstrated by crystal-violet assay and Syto 9 assay) and with no toxic effects on eukaryotic cells (A549; MRC5), tested by MTT assay (Sigma Aldrich).

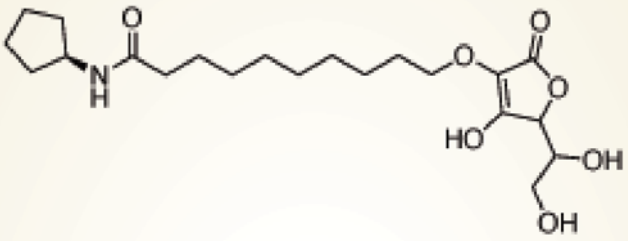

Figure 2. PF28 structure

Conclusions: We identified a compound that is able to reduce the formation of biofilm in *P. aeruginosa* clinical strains. This compound could have a wide application in clinical setting. The possibility to affect biofilm formation in chronically infected patients, such as Cystic Fibrosis patients would improve patient healing and allow to decrease antibiotic drug dosage.

CHARACTERIZATION OF FECAL AND ORAL MICROBIOTA ASSOCIATED WITH LYNCH SYNDROME

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Introduction: The hereditary non-polyposis colorectal cancer (HNPCC), also called Lynch syndrome, is a type of inherited cancer syndrome associated with a genetic predisposition to different cancer types, including colorectal cancer (CRC), endometrium cancer, stomach cancer, breast cancer and several others. It is an autosomal dominant condition associated with germline alterations of genes related to the DNA mismatch repair process (MLH1, MSH2, MSH6, PMS2) and with an estimated prevalence of 1:300 in the general population. In recent years, the development of next-generation sequencing has allowed us to study the composition of the microbiota in different body districts and to investigate its possible role in the onset and development of different types of cancer, including CRC. However, there are still no relevant studies that have investigated its possible involvement in Lynch syndrome-related increased risk of cancer. In this study, we performed the microbiome analysis of fecal and salivary samples of patients affected by Lynch syndrome and their relative age and sex matched controls.
Materials and methods: Total DNA was purified from fecal (16) and salivary (29) samples of patients with Lynch syndrome and matched controls (20 and 34 respectively) using specific DNA extraction kit, and subsequently, from the total DNA, the V3-V4 region of the 16s rRNA gene was amplified. The obtained amplicons were purified using the AMPure XP beads and then a second PCR step was performed in order to attach dual indices and Illumina sequencing adapters to each sample. After a second purification step, the DNA was quantified and pooled in order to obtain a library that was loaded on the MiSeq Illumina system. Sequences with high quality score and length >250bp were used for the taxonomic analysis with QIIME (Quantitative Insights Into Microbial Ecology v1.9.1) software.
Results: In fecal samples, it was not possible to evidence any statistically significant difference between Lynch syndrome patients and their relative controls in alpha and beta diversity indexes. We observed a statistical significant difference in the relative abundance of *Bifidobacterium*, increased in the patients compared to controls (2,09% vs 0,14%), and the family of Lachnospiraceae,

decreased in the patients compared to controls (5,83% vs 9,55%). In salivary samples we found a statistically significantly lower alpha diversity in Lynch syndrome patients. Moreover, the unweighted beta diversity allowed to distinguish the two populations of patients and controls. However, it was not possible to evidence any statistically significant difference in the relative abundance of bacterial taxa between patients with Lynch syndrome and controls.
Conclusions: This is the first study that analyzes the microbiota composition of fecal and salivary samples obtained from patients with Lynch syndrome and their relative age and sex matched controls. The results obtained so far do not show relevant microbiota variations that can allow us to speculate a role of the microbiota in the onset of the disease, which results to be highly influenced by genetic factors. These results in such a specific set of patients highlight the need to always correlate microbiome data to known genetic factors when addressing CRC.

CLONAL CONTAMINATION OF LAYING HENS FARM BY *SALMONELLA ENTERICA* SEROVAR ENTERITIDIS RESPONSIBLE FOR A MULTI-ANNUAL OUTBREAK DEMONSTRATED THROUGH PHYLODYNAMIC RECONSTRUCTION

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Background: In Emilia-Romagna region (Northern Italy), a laboratory surveillance of human salmonellosis based on PFGE/MLVA (and WGS for outbreak confirmation) is in place since 2012. An outbreak caused by *Salmonella enterica* serovar Enteritidis (SE) with 12 human cases was detected in the fall 2016. The epidemiological investigation identified a laying hens farm as the outbreak source and showed the presence within the farm of a contamination by SE with the PFGE/MLVA outbreak profile dating back to 2009. Seventeen additional human cases with the outbreak profile were retrospectively identified within the surveillance database in 2012-2017. Evolutionary analysis based on phylodynamic reconstruction was used to confirm the enduring contamination of the farm and the presence of a long-lasting outbreak.
Materials/methods: SE isolates from human cases and from the source farm were subjected to WGS. Specifically, 29 human isolates and 15 farm-associated isolates (five sampled in 2009-2010 and ten in 2016) were sequenced. SNP-based phylogenetic analysis and phylodynamic reconstruction with molecular clock models in a Bayesian framework were performed to confirm or exclude their clonality. Statistical analyses on the phylodynamic reconstruction were performed to test the robustness of the observed evolutionary signal against date-randomized datasets (DRDs).
Results: Phylogenetic analysis highlighted the presence of a unique genomic cluster including all human and farm isolates. However, SNPs differences within the cluster (up to 16 SNPs) were higher than those usually observed in SE outbreaks. By using a phylodynamic approach, we found that the cluster phylogenetic history can be suitably described through a molecular clock model showing a significant evolutionary signal. By comparing the phylodynamic reconstruction against DRDs, we estimated a very small effective population size confirming the clonal origin of the infection. We found a high level of population drift suggesting the presence of high selective pressure within the source of infection.
Conclusions: Our study suggests that cut-offs based on SNP thresholds, usually implemented to define Salmonella outbreak borders, cannot represent a suitable tool to identify long-lasting outbreaks and persistent clones in herds and food facilities. In these cases, it is critical to complement SNP counts with clustering generated by phylodynamic analyses accounting for the time-span elapsed among isolates.

STUDY OF COLISTIN RESISTANCE RELATED GENES IN *KLEBSIELLA PNEUMONIAE* CLINICAL ISOLATES USING NEXT GENERATION SEQUENCING (NGS) APPROACHES

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Background: Over the last decades, carbapenemase-producing *K. pneumoniae* have become a major health concern worldwide. Colistin is often used as last line treatment for such multi-drug resistance (MDR) bacteria. Colistin is a cationic peptide targeting bacteria membrane and causing cell leakage. Modifications of lipopolysaccharide (LPS) charge can reduce susceptibility to colistin in *Enterobacteriaceae*. Several proteins including *PhoP/Q* and *PmrA/B* systems and *mgrB* transmembrane protein are involved in the molecular mechanism responsible for resistance to colistin. These proteins are involved in regulation of LPS phosphorylation pathway and they are encoded by chromosomal genes. The aim of this study is to understand and investigate chromosomal gene alterations and mutations associated with colistin resistance in MDR *K. pneumoniae* isolates by coupling the use of second and third generation of Next Generation Sequencing (NGS) technologies followed by *in silico* analysis.
Methods: 12 MDR *K. pneumoniae* strains were collected from 10 patients in San Raffaele hospital, Milan. *MgrB*, *Pho* and *Pmr* systems were sequenced using Sanger sequencing. To analyze the whole genome of *K. pneumoniae* we coupled the use of second and third generation NGS techniques. The obtained data were analyzed using specific bioinformatic tools allowing both *de novo* assembly of NGS data for *whole genome sequencing* and study of polymorphisms possibly associated to colistin resistance.
Results and Conclusions: Bioinformatic analysis revealed several modifications in *mgrB, PmrA, PmrB, PhoP* and *PhoQ* such as deletions, insertions and substitutions that can alter the function of these proteins, and finally that can lead to colistin resistance. In the details, *in silico* analysis revealed that modified mgrB gene is present only in colistin resistant *K. pneumoniae* strains.
Further *wet* studies will be performed to validate the role of observed *mgrB* mutations in colistin resistance. Moreover, the use of different generations NGS techniques allowed both whole genome sequencing and high accuracy identification of single polymorphisms.

DIAGNOSTIC ACCURACY OF TOXOPLASMA WESTERN BLOT TEST IN SUSPECTED SEROCONVERSION IN PREGNANCY: A MULTICENTRIC STUDY

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Background: Due to the high sensitivity, the automated screening tests widely employed for *Toxoplasma gondii,* serology can yield false positive results due to cross and/or non-specific reaction. On the other hand, therapy with spiramycin, given most of the time, when IgM are found to be positive, could delay IgG production and mask seroconversion. There is the need of second level test to early detect real toxoplasmic seroconversions.
We planned an European multicentre study with 4 reference center for the diagnosis of toxoplasmosis: (Pitié-Salpétrière Paris, CHU Grenoble-Alpes Grenoble, La Timone Marseille, IRCCS Policlinico San Matteo Pavia) to evaluate the diagnostic accuracy of the new LDBIO-TOXO II IgG/IgM Immunoblot (IgG-IgM WB) by LDBio Lyon - France.
We also looked at the immunodominant antigen in the IgM WB.
Methods: We retrospectively analysed the leftover of laboratory samples stored after clinical diagnosis of 403 sera: 234 corresponding to 96 toxoplasmic seroconversions (2 to 3 sera/patient) and 169 sera corresponding to 69 patients with cross reactions and/or non-specific IgM (1 to 3 sera/patient). All the patients had a documented seroconversion with first IgG /IgM negative results and then either IgG /IgM positive or a false positive result. All anonymised samples were processed in blind by LDBio.
To validate WB we performed two different analyses: concordance (Cohen's kappa) with final diagnosis (seroconversion or false positive) and diagnostic accuracy (sensitivity, specificity etc).
Results: The concordance between IgM and IgG type II WB with the diagnosis was good K= 0,89 and K= 0,89, respectively. In 4 cases the appearance of IgM and 46 cases the appearance of IgG was recorded by WB before the traditional tests. Sensitivity was 100% for IgM WB and 93,8% for IgG WB. Specificity was respectively 87% and 95.7%.

Looking at the most antigenic bands, P30 was recorded in all but one positive sample and P40 in all but five.
Conclusion: The IgM WB not only detected all seroconversions, and even earlier than traditional tests in four cases, but also well discriminated the false positive results.
The definition of immunodominant band will be extremely helpful in the interpretation of the results.

CONGENITAL TOXOPLASMOSIS: OUTCOMES OF NEWBORNS FROM MOTHERS WITH DOCUMENTED SEROCONVERSION OUT OF A MULTICENTER COHORT IN TWO TERTIARY REFERRAL HOSPITALS

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Inytroduction: Primary *Toxoplasma gondii* infection during pregnancy can cause congenital toxoplasmosis (CT) and documented seroconversion (SC) is the only serological evidence of such event. The aim of our study was to describe the newborns outcomes from a multicenter cohort of seroconverted mothers.

Methods: We retrospectively reviewed all *T. gondii* SCs among pregnant women referred to Brescia (Spedali Civili) and Pavia (IRCCS Policlinico San Matteo). We started the data collection from SCs reported in January 2007. We included all pregnancies with estimated/documented delivery at June 2018. Serologic data, amniocentesis results, neonatal outcome, antibiotic treatments and characteristics of CTs were recorded.

Results: 247 pregnant women were included: 12 with periconceptional infection and 235 with documented SC. Our cohort included 3 twin pregnancies with a total of 238 newborns expected. We could not retrieve the pregnancy outcome in 23.5% cases. The observed transmission rate was 25.3%: 46.7% among the 3rd trimester SCs, 8.8% among 2nd trimester SCs and 0/38 among the 1st trimester ones. Among the infected fetuses, 12/46 had clinically apparent CT (26.7%) 11 live births (5 with ocular and 7 with CNS localization – 1 newborn with CNS and retina disease localization) and 1 terminated fetus. The clinically apparent CT rate considering SC trimester epoch was 20% among the 3rd trimester SCs and 45.5% among the 2nd trimester ones. Two miscarriages and 1 more termination of pregnancy were recorded without any information about fetuses infection status. All the infected newborns were treated with 1 year of pyrimethamine-sulfadiazine.

Spiramycin was the mother's first choice treatment (89.8%) and in only 3 cases it was interrupted because of side effects. Six women did not receive treatment and for 4 subjects we didn't have any records.
Amniocentesis was performed in 83/235 cases (35.3%), no complication was recorded and no false positive or false negative results were registered. We did not find any CT among periconceptional infections.
Conclusions: The results are in line with the fetal risks reported in literature for *T. gondii* infection during pregnancy. The treatment was promptly initiated after SC evidence but the delay is longer if we consider the interval from the estimated time of infection. This difference and the high number of missing data on SC outcomes show how is still difficult to guarantee an efficient multidisciplinary management of such complicated pregnancies.

Author Index

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