ISTITUTO PASTEUR ITALIA
FONDAZIONE CENCI BOLOGNETTI

2019 Annual Report

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FOREWORD

The **Istituto Pasteur Italia - Fondazione Cenci Bolognetti**, the italian member of the Institute Pasteur International Network (32 institutes worldwide), is a private *non-profit* foundation established according to the terms of the bequest of princess Beatrice Cenci Bolognetti with the purpose to create a Center of Biomedical Research with the same mission and values of *Institut Pasteur* in Paris.

The **Istituto Pasteur Italia** research activity is committed to biomedicine, with particular references to infectious diseases, drug design, molecular medicine extended to innovative therapies (e.g. cancer immunotherapy; therapy of genetic diseases; regenerative medicine). The **funding of research projects** is possible thanks to the income from the donated real estates and thanks also to donations from citizens. In 2019 the Institute has invested a total of 754.000 euros to fund **high level research projects in different areas** (microbiology, virology, molecular genetics, molecular biology, cellular and molecular immunology as well as biology of malaria and of food-borne diseases) and to support young researchers with **fellowships** (i.e. and PhD courses to have experience abroad and to return in Italy).

Istituto Pasteur invested in the Research Projects on I**mmunotherapies for Cancer and Infectious Diseases** carried on at **Laboratorio Pasteur Italia,** at its fifth year of establishment and directed Dr. John Hiscott. It also continue to support the unit supervised by Dr Stefania Uccini carrying out activities to improve the quality of tumor diagnosis of Iraqi children and supporting scientific education of the Baghdad's Hospital medical staff members.

The 2019 also saw the beginning of 13 new research projects that were funded to be carried out in affiliated laboratories of Sapienza University Departments. The funded research projects were selected through an international peer review process thus ensuring **scientific excellence**. The researches focus on the study of therapies for the treatment of infectious diseases, genetic diseases, cancer and neuromuscular degenerative disorders.

Istituto Pasteur Italia also carried on still ongoing **collaborations with the International Network of Pasteur Institutes** (32 Institutes worldwide): such as the "Seed International research Projetcs" (funded by Istituto Pasteur Italia); the *Programmes Transversaux de Recherche*, the *Actionnes Concertée International Pasteuriennes* and the *Grand Programme Fedéreaux* – all funded by the Institut Pasteur of Paris. These Programs bring together researchers, engineers and technicians within the Paris campus and the International Network and work towards a shared discovery research goal, also to develop synergy and allow further collaborations.

The scientific excellence reached over 2019 is demonstrated by high quality publications in peer-reviewed scientific journals, for a cumulative impact factor: 854,912 (the number is the result of 2019 publications derived by the mentioned first year projects as well as by studies funded by the Institute in the past years).

Last but not least, the Institute has as always also been active in promoting **educational programs** and **scientific communication.** First and foremost we hosted the **V International Course on Viruses and Human Cancers** organized in collaboration with the Pasteur Institute of Paris. Moreover, the Institute has carried on the well-established **dissemination project** for secondary schools involving students of Rome, Civitavecchia, Monterotondo, Caserta, Lecce and Corfu (Greece) and published a book about antimicrobial resistence - one of the most urgent health threats of our time - coupling Science with Comics.

From 12 to 15 June 2019, the Pasteur Italia Institute hosted and coordinated the activities of STAPA International Retreat - in collaboration with Young Italian Scientists Of Pasteur and YouPi (Young researchers of the Hellenic Pasteur Institute).
Every year StaPa (Association for Young Researchers of the Institute Pasteur in Paris) organizes and promotes retreats to encourage young researchers to present their work and share their own experiences and opinions on research.
These 26 young researchers from the Pasteur Italia Institute - Cenci Bolognetti Foundation and "Sapienza" University of Rome, 32 young researchers from the Institut Pasteur in Paris and 7 young researchers from the Hellenic Pasteur Institute, have shared scientific and social aspects through a rich program of conferences and meetings, thus creating an interdisciplinary and international knowledge network.

This Annual Report documents the results obtained during the year 2019 thanks to the enthusiasm and the effort of the Italian “Pasteur” community.

Luigi Frati
President

Angela Santoni
Scientific Director

RESEARCH PROJECTS

1. **LABORATORI PASTEUR ITALIA**

- **Immunotherapies for cancer and infectious diseases (PI: J. Hiscott)**
- **Diagnosis for *EBV-related lymphoproliferative diseases* (PI: S. Uccini)**

2. **SAPIENZA UNIVERSITY (IPI AFFILIATED LABORATORIES)**

- **"Anna Tramontano" Research Projects**
- **Under 45 Research Projects**
- **"Teresa Ariaudo" Research Projects**

3. **COLLABORATIONS WITHIN THE INTERNATIONAL PASTEUR NETWORK**

- **Seed International Research projects (funded by IP Italia)**
- **Research Projects funded by IP Paris (PTR; ACIP and GFP)**

4. **SCIENTIFIC BOARD RESEARCH PROJECTS**

IMMUNOTHERAPIES FOR CANCER AND INFECTIOUS DISEASES

Director of Research: John Hiscott

IMMUNOTHERAPIES FOR CANCER AND INFECTIOUS DISEASES

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This research program has focused for a number of years on the early innate immune events involved in the host response to RNA virus infection, with the long-term objective to utilize knowledge of the immune response against virus infection to develop novel immunotherapeutic approaches for the treatment of infectious diseases and cancer. Our goal is relevant for the translational development of novel antiviral and adjuvant compounds to augment immunity against diverse viral pathogens, including influenza, dengue, and chikungunya. This objective is also important for the development of oncolytic virus therapies for cancer, since defects in innate antiviral signaling in tumor cells contribute to the selective growth of replicating oncolytic viruses in cancer versus normal tissues. Below the main research themes of the laboratory are summarized.

1. Oncolytic virotherapy of pancreatic cancer

Oncolytic virotherapy represents a promising experimental cancer strategy, based on the use of genetically modified viruses to selectively infect and kill cancer cells. Vesicular stomatitis virus (VSV) is a prototypic oncolytic virus that induces cancer cell death through activation of the apoptotic pathway, although intrinsic resistance to oncolysis is found in some cell lines and many primary tumors, as a consequence of residual innate immunity to the virus. In the effort to improve OV therapeutic efficacy, we previously demonstrated that different agents, including histone deacetylase inhibitors (HDIs), functioned as reversible chemical switches to dampen the innate antiviral response and improve the susceptibility of resistant cancer cells to VSV infection. In recent studies, we demonstrated that the NAD+-dependent histone deacetylase sirtuin 1 (SIRT1) plays a key role in the permissivity of prostate cancer PC-3 cells to VSVΔ51 replication and oncolysis. HDI-mediated enhancement of VSVΔ51 infection and cancer cell killing directly correlated with a decrease of SIRT1 expression. Furthermore, pharmacological inhibition as well as silencing of SIRT1 by siRNA was sufficient to sensitize PC-3 cells to VSVΔ51 infection, resulting in augmentation of virus replication and spread. Mechanistically, HDIs such as Vorinostat and Resminostat up-regulated the microRNA miR-34a that regulated the level of SIRT1. Altogether, our findings identify SIRT1 as a viral restriction factor that limits VSVΔ51 infection and oncolysis in prostate cancer cells. *(J. Virology, 2019).*

With the support of Associazione Italiana Ricerca sul Cancro (AIRC, 2020 – 2024), as well as a Fondazione Veronesi Fellowship awarded to Dr. Evelyne Tassone, we are now beginning a new and challenging project; we will investigate the potential of a new recombinant VSV expressing miR-34a, designed to increase sensitivity of tumor cells to

OV therapy, together with immune checkpoint blockade therapy (anti-PD1), in the experimental treatment of pancreatic ductal carcinoma (PDAC), one of the most deadly forms of cancer. These studies are based on the rationale that a combination of oncolytic viro-immunotherapy may represent a promising experimental strategy for the following reasons: 1) VSV has been shown to effectively kill the majority of human PDAC cell lines, both *in vitro* and *in vivo*, although cell lines that retained functional type I IFN responses were resistant to VSV replication and oncolysis; 2) improvements in cancer immunotherapies have shown promising results for many malignancies but to date these advances have not extended to pancreatic cancer using single-agent immune checkpoint blockade, such as anti-PD-1 and anti-CTLA-4; and 3) the failure of single agent immunotherapy is likely attributable to multiple mechanisms of immune escape - low tumor antigenicity, impaired antigen recognition, an immunosuppressive tumor microenvironment (TME) and a small number of tumor infiltrating lymphocytes (TILs) in the dense stromal architecture - all combining to maintain a non-inflamed, non-immunogenic tumor phenotype in PDAC. These observations provide a rationale for the combined use of OV therapy and checkpoint inhibitors to convert a non-inflamed tumor environment into an inflamed site permissive for checkpoint inhibitor therapy. This project will therefore investigate how oncolytic rVSVΔ51-miR34a changes host immunity and the tumor-microenvironment to permit successful immunotherapy with anti-PD-1. This knowledge will be used to design logical combinations with other immunotherapies that will be tested in a selection of immunocompetent murine tumor models.

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2. Sequence-optimized RIG-I agonist M8 induces immunogenic cell death of cancer cells and dendritic cell activation

RIG-I is a cytosolic RNA sensor that recognizes short 5' triphosphate RNA, commonly generated during virus infection. Upon activation, RIG-I initiates antiviral immunity, and in some circumstances, induces cell death. Because of this dual capacity, RIG-I has emerged as a promising target for cancer immunotherapy. In previous studies, a sequence-optimized RIG-I agonist (termed M8) was identified for its ability to stimulate a robust innate immune response capable of blocking viral infection and functioning as an adjuvant in vaccination strategies of influenza antigens. During the past year, we demonstrated the potential of M8 as an anti-cancer agent by analyzing its ability to both induce cell death and activate the immune response. In multiple cancer cell lines, M8 treatment strongly activated caspase 3-dependent apoptosis. Cell death induced by M8 was characterized by the expression of the immunogenic cell death markers - calreticulin and HMGB1 - as well as high levels of CXCL10, a marker of inflammation. M8 induction of the RIG-I pathway in cancer cells favored dendritic cell phagocytosis and induction of co-stimulatory molecules CD80 and CD86, together with increased expression of IL12 and CXCL10. Altogether, these results highlight the potential of RIG-I agonist M8 for cancer immunotherapy, by inducing immunogenic cell death and activating immunostimulatory signals that can synergize with current therapies *(Cancer Immunology & Immunotherapy, 2019).* Preliminary experiments further suggest a

synergistic effect of RIG agonists with immune checkpoint inhibitors. With its potential as an antiviral and anti-cancer agent, several patents have been awarded for RIG-I agonist use; in 2019, an additional patent was filed by Istituto Pasteur Italia to protect the use of M8 as an anti-cancer compound.

3. Activation of latent HIV-1 T cell reservoirs with a combination of innate immune and epigenetic regulators

The presence of T cell reservoirs in which HIV establishes latency by integrating into the host genome represents a major obstacle to a HIV cure and has prompted the development of different strategies aimed at eradication of HIV from latently infected cells. The "Shock and kill" strategy is one of the most pursued approaches directed towards the elimination of viral reservoirs; although several Latency-Reversing Agents (LRAs) have shown promising reactivation activity, they have failed to eliminate the cellular reservoir. Here, we evaluated a novel immune-mediated approach to clear the HIV reservoir, based on the combination of innate immunity stimulation and epigenetic reprogramming. The combination of the STING agonist cGAMP and the FDA-approved histone deacetylase inhibitor Resminostat resulted in a significant increase in HIV proviral reactivation and specific apoptosis in HIV-infected cells *in vitro*. A reduction in HIV-harboring cells was also observed in CD4+ T central memory (T_{CM}) cells in a primary cell model of latency, where Resminostat alone or together with cGAMP induced high levels of selective cell death. Finally, high levels of cellular-associated HIV-RNA were found in PBMCs obtained from individuals on suppressive ART treated with Resminostat or cGAMP, although no synergistic effect was detected with the combination. Collectively, these results represent a promising step towards HIV eradication by demonstrating the potential to reduce the viral reservoir and induce specific death of HIV-infected cells. *(J. Virology 2019).*

4. Metabolic regulation of the interferon antiviral response during Dengue virus infection of dendritic cells

Dengue virus (DENV), the leading arthropod-borne viral infection in the world, infects more than 300 million people worldwide, leading to 50,000 deaths annually. While the parameters contributing to dengue immunopathogenesis remain unclear, the collapse of redox homeostasis and damage induced by oxidative stress has been correlated with the development of chronic inflammation and progression towards the more severe forms of disease. Previously, we identified a critical role for the transcription factor NF-E2-related factor 2 (Nrf2) in the negative regulation of both antiviral and inflammatory gene responses to DENV infection. In the present study we demonstrate that the accumulation of reactive oxygen species (ROS) late after DENV infection of primary human monocyte-derived dendritic cells resulted from a disruption in the balance between oxidative stress and the Nrf2-dependent antioxidant response. The DENV protease complex NS2B3 strategically targeted Nrf2 for degradation in a proteolytic-

independent manner; rather NS2B3 licensed Nrf2 for lysosomal degradation. Impairment of the Nrf2 regulator by the NS2B3 complex inhibited the antioxidant gene network and contributed to the progressive increase in ROS levels, along with an increased virus replication and inflammatory or apoptotic gene expression. Interestingly, similar alterations were identified in the transcriptome profiles of healthy versus DENV-infected monocytes from a cohort of Brazilian patients; expression of Nrf2-dependent genes was decreased, whereas inflammatory cytokines and apoptotic markers were increased in patients with DF and severe DF compared to healthy controls. Metabolic re-programming of the antioxidant response during DENV infection potentially establishes metabolic conditions for ROS accumulation and oxidative stress that aggravates DENV pathogenesis. Collectively, these data identify that Nrf2 and the anti-oxidant gene network as important regulators of the innate antiviral and inflammatory response, and as a target for DENV-mediated metabolic re-programming of the host response to infection. *(Nature Microbiol., submitted).*

Beginning in May 2019, Istituto Pasteur Italia launched a new EU Horizon 2020 project, INITIATE (innate-immunometablism as an antiviral target), an Innovative Training Network funded by a Marie Skłodowska-Curie Action grant of the European Commission. The INITIATE network will investigate the interaction between pathogenic viruses, the human immune system and host metabolism at the cellular level. The ultimate goal is to develop new strategies to treat and control serious human viral infections. Ten European leading research institutes and three pharmaceutical companies established the consortium and together they structured a research network that includes 15 newly recruited, international PhD students. In addition, four partner organizations joined the project to provide further state of the art scientific expertise, training and education. The project is coordinated by Dr. B. van den Hoogen of the Department of Viroscience at Erasmus MC, Rotterdam in The Netherlands and Prof. J. Hiscott of Istituto Pasteur Italia in Rome.

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A COLLABORATIVE WORK TO IMPROVE THE QUALITY OF TUMOR DIAGNOSIS OF IRAQI CHILDREN AND TO SUPPORT SCIENTIFIC EDUCATION OF THE MEDICAL STAFF MEMBERS

Director of Research: Stefania Uccini

A COLLABORATIVE WORK TO IMPROVE THE QUALITY OF TUMOR DIAGNOSIS OF IRAQI CHILDREN AND TO SUPPORT SCIENTIFIC EDUCATION OF THE MEDICAL STAFF MEMBERS

STEFANIA UCCINI
RESEARCH AREA: EBV-RELATED LYMPHOPROLIFERATIVE DISEASES

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The project is part of a long-lasting collaboration between the Department of Clinical and Molecular Medicine of Sapienza University and the Children's Welfare Teaching Hospital of Baghdad School of Medicine, Iraq. The collaborative work started in 2007 and is stll ongoing. It concerns the histologic reviewing for a second opinion of biopsies and surgical samples of Iraqi children with tumors, in order to confirm the diagnoses made in Iraq.
In 11 years of second opinion, more than 1350 diagnoses of pediatric oncology were made, concerning all types of pediatric tumors such as lymphoma, Wilms tumor, neuroblastoma, rabdomyosarcoma, Ewing sarcoma, germ cell tumors, retinoblastoma, hepatoblastoma and other tumors.
Research collaborative studies started investigating EBV-related malignant lymphomas which are relatively common in Iraqi children. We reported that 86% of the classic Hodgkin lymphoma are EBV positive in contrast to what reported in Western countries accounting for less than 30%. Moreover, EBV is present in 5% of diffuse large B cell lymphoma occurring in nonimmunocompromised children.
In a subsequent study, we confirmed the strict association between EBV infection and malignant lymphomas in Iraqi children, describing a large series (125 cases) of Burkitt lymphoma (BL) diagnosed in children living in a geographic region not involved by malaria.
More recently, expression of the latent membrane protein-1 (LMP1) of Epstein-Barr virus (EBV) was investigated in 153 cases of EBV+ classic Hodgkin lymphoma (cHL); 120 cases were pediatric patients (< 14 years of age) from Iraq, and 33 cases were adult patients from Italy. We described for the first time the presence of LMP1 protein in EBV-encoded RNA (EBER)-negative follicular dendritic cells (FDCs) of reactive germinal centers (GC) associated with EBV+ cHL. Presence of LMP1+ GCs was independent of geographic region and age of patients. GC cells with LMP1+ FDCs were surrounded by numerous EBV-infected cells which were positive for EBER, LMP1, and CD30. Double immunolocalization analysis revealed that LMP1 was associated with CD63, an

exosomal marker, and with CD21. We suggested the possibility that peri-follicular EBV-infected cells release LMP1 protein, perhaps through exosomes, and that the protein is then captured by FDCs and is presented to EBER-negative GC B cells.
Our contribution provided information useful for treatment of children with malignant lymphoma. In fact, since 2000, an adapted LMB 96 protocol was implemented at the Children's Welfare Teaching Hospital in Baghdad for the treatment of childhood B-cell non-Hodgkin lymphoma. The first experience (2000-2005) demonstrated efficacy and feasibility of this protocol in Iraq. In 2006, further adjustments were made in the attempt to reduce therapy-related toxicities. The outcome of the second cohort of 190 children (2006-2010) and the comparison with the previous study were reported. Out of the 180 treated patients, 120 achieved a complete response. Therapeutic group-B in the second cohort showed better outcome, although not significant, compared to the first one. However, therapy-related mortality remained high.
In conclusion, our collaborative work can support Iraqi colleagues in daily practice helping their efforts to re-establish high quality medicine in Iraqi Health System.
Our contribution may be also helpful in improving the medical education and in training young medical doctors, helping them to break the cultural isolation caused by the long-lasting war in which they lived and are still living.

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RESEARCH PROJETCS CARRIED OUT IN AFFILIATED LABORATORIES AT SAPIENZA UNIVERSITY OF ROME

"ANNA TRAMONTANO" RESEARCH PROJETCS – CALL 2018
3 YEARS PROJECTS LED BY UNDER 60 YEAR OLD RESEARCHERS

SECOND YEAR REPORTS

TARGETING MYC TRANSLATION IN COLORECTAL CANCER

GIANLUCA CANETTIERI
RESEARCH AREA: GENETICS, BIOLOGY AND PATHOPHYSIOLOGY OF EUKARYOTES

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Colorectal cancer (CRC) is a major cause of death from cancer worldwide. Despite the progresses made with early diagnosis and improvement of therapeutic protocols, the prognosis of the advanced stages is still poor. For this reason, understanding the molecular determinants of CRC tumorigenesis represents an indispensable step to find novel therapeutic opportunities. Several genes and pathways have been found mutated in CRC and most of them converge on the activation of MYC, thus making this oncogene an attractive therapeutic target. However, attempts to find direct MYC inhibitors have been disappointing, suggesting that alternative strategies, aimed at reducing MYC expression or activity are preferable options. One avenue is the inhibition of MYC translation, although inhibitors of general translation, such as PI3K/mTor inhibitors, have shown the paradox effect to increase MYC translation via non-canonical, compensatory mechanisms. Our work has led to the identification of MTR (MYC translational regulator), a regulator of MYC expression that is essential for the growth of CRC. Our data indicate that MTR promotes MYC translation by binding a G-rich element in the coding sequence of MYC, thereby resolving stable mRNA structures and relieving ribosome stalling. Moreover, we have found that this protein is required for the translational upregulation of MYC in response to activation of the Wnt/beta catenin pathway, likely through a stabilization-mediated mechanism.
During the second year of the project we have generated mice with homozygous conditional deletion of MTR in the intestine and have crossed them with CRC-prone mice (APC/Min+). We have also generated CRC cells (HCT116, HT29) carrying homozygous deletion of the MTR protein with CRISPR-Cas9 or expressing specific shRNA through lentiviral-mediated knockdown and are performing molecular analyses. Notably, tumor specimens from MTR KO mice and CRC cells show reduced levels of MYC and impaired tumor growth.
We have performed RNAseq analyses of MTR KO vs WT cells and found several differentially regulated transcripts. Importantly, the levels of MTR strongly correlate with Wnt/beta catenin pathway activation, suggesting a concerted transcriptional-translational regulation of MYC expression by this developmental pathway.

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CHARACTERIZATION OF THE ROLE OF SEPARASE IN THE REGULATION OF LAMINS AND RAD50

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Our proposal aims at exploiting a combination of genetic and molecular approaches in both Drosophila and human cells to study an unanticipated relationship between the protease Separase, Lamins and the DNA damage protein Rad50. We found indeed that Separase interacts with Lamins and the Rad50 and that both factors accumulate in *Separase (Sse)* mutants indicating that Separase is required to maintain a proper turnover of these proteins.

In this second year of funding we made progresses in understanding how Separase fulfills this regulation. We have previously shown that loss of Separase activity determines reduction of Heterochromatin Protein 1 (HP1) both in *Drosophila* and human fibroblasts (Cipressa *et al.* 2016). As it is known that HP1 affects lamin regulation we wanted to investigate whether decreased HP1 protein levelS in *Sse* mutants could account for lamin perturbation. To this aim, we restored HP1 expression in *Sse* mutant by introducing a Red Fluorescent Protein tagged HP1 (RFP-HP1) encoding construct through meiotic recombination. Western blotting analysis on larval brains from control Or-R, *Sse* mutants and recombinant *Sse-RFP-HP1* showed that lamin perturbation is not rescued by increasing HP1 expression thus indicating that Separase regulates lamins in a HP1 independent manner. In addition, we found that Lamins depletion in larval neuroblasts does not affect HP1 protein expression, suggesting that Separase acts in a direct way on both HP1 and lamins to regulate their cellular levels. These results indicate that the increase of Lamin C in *Sse* mutants is directly linked to the loss of Separase activity. Collectively our preliminary data indicate that Separase acts as a positive regulator of HP1 while it negatively regulates lamin C.

In our proposal, we have also envisaged that Separase could regulate processing of Lamin C. To confirm our hypothesis we first sought to get further insights on the expression and localization of Lamin C and of its putative products of processing. Thus, we expressed a recombinant Lamin C carrying the V5 and FLAG tags that have been fused in frame to the N and C termini of the protein, respectively (V5-LamC-FLAG). Western blot analyses showed that both anti-V5 and –FLAG antibodies, in addition to a full length protein (~70 KDa), recognized Lamin C fragments of ~30KDa and ~40 KDa

that retained either the N- or C-terminus of the protein, respectively, indicating that Drosophila LaminC indeed undergoes a physiological proteolytic processing. Preliminary immunofluorescence analyses indicated that ~30KDa and ~40 KDa Lamin C fragments localized differently during interphase and that the N-terminus containing fragment is retained in the cytoplasm forming distinct foci after the nuclear envelop breakdown. We are currently carrying out experiments to understand whether Separase has a role in the production and localization of these fragments.

Finally, we have generated a V5-Rad50-FLAG recombinant protein that we expressed in S2 cells. Western Blot analysis showed that this protein, unlike lamin C, is not subjected to an obvious processing when Separase is either present or depleted. However, we found that levels of V5-Rad50-FLAG dramatically increased upon siRNA-mediated Separase depletion indicating that the Rad50 recombinant protein could provide substantial information to address how Separase regulates Rad50.

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AUTOPHAGY MANIPULATION AS A STRATEGY TO COUNTERACT EBV- AND KSHV-DRIVEN MALIGNANCIES

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One of the main aspects of the studies from our laboratory is to unveil the mechanisms through which EBV and KSHV, human oncogenic gammaherpesviruses, reduce immune response, effect that may indirectly contribute to their oncogenic potential. We have shown that the reduction of autophagy by gammaherpesviruses plays a major role in the impairment of dendritic cell (DC) formation from monocyte precursors. Indeed, in the case of EBV, as a consequence of autophagy reduction and p62 accumulation, the stabilization of NRF2 increased, leading to the up-regulation of the anti-oxidant response. The up-regulation of the anti-oxidant enzymes reduced ROS whose production, increased by GM-CSF and IL-4, was essential for monocyte differentiation into DCs (Gilardini Montani et al. Autophagy 2019). The reduction of autophagy and ROS was also involved in the impairment of monocyte differentiation into macrophages mediated by KSHV (Gilardini Montani et al. Int. J Biochem. Cell Biol. 2019), virus previously found to alter DC formation (Santarelli et al. Autophagy 2016). We are currently investigating whether, besides interfering with monocyte differentiation, KSHV infection of differentiated macrophages could affect their polarization into M2. These are alternatively differentiated macrophages that promote instead of fighting cancer, thus skewing mascrophage polarization into M2 could be an important strategy contributing to the oncogenic potential of this oncovirus. The interplay between autophagy and ROS was then investigated in the context of EBV-driven cancerogenesis. We found that in B cell immortalization into LCLs induced by EBV, the activation of signal transducer and activator of transcription 3 (STAT3), interleukin-6 (IL-6) and reactive oxidative species (ROS) production as well as the reduction of autophagy played a key role. Indeed, the use of quercetin that counteracted all these virus-induced effects was able to prevent B cell immortalization and LCL formation (Granato et al. Biomolecules 2019), according to what was previously hypothesized (Cirone M Viruses 2018). Given that autophagy is strongly interconnected with ER stress/UPR activation, as autophagy reduction increases ER stress, we are currently investigating whether this interplay could contribute to KSHV-induced transformation of HUVEC into spindle cells that resemble KS cells. Indeed, by a general revision of the literature of this field, we have highlighted how autophagy manipulation could represent a more general strategy to interfere with

tumorigenesis, also other than that virus-induced (D'Orazi et al Cancers 2019 and Cirone et al J.Exp. Clin. Cancer Res. 2019), as for example the cancerogenesis driven by mutant p53 (Gilardini Montani et al Cancers 2019). Interestingly, we have shown that the manipulation of ER stress by using the chemical chaperone 4-PBA could represent a valid strategy to prevent the impairment of DC formation from monocytes, as in the case of HHV-6 infection that reduced autophagy in primary monocytes. The results obtained in this study indicated that ER stress induction by virus infection of immune cells, worsened by autophagy reduction, could contribute to immune dysfunction and represent a mechanism of viral immune escape (Romeo et al Virus Res 2019). As autophagy dysregulation and viral infections may be involved in the pathogenesis of Alzheimer's Disease (AD) (Romeo et al- Nur Reg Res 2019), we have more recently investigated whether HHV-6, being a neurotropic virus, could infect and dysregulate autophagy/UPR interplay in neurons and glial cells and through this mechanism affect amyloid beta secretion and Tau protein phosphorylation, two hallmarks of AD. We have recently published that HHV-6 infection led to a reduction of autophagy in both cell types, inducing an aberrant activation of PERK arm of UPR leading to an increased amyloid beta secretion and Tau protein phosphorylation (Romeo et al. BBA Dis. 2019), suggesting that manipulating virus infection and autophagy/UPR interplay could be promising in counteracting onset/progression of AD.

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DYNAMICS OF INTRA-CHROMOSOMAL GENE CONVERSION BETWEEN PALINDROME ARMS OF THE HUMAN Y CHROMOSOME

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The Male Specific region of the human Y chromosome (MSY) is characterized by the presence of 8 near-identical 'pseudo-diploid' palindromes, designated as P1–P8, which exhibit a strong self-recombinational activity. Palindromic sequences are made up of inverted repeats (palindrome arms), separated by a non-duplicated spacer. These 'pseudo-diploid' elements, which in total span 5.7 Mb, exhibit more than 99.9% similarity between arms, due to the homogenizing effect of arm-to-arm gene conversion (Trombetta et al. 2017; Trombetta and Cruciani 2017): a type of recombination which, unlike the crossing-over, involves the non-reciprocal transfer of genetic information from a "donor" sequence to a highly similar "acceptor" one. The main effect of gene conversion is to increase the sequence similarity between the arms and it has been suggested that it was acquired to maintain the structural integrity of multi-copy genes predominantly expressed in the testis, which are involved in the male-fertility, in order to preserve their functionality over time. Therefore, it has been proposed that gene conversion evolved as a mechanism to retain the ancestral state of gene sequences: a de novo mutation on a palindrome arm is preferentially back mutated to the ancestral state rather than transmitted to the other arm.
Despite its general importance, little is known about the dynamics of gene conversion within these peculiar structures. The detection of gene conversion effects is possible thanks to the presence of single nucleotide differences between the two palindrome arms (Paralogous Sequence Variants; PSVs). When a PSV does exist ('pseudo-heterozygous' state), the observation in other chromosomes of the two other possible 'pseudo-homozygous' genotypes, indicates that gene conversion must have occurred during the evolution of the examined sequences. However, this evidence tells us nothing about how many independent conversion events generated the observed genotypes, but the availability of a detailed Y chromosome phylogeny, defined by stable single nucleotide polymorphisms (SNPs) of the X-degenerate region, allows the evolutionary relationships of palindromic sequences to be investigated. The genetic diversity analysis of palindrome arms within this phylogenetic context can provide an estimate of the minimum number of conversion events, the conversion rate, the conversion tract length, the directionality of the events (ancestral to derived or vice-versa) and if the gene conversion is biased toward a particular base (G/C or A/T bias).

Results:

To shed light into the evolutionary dynamics of the human Y chromosome palindromes, we performed a target enrichment and Next Generation Sequencing (NGS) of P6-P7 and P8 palindromes in 157 worldwide distributed unrelated males, chosen to represent the most divergent evolutionary lineages of the MSY. At first, we assessed the phylogenetic relationships existing among our samples, through a bioinformatic analysis of deep-sequencing data of 3.3 Mb of the X-degenerate region (D'Atanasio et al. 2018; Finocchio et al. 2018), from which we obtained a detailed and reliable phylogeny based on 7,240 bi-allelic markers. Then, we used this stable phylogeny to temporally frame the mutations and gene conversion events observed in the palindromes.

During the first year of the project we reported the data about P7 palindrome (the shortest one) while in the second year we analysed P6 (110 kb each arm) and P8 (38 kb each arm) palindromes, for which we obtained respectively NGS data for 35,156 bp (each arm) and 17,339 bp (each arm), after removing the repetitive elements.

Since the MSY palindromes are made up of nearly identical duplicated sequences and they are often involved in genomic rearrangements, variant calling is not a trivial task. In particular, it may be possible that one palindrome arm is lost by deletion, thus the apparent 'pseudo-homozygous' states can be actually a 'pseudo-hemizygous' one. For this reason, we evaluated the presence of both P6 and P8 arms in each sample through 4 boundary-specific PCRs overlapping the sequences between arms and unique regions. In order to detect possible deletion/duplication events within palindrome arms, we also performed a NGS depth analysis, standardizing the depth value of each position of P6 and P8 for the average depth value of the non-duplicated 3.3 Mb of the MSY, the same that we used to reconstruct the phylogeny. The boundary-specific PCRs approach didn't provide evidence for lack of palindrome arms, but the NGS depth analysis revealed a ~1.3 kb 'pseudo-homozygous' deletion within P6 palindrome, occurred in two phylogenetically related samples (Hg A2'PN3). We checked by Sanger sequencing the region around the deletion and we found two 58 bp direct repeats (DRs) upstream and downstream the deletion, so we assumed that the deletion was possibly generated by a homologous recombination event between the DRs, followed by an arm to arm gene conversion occurred in the common lineage of the two samples.

The genetic diversity of P6 and P8 palindrome arms was assessed using standard bioinformatic tools (SAMtools, VCFtools, SNPEff). We obtained a list of putative variants which underwent filtering criteria, set on the basis of the peculiar 'pseudo-diploid' features of paralogue sequences. These bioinformatic analyses and the subsequent experimental validation resulted in the identification of 118 PSVs in P6 palindrome and 72 PSVs in P8 (Figure 1). Most of the PSVs found in P8 palindrome fall within a region that shares a high sequence similarity with 4 sequences on the X chromosome (which span from 10 to 18 Mb), and many of them are possibly introduced by X-to-Y gene conversion events (Figure 1).

These variant sites show both 'pseudo-heterozygous' and 'pseudo-homozygous' states. We detected in P6 and P8 palindromes 80 and 46 gene conversion events respectively, and in particular we found evidence of a possible gene conversion tract-length longer than 9 kb, due to a single co-conversion event that involved 4 PSVs, occurring in P6 palindrome. For both palindromes we observed a slight excess of conversion events leading to the derived 'pseudo-homozygous' state rather than the ancestral 'pseudo-homozygous' one: this result suggests that not necessarily gene conversion acts as a mechanism to retain the ancestral state of the variants, as previously suggested. Furthermore, we tested for a bias of gene conversion that could lead to the fixation of G/C or A/T base pair as a result of the gene conversion events, and we found a significant excess of events leading to G/C bases for both P6 and P8 palindromes.

By mapping gene conversion events within our stable phylogeny, we calculated a gene conversion rate with a method used for the first time in this study and we obtained for P6 palindrome a figure of 7.74×10-6 events/PSV/year and of 4.43×10-6 events/PSV/year for P8 palindrome; the latter is really close to the rate calculated for P7 palindrome (4.31×10-6 events/PSV/year), and this result suggests that P6 palindrome seems to act more rapidly in solving PSVs by gene conversion.

Finally, we estimated the mutation rate for both palindromes and we found respectively a rate of 6.91×10-10 (P6) and 9.21×10-10 (P8) events/base/year. The former is really close to that reported for P7 palindrome (6.90×10-10 events/base/year), while P8 mutation rate resulted to be the highest among those calculated for the three palindromes, mainly due to the X to Y gene conversion contribution.

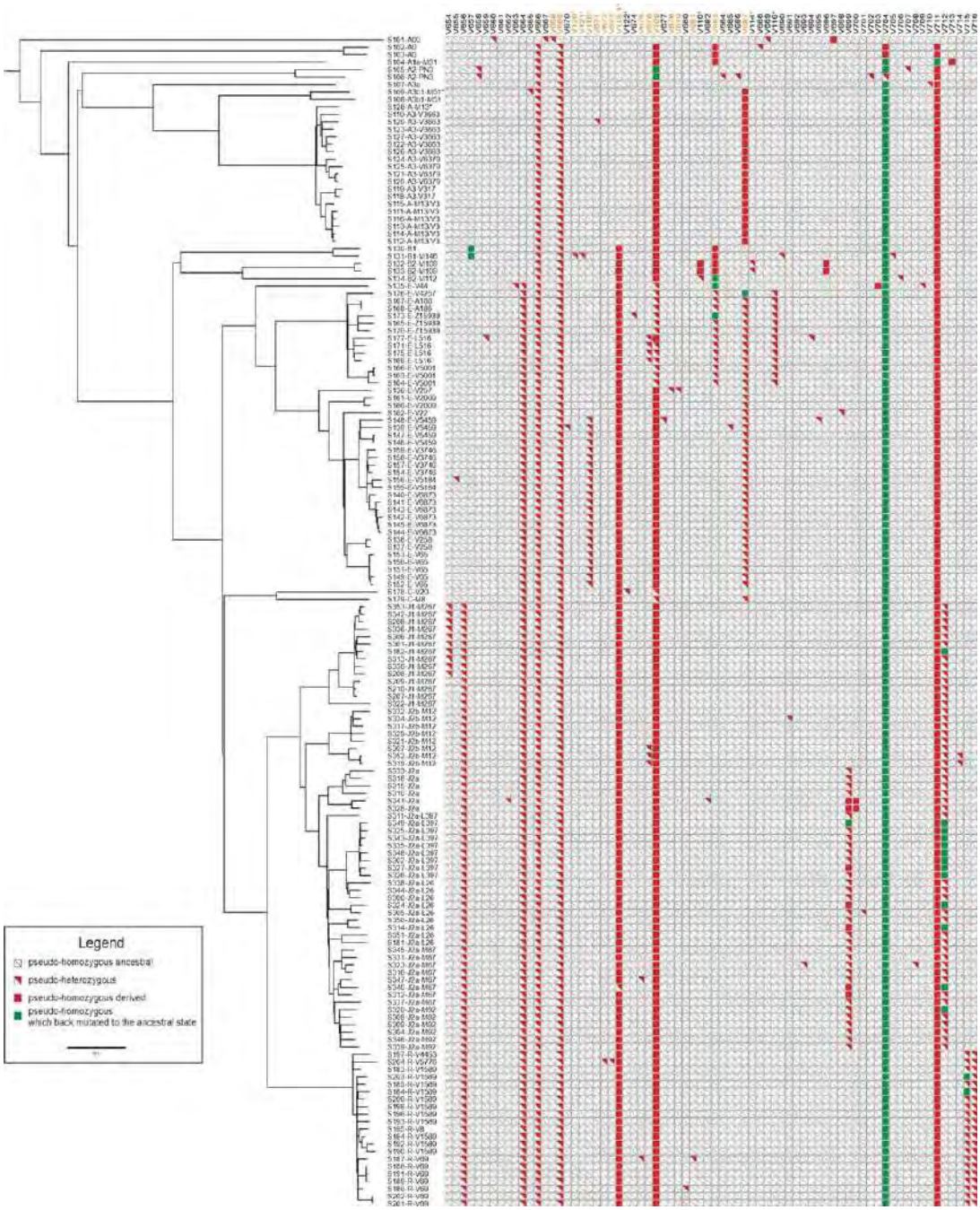

Figure 1: 'Pseudo-diploid' sites identified in the P8 palindrome arms (chrY: 16093532-16131537 and chrY: 16134952-16172355, GRCh37/hg19) by sequencing 157 Y chromosomes. To the left is reported the Y chromosome tree showing the phylogenetic relationships of the chromosomes analysed. PSV names are given at the top. In orange are indicated the PSVs of the X-Y homologous region, probably introduced by X-Y gene conversion. Each square is divided into two triangles, representing the paralogous sites in the two arms of the palindrome.

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REGULATION OF VITAMIN B6 METABOLISM AND BIOAVAILABILITY IN EUBACTERIA

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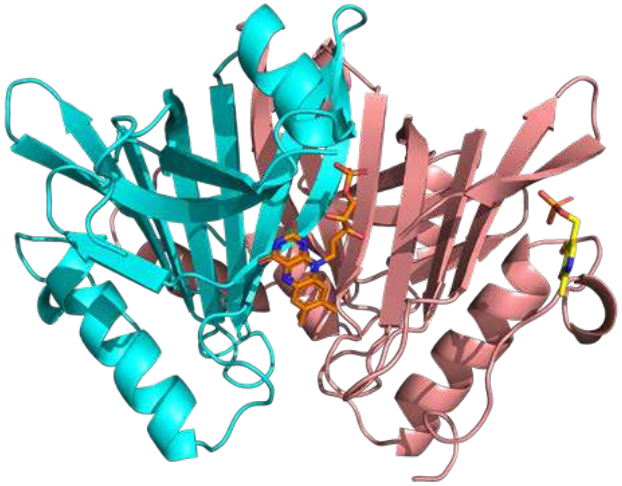
Introduction - Vitamin B_6 plays a fundamental role in physiology and virulence of microorganisms, as well as in human health and disease. The aim of our research project is to clarify some crucial but obscure aspects of vitamin B_6 metabolism in bacteria and humans. The term vitamin B_6 refers to six interconvertible vitamers, pyridoxine (PN), pyridoxal (PL), pyridoxamine (PM) and their phosphorylated forms, which play essential biological functions, the best-known of which is the catalytic activity of pyridoxal 5'-phosphate (PLP), an essential cofactor for dozens of enzymes. Because of its aldehyde group, PLP is a very reactive molecule that readily combines with thiols and amines; therefore, it is potentially toxic, and its cellular concentration must be kept at a low level. At the same time, large amounts of the cofactor are needed to saturate PLP-dependent enzymes and satisfy cell needs. How these requirements are met is yet largely unknown. In humans, homeostatic unbalance of PLP is responsible for severe neurological disorders and is implicated in cardiovascular diseases and cancer. Understanding how PLP homeostasis is maintained and how this vitamer is delivered to the apoenzymes that require it as cofactor is the main target of our research project.
Main findings of the first year of the project. A detailed kinetic characterisation of *E. coli* pyridoxine 5'-phosphate oxidase (PNPO), an FMN-dependent enzyme which converts PNP and PMP to PLP, led to the discovery of an allosteric feedback inhibition mechanism by which PLP levels control the activity of this enzyme. The location of the allosteric site in the enzyme three-dimensional structure was investigated by molecular docking (by our collaborator Prof. Martin Safo) and site-directed mutagenesis experiments. A possible site was located, close to the interface of the dimeric structure of PNPO and outlined by residues R23, R215 and F202. Studies on the PLP-carrier protein YggS from *E. coli*, whose regulatory function in PLP homeostasis is well known in bacteria, yeast and humans, were also carried out. Docking studies on YggS and serine hydroxymethyltransferase (SHMT, a PLP-dependent enzyme) suggested a possible model of interaction between the proteins to form a complex. On the basis of this information, several mutant forms of YggS, concerning residues at the possible protein-protein interface, were produced and two of them (E134A and K229A) were found not able to complement a Δ*yggS E. coli* strain (experiments carried out by our collaborator Prof. Valerie De Clergy-Lagard). Since E134 and K229 are on the surface of the protein, far from the active site where PLP binds, this result indicated that E134 and K229 may be involved in the interaction between YggS and apo PLP-dependent enzymes. A

homology-based survey on all available bacterial proteomes was carried out to analyse the distribution of DXP-dependent and -independent vitamin B_6 biosynthetic pathways in Eubacteria. Interestingly, we found that the distribution of the *pdxJ* gene, encoding PNP synthase and indicating the presence of the DXP-dependent pathway, is not limited to γ-proteobacteria as previously reported (Mittenhuber, G. 2001, *J. Mol. Microbiol. Biotechnol. 3*, 1-20), but is extended to FCB, PVC and Terrabacteria groups.

Second year of the project. *Studies on PNPO* - Crystallographic studies were carried out by our collaborator Dr. Andrea Ilari (Istituto di Biologia e Patologia Molecolare, Consiglio Nazionale delle Ricerche) on a quadruple mutant form of *E. coli* PNPO (K72I/Y129F/R133L/H199A), which is unable to bind substrates at the active site. This multiple mutant form was devised, produced and characterised so as to make sure that PNPO maintained its native structure and at the same time the capability to tightly bind PLP at the allosteric site (but not at the active site). High resolution crystallographic structures of this mutant were obtained in the presence and absence of PLP. In the latter case, PLP electronic density was clearly visible only in one monomer of the dimeric protein, at the site previously predicted by molecular docking and mutagenesis studies (Fig. 1). Interestingly, a whole domain of the PLP-binding monomer was not visible in the electron density map, indicating a high flexibility of this region. On the basis of this crystal structure, more focused site-directed mutagenesis studies were carried out, confirming the coincidence between the newly identified PLP-binding site and the allosteric feedback inhibition site highlighted by kinetic studies. A manuscript reporting these results is in the process of being published. The second part of the work on PNPO focused on the characterisation of the human enzyme, which is structurally very similar to its *E. coli* counterpart and is also inhibited by PLP. Our studies indicated that a similar allosteric feedback inhibition observed in the *E. coli* PNPO is present in the human enzyme. However, in this case the ternary enzyme-substrate-PLP complex is productive (although with reduced rate constant with respect to the enzyme-substrate complex) and inhibition is also due to PLP binding at the active site. Although the allosteric site on the human PNPO has not yet been identified, its location is probably the same as that identified in the *E. coli* enzyme. We are presently investigating this aspect. If this hypothesis will be confirmed in the future, we will be able to look for variants of the allosteric PLP binding site in the human population, that are already known and not yet associated with a dysregulation of vitamin B_6 metabolism. Furthermore, in our investigations on human PNPO, we turned out

Figure 1. Crystal structure of *E. coli* PNPO quadruple mutant with PLP (yellow sticks) bound at the allosteric site.

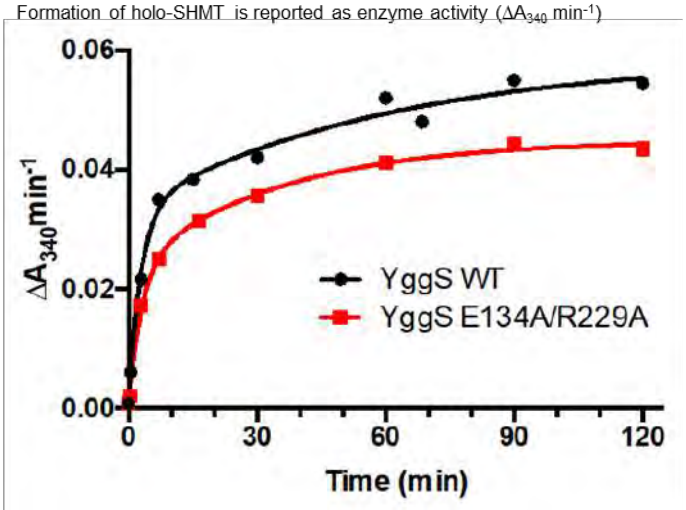


attention to the molecular basis of neonatal epileptic encephalopathy, a severe and rare disease caused by mutations of the PNPO encoding gene. We concentrated our studies on missense mutations G118R, R141C, R225H, R225C, R116Q/R225H and X262Q. We found that all mutations lead to substantial alteration of the kinetic parameters of the reaction catalysed by the enzyme, with increase of K_M and decrease of k_{cat}, particularly pronounced in the case of G118R and R116Q/R225H mutants. The R141C, X262Q and R116Q/R225H mutants were also characterised by a marked decrease of thermal stability. Affinity for the FMN cofactor was drastically decreased by R141C and X262Q mutations. On the other hand, PLP binding at the allosteric site was found substantially unaltered in all mutant forms with respect to the WT enzyme. Our findings on human PNPO are in the process of being published.

Studies on human pyridoxal kinase - Pyridoxal kinase (PDXK) catalyses the ATP-dependent phosphorylation of B_6 vitamers, directly producing PLP or generating PNP and PMP, which are then oxidised to PLP by PNPO. PDXK is encoded by the highly conserved *pdxK* gene, which is also present in *E. coli*, and located in humans on the 21q22.3 chromosome. In collaboration with Dr. Fiammetta Vernì (Sapienza University of Rome) and using Drosophila as animal model, we investigated whether PDXK variants present in the human population may impact on DNA integrity and be considered predictive of an increased cancer risk. In Drosophila, mutations in the *pdxK* gene cause chromosome aberrations (CABs) and increase glucose content in larval hemolymph. Both phenotypes are rescued by the expression of wild human PDXK. We expressed, in *dPdxk*^{1} mutant flies, four PDXK human variants that were predicted to be damaging: three of which (D87H, V128I and H246Q) listed in databases, and one (A243G) found in a genetic screening of patients with gestational diabetes. Differently from human wild type PDXK, none of the variants was able to completely rescue CABs and glucose content elicited by the *dPdxk*^{1} mutation. Biochemical analysis of D87H, V128I, H246Q and A243G proteins revealed reduced catalytic activity and/or reduced affinity for PLP precursors which justify this behavior. Although these variants are rare in the human population and carried in heterozygous condition, our findings suggest that in certain metabolic contexts and diseases in which PLP levels are reduced, the presence of these PDXK variants even in heterozygous condition could threaten genome integrity and increase cancer risk.

Studies on the PLP transfer capability of YggS – A novel discontinuous assay was set up to monitor PLP transfer from *E. coli* YggS to *E. coli* apo-SHMT. Experiments in which holo-YggS was mixed in equimolar amounts with apo-SHMT showed that PLP transfer takes place with biphasic kinetics. When the equilibrium is

Figure 2. PLP transfer from YggS WT and mutant forms to WT apo-SHMT. Formation of holo-SHMT is reported as enzyme activity (ΔA_{340} min^{-1})



reached, only 10% of the PLP originally bound to YggS is transferred to SHMT. Measurements of PLP binding equilibria carried out on the single proteins showed that the B_6 vitamer binds tighter to YggS (Kd = 1 μM) than to SHMT (Kd = 5 μM), accounting for the observed partial transfer of PLP from YggS to apo-SHMT. When holo-YggS was incubated with PLP phosphatase, which catalyses the hydrolysis of free PLP, a biphasic conversion of PLP into PL was observed, indicating a heterogeneous binding of PLP to YggS. Size exclusion chromatography experiments on YggS showed that protein subunits mainly exist in the monomeric form (65%) but are also associated as a dimer (35%). Monomeric and dimeric forms are not in rapid equilibrium. This heterogeneity in quaternary structure may be responsible for the observed biphasic kinetics of PLP transfer to apo-SHMT and of PLP hydrolysis by PLP phosphatase. Mutant forms of YggS (E134A/K229A double mutant) and SHMT (D89A/K354A double mutant) were produced in which the charged residues putatively involved in the formation of a complex between the two proteins, indicated by molecular docking studies, were replaced by Ala residues. Experiments with these forms are under way (Fig. 2).

Studies on the physiological role of YggS - A *ΔyggS E. coli* strain was characterized with respect to its growth capability in different liquid media. No differences were noticed with respect to the WT strain, except a more pronounced PN toxicity, which had been previously observed by other authors. A novel observation was made when 4-deoxypyridoxine (0.1 mM), a metabolic poison that mimics vitamin B_6 inhibiting PLP-dependent enzymes, was added to the growth minimal medium. In this case, a much more pronounced toxicity of 4-deoxypyridoxine was observed with the mutant *ΔyggS* strain. This result may be interpreted assuming that YggS is able to bind 4-deoxypyridoxine or its derivatives, acting as a detoxifying agent. An alternative hypothesis is that the presence of YggS in the cell, constituting a large PLP reservoir, counteracts the toxic effect of 4-deoxypyridoxine. Understanding which hypothesis is correct may shed light on the physiological role of YggS. We also produce a *B. subtilis ΔylmE* strain, lacking the gene which is homologous to *E. coli yggS*. Analysis of the growth capability of this strain did not reveal any differences with respect to its WT counterpart.

Studies on *Bacillus subtilis* vitamin B_6 biosynthesis pathway – *Bacillus subtilis* follows the DXP-independent pathway to synthesize PLP. During our bioinformatics studies, we noticed that the genome of this organism lacks a gene annotated as *pdxH* (encoding PNPO). This is not surprising, as the DXP-independent pathway does not require PNPO activity to produce PLP. We produced a *B. subtilis ΔpdxS* strain, lacking the gene encoding PLP synthase. Expectedly, this strain was not able to grow on a minimal medium but grew normally upon supplementation with PL. Surprisingly, this knockout strain was also able to grow upon supplementation with PN, indicating the presence of an enzyme catalysing the oxidation of PNP to PLP. Bioinformatics analysis of the *B. subtilis* genome revealed the presence of three genes encoding FMN-dependent proteins which are homologous to *E. coli* PNP and are yet not annotated as PNPO. We cloned, expressed these genes and measured the PNPO activity of the cell extracts. Our results suggest that one of the genes may actually encode a novel PNPO, which has

significant structural differences with respect to *E. coli* PNPO. We are going to purify this protein to homogeneity and characterize it with respect to its functional and structural properties.

Studies on gene expression in *E. coli* vitamin B_6 biosynthesis pathway – *E. coli* wild type (BW25113) and knock out strains lacking one of the main genes involved in vitamin B_6 metabolism (*pdxH*, *pdxJ*, *pdxB*, *pdxK*, *pdxY*, *yggS* and *ybhA*) were characterized with respect to their capability to grow in minimal liquid medium, supplemented or not with either PL of PN at different concentrations. Comparison of growth curves showed that *pdxH*, *pdxJ* and *pdxB* are essential for PLP biosynthesis, as the corresponding KO strains are not able to grow unless supplemented with the appropriate B_6 vitamers. The expression level of all genes, in exponential and stationary growth phase, under different growth conditions (minimal or reach medium in the presence or absence of B_6 vitamers and casamino acids), were measured. All genes were maximally expressed during the exponential growth phase. Surprisingly, neither PL nor PN had any significant effect on the expression level of all genes. On the other hand, the presence of casamino acids (which are believed to induce a PLP request by the cell) had the effect to reduce the expression of *pdxB*, *pdxK* and *yggS* genes, in either growth phases. These results indicate that the regulation of vitamin B_6 metabolism is more complex than we expected, and suggest that PLP homeostasis is very efficiently regulated at the level of enzyme activity, so as to maintain the cellular concentration of this cofactor constant, even when PL or PN are supplied from the extra-cellular environment. We are now devising novel methods to abruptly perturb the cellular concentration of PLP, so as to look at the regulation of gene expression.

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EXPLOITATION of NOVEL GENOMIC RESOURCES to DEVELOP MOLECULAR TOOLS for GENOTYPING AFRO-TROPICAL MALARIA VECTORS and STUDY ECOLOGICAL SPECIATION

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The main aim of this project is to exploit novel genomic approach/resources provided by the "*Anopheles gambiae* 1,000 Genome project" (Ag1000G; www.malariagen.net/projects/ag1000g) to develop and validate novel tools to overcome the limitation of current approaches in identifying signatures of introgression within the genomes of the two major Afrotropical malaria vectors species of the *Anopheles gambiae* complex, *A. gambiae* s.s. and *A. coluzzii* (1), as well as in karyotyping polymorphic paracentric chromosomal inversion with known or possible adaptive value (2). In addition, we studied the ecology and behaviour malaria vector in a village of Burkina Faso where Long Lasting Insecticide Nets (LLINs) are implemented to protect the population from malaria transmission (3). Finally, we used ddRAD sequencing approach to infer the invasion history and migration patterns of *Ae. albopictus* (the invasive Tiger Mosquito responsible of >500 human infection by Chikungunya virus in Lazio and Calabria regions in 2017) in Italy and other European Countries (4).

1- Development of a PCR-assays to identify signatures of introgression in *A. gambiae* and *A. coluzzii*.

At the beginning of the project, and based on Ag1000G data, we developed an Agena iPLEX mass-spectrometry assay to genotype species-specific variants covering all the chromosomal arms in order to make available to the research community a tool to investigate fine-scale levels of recombination and introgression along the genome. This identifies 26 SNPs with calculated Allele Frequency Difference (DAF) from Ag1000G phase-1 data. The assay was validated by genotyping 343 individual mosquitoes from the Ag1000G Phase-1 samples, with results consistent with those obtained by Illumina sequencing.

We also developed a more cost-effective PCR approach for two autosomal markers identified among the SNPs included in the MassArray assay, offering the possibility to genotype large numbers of specimens and to detect the possible presence of admixed or

hybrid specimens, which would not be identified by conventional PCR approaches based on chromosome-X markers commonly used to identify *A. coluzzii* and *A. gambiae*. We focused on two autosomal SNPs situated on chromosome-3 (3R:42848; 3L:129051). We validated the PCR-approach on 106 specimens from the High-Hybridization-Zone in Guinea Bissau already genotyped by Agena iPLEX mass-spectrometry approach. A manuscript summarizing the above data is in preparation.

These above results were the product of a joint effort of the present project and of EXGENMAL Inter-Institut Pasteur Concerted Action funded to Dr. Beniamino Caputo. This project aims to analyse introgression between *A. gambiae* and *A. coluzzii* along the west-Africa coast and its effect on malaria transmission. More in detail, the study is focused on a coastal population and inland population from Senegal (where high and low hybridization levels are expected, respectively) and a coastal and inland populations from Cote d'Ivoire (where complete isolation between the two species is expected). Preliminary genotyping of the populations based on conventional markers on chromosome-X and on the two above mentioned SNPs on chromosome-3 showed results conflicting with some of the expectations. We found little signature of hybridization (i.e. <4% of IGS, 3R and 3L heterozygous genotypes) in the two inland villages, as expected. However, frequency of IGS, 3R and 3L heterozygous genotypes is higher in the coastal village in Cote d'Ivoire (<30%) than in the Senegalese one (<4%). Further analyses are in progress to confirm this results - which suggest that the area of hybridization between *A. gambiae* and *A. coluzzii* along west African coast may extend from the far west to >1,000 km eastwards – and assess the epidemiological consequences of this pattern.

2- Development of novel non-cytological tools for the karyotypization of chromosomal paracentric inversion in *Anopheles gambiae* and *Anopheles coluzzii*.

Chromosomal inversions are fundamental drivers of genome evolution and chromosomal inversion polymorphisms play an important role in adaptation to environmental heterogeneities. For mosquito species in the *A. gambiae* complex, paracentric inversion polymorphisms are abundant and are associated with ecologically and epidemiologically important phenotypes. Improved understanding of these traits relies on determining mosquito karyotype, which currently depends upon laborious cytogenetic methods whose application is limited both by the requirement for specialized expertise and for properly preserved adult females at specific gonotrophic stages. In collaboration with the group of prof. Nora Besansky (Notre-Dame University, IN, USA), we 2.1) developed an in silico karyotyping approach based on Ag1000G data, and 2.2) applied exploited proximity-ligation sequencing (Hi-C) data to molecularly fine-map breakpoints of *A. coluzzii* and *A. gambiae* inversions. Further studies are ongoing to develop PCR-based methods for molecular karyotyping of most common inversions (2Rb and 2Rc).

2.1 - We developed sets of tag single nucleotide polymorphisms (SNPs) inside inversions whose biallelic genotype is strongly correlated with inversion genotype. We leveraged 1,347 fully sequenced *A. gambiae* and *A. coluzzii* genomes in the Ag1000G database of natural variation. Beginning with principal components analysis (PCA) of population samples, applied to windows of the genome containing individual chromosomal rearrangements, we classified samples into three inversion genotypes, distinguishing homozygous inverted and homozygous uninverted groups by inclusion of the small subset of specimens in Ag1000G that are associated with cytogenetic metadata. We then assessed the correlation between candidate tag SNP genotypes and PCA-based inversion genotypes in our training sets, selecting those candidates with >80% agreement. Our initial tests both in held-back validation samples from Ag1000G and in data independent of Ag1000G suggest that when used for in silico inversion genotyping of sequenced mosquitoes, these tags perform better than traditional cytogenetics, even for specimens where only a small subset of the tag SNPs can be successfully ascertained (Love et al. 2019).

2.2 - We demonstrated that proximity-ligation sequencing (Hi-C) data allows to map breakpoints at the molecular level and that this approach is widely applicable for robust identification and fine-mapping inversion breakpoints in species whose inversions have heretofore been challenging to characterize. We apply our method to interrogate the previously unknown inversion breakpoints of 2Rbc and 2Rd in *An. coluzzii*. We found that inversion breakpoints occur in large repetitive regions, and, strikingly, among three inversions analyzed, two breakpoints appear to be reused in two separate inversions. These breakpoint-adjacent regions are strongly enriched for the presence of a 30 bp satellite repeat sequence. Because low frequency inversion breakpoints are not correlated with genomic regions containing this satellite, we suggest that interrupting this particular repeat may result in arrangements with higher relative fitness. Additionally, we use heterozygous individuals to quantitatively investigate the impacts of somatic pairing in the regions immediately surrounding inversion breakpoints (Corbett-Detig et al. 2019).

3- Analysis of *Anopheles coluzzii* and *Anopheles arabiensis* behavioural plasticity in a LLIN-protected Sudanese-savannah village in Burkina Faso.

Despite the overall major impact of Long Lasting Insecticide treated Nets (LLINs) in eliciting individual and collective protection to malaria infections, some sub-Saharan Countries, including Burkina Faso, still carry a disproportionately high share of the global malaria burden. We analysed the possible entomological bases of LLIN limited

impact by focusing on a sample (1,080 *A. coluzzii*, 880 *A. arabiensis*) collected in 2015 by Human Landing Catches (HLCs) in a LLIN-protected village in the Plateau Central region of Burkina Faso. A very high estimated median hourly Entomological Inoculation Rate (EIR 1.4 infective bites/person/hour) for unprotected inhabitant/volunteer was observed in the village. The value is in line with the literature data available for *A. gambiae* s.l. in the same geographical area before LLIN implementation and highlights high levels of malaria transmission in the study village. Lack of the night hour biting peak typical for *A. gambiae* s.l. in the absence of bednet protection and lack of preference for indoor-biting activity suggests the capacity of both *A. coluzzii* and *A. arabiensis* to adjust their host-seeking behaviour to bite humans despite bednet protection, accounting for the maintenance of high rates of mosquito infectivity and malaria transmission. These results – despite being limited to a local situation in Burkina Faso – may represent a paradigmatic example of how high densities and behavioural plasticity in the vector population may contribute to explain the limited impact of LLINs on malaria transmission in holo-endemic Sudanese savannah areas in West Africa (Perugini et al., submitted).

4- Analysis of *Aedes albopictus* invasion history and migration patterns in Europe.
In the last four decades, the Asian Tiger mosquito, *Aedes albopictus*, vector of several human arboviruses, has spread from its native range in South-East Asia to all over the world, largely through the transportation of its eggs via the international trade in used tires. Albania was the first country invaded in Europe in 1979, followed by Italy in 1990 and other Mediterranean countries after 2000. We analysed a panel of >100,000 single nucleotide polymorphisms (SNPs) obtained for 11 European populations by sequencing of double-digest Restriction site-Associated DNA (ddRADseq) to infer the species invasion history and migration patterns. The obtained dataset was combined with 20 samples, previously analysed using the exact same approach and coming from both, the native and invasive range worldwide. This allowed us to interpret our results using a broader spatial and historical context. The emerging evolutionary scenario complements the results of other studies in showing that the extraordinary worldwide expansion of *Ae. albopictus* has occurred thanks to multiple independent invasions by large numbers of colonists from multiple geographic locations in both native and previously invaded areas, consistently with the role of used tires shipments to move large numbers of eggs worldwide. In particular for Europe our results suggest the occurrence of at least two independent invasions, with Albania and northern Italy invaded by populations from Japan and the USA, and Greece invaded by populations from Southern Asia. By analyzing mosquitoes from nine sites across ~1,000-km transect in Italy, we were also able to detect a complex interplay of drift, isolation by distance mediated divergence, and gene flow in shaping the species very recent invasion and range expansion, suggesting

overall high connectivity, likely due to passive transportation of adults via ground transportation, as well as specific adaptations to local conditions (Pichler et al. 2019).

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HEDGEHOG/GLI SIGNALING REGULATORY NETWORKS IN COLORECTAL CANCER STEM CELLS

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Summary: state of the art and research aims
Colorectal (CR) cancer is a heterogeneous disease that, despite advances in the molecular mechanisms underlying oncogenic features, represents one of the leading causes of cancer-related morbidity and mortality. CR- Cancer stem cells (CR-CSCs) are a subpopulation of CR with unique properties, a reservoir of cancer cells involved in drug resistance and consequently in CR relapse. The Hedgehog-GLI (HH-GLI) signalling is a key developmental pathway and a master regulator of the stem cell phenotype. HH-GLI misactivation has been described in several types of cancer. We hypothesize that HH-GLI could have a role in CR-CSCs maintenance and in mechanisms of drug resistance. Therefore, our project aims to characterize the HH-GLI signalling in CR-CSCs in order to shed light on its putative role in drug resistance.

During the second year of the project, we carried out experiments as planned in the proposed research project.

Specifically, in CR-cells characterized by the expression of the HH-GLI signaling pathway (as reported in the first year) we used a new lentiviral mediated short hairpin vector (shGLI1) for the genetic inhibition of GLI1. At this stage of the project, we added to the primary CSC cells other models derived from commercial cells lines that recapitulate the heterogeneity both in the genetic background and in the origin from primary or metastatic site as included in the present research project.
This strategy allowed us to select infected cells and overcome the low infection rate of CR-CSC that we reported in the first year.
CR-CSC were infected with shGLI1 and the result showed that the specific silencing of GLI1 is able to significantly impair cell viability and decrease the cell growth rate in cells with different genetic background (BRAF, KRAS WT and BRAF, KRAS mutated).
These results confirm the data obtained with the pharmacological inhibition of GLI1 using both Arsenic Trioxide (ATO) and GANT61 that we reported in the first year.
Altogether this set of experiments highlight that HH-GLI signalling plays a major role in the control of our cellular models.

Thus, we used these models to investigate the molecular mechanism involved in CR-cells chemoresistance.
To this end we first investigated the role of the HH-GLI signalling in the cell growth of both CR-CSC and CRC-cells, treated with current approved therapeutic regimen (5-fluorouracil and Oxaliplatin (FOXA)). The exposure of CR-cells to increasing doses of FOXA (from 0 to 10 μM) was associated with induction of GLI1 expression and increased staining of GLI1 in the nuclei of treated cells suggesting an active role of GLI1 in chemoresistance.
We then investigated the efficacy of GLI1 inhibition combined with FOXA and we found that CR-cells previously exposed to GLI1 inhibition showed impaired cell viability that was significantly higher than FOXA alone. These results indicate that GLI inhibition is able to promote a more effective activity of FOXA. Interestingly, the combined treatments induced CR-cells death as evaluated by the measurement of the cleaved PARP (c-PARP) levels.
Subsequently, we proceeded to investigate whether GLI1 could sustain the expression of molecules involved in drug resistance. ATP-binding cassette (ABC) transporters are a family of transmembrane proteins that recognize a wide range of substrates and use the energy from ATP hydrolysis to translocate molecules across membranes, irrespective of the concentration gradient.
Cytotoxic drugs are substrates of ABC transporters and high expression of some ABC transporters (i.e. ABCB1, ABCC1 and ABCG2) correlate with malignant phenotype and/or with bad prognosis in several types of cancer. We then performed gene expression analysis of all ABC transporters in CR-cells before and after GLI1 inhibition. Thirty-six ABC transporters were expressed and, among these, ten were significantly down-modulated in GLI1 silenced cells. To investigate whether HH-GLI signalling control the modulated ABC transporters at the transcriptional level, we analysed their promoter regions, 1000 base pairs upstream the transcription start site (TSS) looking for canonical and non-canonical GLI binding sites.
Chromatin immunoprecipitation (ChIP) experiments showed recruitment of GLI1 on the promoter of 6 ABC transporter considered (ABCA2, ABCB1, ABCB4, ABCB7, ABCC2, ABCG1 and ABCG2).
In order to further investigate the role of HH-GLI signalling and ABC transporters in CR chemoresistant cells, we induced chemoresistance in CR-cells by chronic and increasing FOXA treatments (from 1 to 10 μM for 5 weeks). In FOXA resistant cells GLI1 were significantly up-regulated together with ABCB1, ABCB4, ABCC2, ABCG1 and ABCG2 expression levels.
Taken together, our experiments demonstrated that a subset of ABC transporters were regulated by HH-GLI signalling, their expression levels were increased in a chemoresistance promoting condition, and this modulation was HH-GLI dependent.
These findings provide proof of principle for the evidence of GLI1 function in CRC chemoresistance.
To further validate the role of GLI1 in CR chemoresistance, we decided to set up organoids from CR-CSCs. Organoid culture allows cells to form tridimensional

aggregates and structures that recapitulate in vivo growth, with the advantage of a faster growth with respect to in vivo experiments. A number of cells comprised between 5000 and 20000 were suspended in 500 ul of Matrigel (Corning), allowed to settle and let grow undisturbed until structures appear. The timing of organoid formation was highly variable, depending on the cell type. We performed drug-mediated GLI1 inhibition in growing organoids and we observed that treated cells formed significantly smaller organoids.
Based on these results we further proceeded with the validation of the in vitro results, described above, using both new generated organoids and CR-CSC xenograft tumors. Specially we treated both models with single and combined treatments and ABC transporters along with effects on tumor growth and CR-cells apoptosis are under evaluation.

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2) Manuscript under evaluation

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HARNESSING REPLICATION STRESS TO UNDERSTAND AND TACKLE MYCN-DEPENDENT TUMORS

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Genome integrity needs to be preserved for the propagation of genetic information. To this end, an integrated set of molecules sense and repair DNA damages, while signaling for cell cycle checkpoints and/or apoptosis. Dysfunction of these machineries promotes accumulation of DNA lesions and/or aberrant DNA damage responses (DDRs), causing cell death or genetic instability and tumorigenesis. DDR-defective syndromes due to mutations in DDR genes (*e.g.*, *ATM*, *TP53*, *BRCA1/2*, *NBS1*, *WRN*, *BLM*, FA genes) are associated with genomic instability and inherited predisposition to cancer, plus developmental/degenerative disorders of the nervous system, which appears particularly vulnerable to DNA distress.

Activation of many oncogenes results in replication stress (RS), "a systemic state that leads to collapse of DNA replication forks" (according to T. Helazonetis), and a primary source of endogenous DNA damages. The DDR initiated by oncogenic stress may eventually end up in cell death or senescence, preventing cell transformation, and thus representing an anticancer barrier. On the other end, pharmacological inhibition of specific DNA repair mechanisms is synthetic lethal with loss of function mutations in DNA repair genes sometimes occurring in cancer cells. This approach is being successfully used to induce cancer cell specific death (i.e., the use of PARP inhibitors in BRCA defective tumors), raising an apparent paradox: DNA repair pathways might be both essential tumor suppressors and/or targets for anti-cancer therapies.

A better understanding of how this apparent paradox impinge on developmental and neoplastic diseases of the nervous system may offer great opportunities for their comprehension and possibly for their therapy and represents the main focus of our most recent and ongoing work.

To address these issues, we focused on MYCN. This is an essential component of the SHH-pathway required for the expansion of neuronal progenitors during development of the nervous system and an oncogene frequently deregulated in tumors, such as medulloblastoma and neuroblastoma. We had shown that MYCN induces replication stress and replication-born DNA damage (Petroni et al., 2015; Petroni et al., 2016). At the same time, it controls the expression of a complex pattern of DNA damage/repair

proteins to restrain the deleterious consequences of RS. In particular, we demonstrated that induction of each component of the MRE11/RAD50/NBS1 (MRN) complex by MYCN is required to control oncogene-dependent RS, in primary cerebellar granule cell progenitors (GCPs), but also in neuroblastoma cells (Petroni et al., 2016; Petroni et al., 2018)

Based on these observations, and in agreement with our project, we are now developing a series of animal and cellular models to address the relevance of the MRN complex and its functional interaction with the SHH-MYCN pathway in nervous system development and tumorigenesis.

As part of this project, we recently developed a SHH-MYCN-dependent primary culture of GCPs, named S-cNS (for SAG-dependent cerebellar neurospheres). These cells can grow up to 4-5 weeks as neurospheres, remaining exquisitely dependent on SHH signal, and can be induced to differentiate into granule cells. This model helped us establishing that a well-known tumorigenic mutation of the Smo transducer, which supports SHH-MYCN-dependent tumorigenesis in transgenic animals, is unable to promote cell-autonomous growth of GCPs, at variance with Ptch1-KO (Petroni et al., 2019).

More in general, this model represents a very important tool to investigate on physiological and tumorigenic conditions affecting GCPs. Indeed, we derived S-cNS from a number of animal models relevant for this project. In particular, we generated mice models to address the effect of inactivating the MRN complex during cerebellar development and SHH-dependent medulloblastoma. Interesting observations indicate a haploinsufficient role of NBS1 in medulloblastoma development. Paradoxically, however NBS1 defect seems to be epistatic for SHH-MYCN pathway. The molecular details of these finding are being addressed.

Following up on the idea that targeting RS can be exploited for the therapy of tumors bearing activated MYC-oncogenes, such as neuroblastoma and medulloblastoma, we evaluated the effects of the combined inhibition of PARP and CHK1. While the results of initial *in vitro* studies had already been reported (Colicchia et al., 2017) we have further investigated the effect of this combination in preclinical animal models, such as subcutaneous and orthotopic MYCN-amplified xenografts of human neuroblastoma, as well as a spontaneous medulloblastoma mouse model. Results are consistent with previous *in vitro* studies and will be reported in a publication, shortly.

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DEVELOPMENT OF NOVEL PEPIDE-BASED FORMULATIONS AND NANO/BIO-MATERIALS AGAINST PULMONARY AND OCULAR SURFACE MICROBIAL INFECTIONS

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The rising number of bacterial infections that have become resistant to the available antibiotics is one of the most serious life threats worldwide and is expected to lead to almost 1 million deaths/year, globally, by 2050, killing more than cancer. From this scenario, the discovery of alternative strategies to address the vital problems of infectious diseases is highly demanded. Antimicrobial peptides (AMPs) of innate immunity or their derivatives represent promising compounds to develop novel anti-infective agents with alternative mechanisms to drugs in clinical use.
In recent years, our research group has focused on the opportunistic bacterial pathogens *Staphylococcus aureus* and *Pseudomonas aeruginosa*. The ability of these microorganisms to persist in hostile conditions is primarily associated with their tendency to transform from a drug-tolerant planktonic to a more dangerous and treatment-resistant sessile life form, called biofilm. They can easily colonize host tissues which are most exposed to the external environment, such as the respiratory tract, especially in cystic fibrosis (CF) patients or the ocular surface.

Our research group identified amphibian skin-derived AMPs (e.g. esculentins and temporins) which rapidly kill *S. aureus* and *P. aeruginosa* with a membrane perturbing activity that limits the induction of resistance. This is in contrast with traditional antibiotics that are active only against the planktonic form of bacteria and generally recognize specific targets or biochemical pathways, making it easier for the microbes to become resistant to them. One of these AMPs, i.e. Esc(1-21) and particularly its diastereomer Esc(1-21)-1c carrying two D-amino acids, i.e. D-Leu14 and D-Ser17 (Esc peptides), were found to cause significant bacterial clearance in mouse models of acute *P. aeruginosa* pulmonary infection and *Pseudomonas*-induced keratitis. Nevertheless, a relevant aim which needs to be achieved for the usage of AMPs in therapy includes a proper delivery system to the target site. To this goal, the production of peptide-loaded polymeric nanoparticles (NPs) is an attractive approach to assist peptide release at lung.

In this context, we encapsulated Esc peptides inside biodegradable polymeric NPs made of poly(lactic-co-glycolic) acid (PLGA) and engineered with the hydrophilic polymer poly-vinyl alcohol (PVA). Because of their optimal size, morphology, and neutral/hydrophilic surface, the developed NPs were found to be aerosolizable without showing any

mucoadhesive property nor interacting with components of the extracellular matrix of Pseudomonas biofilm, i.e., polymannuronic acid, which can prevent NPs from reaching the target bacterial cells. Improved peptide transport through artificial CF mucus and simulated bacterial extracellular matrix was achieved in vitro. Importantly, encapsulation of AMPs in NPs resulted in improved efficacy in inhibiting *P. aeruginosa* growth *in vitro* and *in vivo* in the long term. A single intratracheal administration of Esc peptide-loaded nanoparticles in a mouse model of *P. aeruginosa* lung infection caused a 3-log reduction of pulmonary bacterial burden up to 36 h (Fig. 1).
In summary, our data have provided the first evidence of the success of PVA-engineered PLGA nanoparticles as valuable nanocarriers to assist the delivery of AMPs in the conductive airways as well as to extend and increase their therapeutic effect against *P. aeruginosa* lung infections compared with AMPs in their free soluble form.

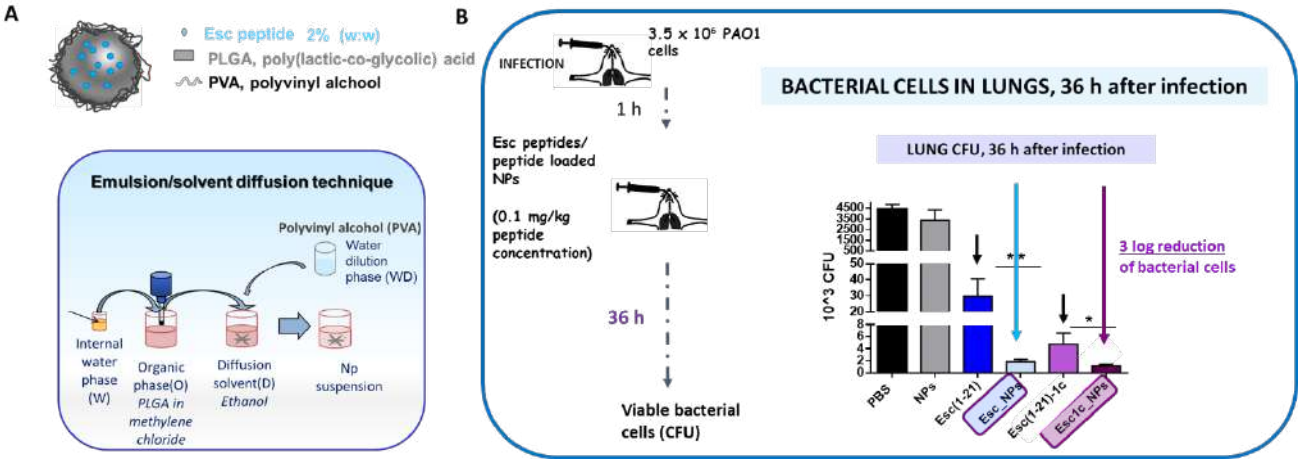

Fig. 1. A: Schematic representation of PVA-PLGA NPs production. B: Efficacy of Esc peptide-loaded NPs in reducing the number of lung bacterial cells in a mouse model of acute pulmonary infection in comparison to the free Esc peptides, unloaded NPs and vehicle (PBS).

Notably, prevention of *P. aeruginosa* biofilm formation already in its early stages could be even more advantageous for counteracting its infections. Interestingly, we found that the diastereomer Esc(1-21)-1c can prevent Pseudomonas biofilm formation in comparison to the parent peptide and two clinically-used conventional antibiotics, i.e. colistin and aztreonam when used at dosages below the minimal growth inhibitory concentration. This was correlated to the peptide ability to hamper the three different types of bacterial motility (swimming, swarming and twitching) and to reduce the production of virulent metabolites, e.g. pyoverdine and rhamnolipids (Fig. 2).

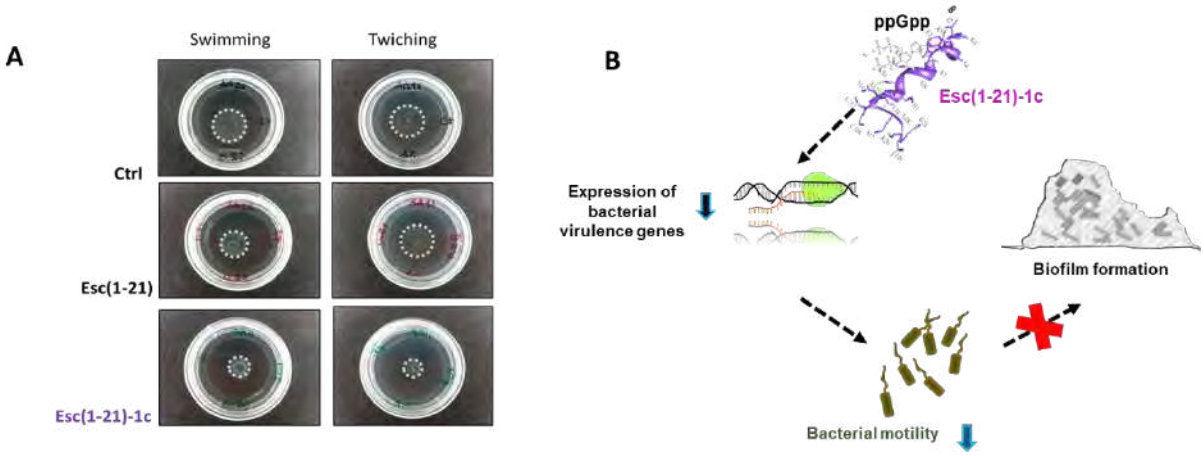

Fig. 2. A: Effect of Esc peptides on the motility of *P. aeruginosa* in comparison to untreated bacterial cells (Ctrl). The diastereomer clearly inhibited the swimming and twitching motility. B: Schematic representation of one possible mechanism of bacterial biofilm inhibition by Esc(1-21)-1c. Prolonged peptide interaction with ppGpp would reduce the availability of free nucleotides, lowering the expression of virulence genes. This would lead to inhibition of biofilm formation.

Our results have suggested that the presence of only two D-amino acids in Esc(1-21)-1c is sufficient to downregulate the expression of biofilm-associated genes, presumably as a result of a prolonged interaction with the bacterial signalling nucleotide ppGpp compared to the all-L Esc(1-21), which may be rapidly degraded. Overall, these studies should assist efficient design and optimization of new antiinfective agents with multiple pharmacologically beneficial properties.

Remarkably, we have to consider that the airway epithelium is seriously damaged upon pulmonary *P. aeruginosa* infection. We previously found that Esc peptides are able to stimulate migration of bronchial epithelial cells, suggesting their ability to accelerate healing of infected injured lung tissue. Interestingly, we have demonstrated that promotion of cell migration occurs by an indirect activation of epidermal growth factor receptor mediated by metalloproteinases.

As stated above, *Staphylococcus aureus* is another human pathogen causing a wide range of serious infections. Notably, Nature is undoubtedly an invaluable source of bioactive molecules with an ample chemical diversity. Beside AMPs, a class of natural compounds with a variety of biological activities is represented by alkaloids, important secondary metabolites produced by a large number of organisms e.g. bacteria, fungi, plants, and animals. By screening compounds retrieved from a unique *in-house* library, we identified a heterodimer β-carboline alkaloid, nigritanine, with a potent anti-

Staphylococcus action without being toxic *in vitro* to mammalian red blood cells and human keratinocytes. The analysis of the antibacterial activity related to the nigritanine scaffold provided new insights in the structure-activity relationships (SARs) of β-carboline, highlighting that dimerization improves its antibacterial activity. On the basis of these data, nigritanine can be considered as a valuable template for the development of new antimicrobial molecules to treat *S. aureus*-associated infections.

In parallel, we enlarged our knowledge on the potent antimicrobial analog of temporin L, [Pro^3]TL, in which glutamine at position 3 is substituted with proline, by performing SAR studies on a series of analogs in which position 3 is substituted with non-natural proline derivatives. Non-natural proline analogs with substituents at position 4 of the pyrrolidine ring were also considered. Antimicrobial and cytotoxicity assays along with circular dichroism and NMR spectroscopic analyses for the selected compounds were carried out. The peptides endowed with the highest antibacterial activity and lowest cytotoxicity were additionally evaluated for their effect against some representative veterinary microbial strains. We identified a novel lead compound carrying a cis-4-amino-L proline (Fig. 3), with interesting properties for the development of new anti-infective agents.

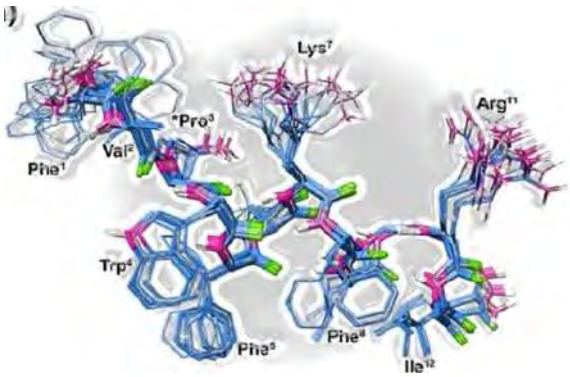

Fig. 3. S20 lowest-energy conformers of Temporin L analog carrying a cis-4-amino L proline at position 4, in sodium dodecylsulfate.

Finally, we started performing studies to better understand the target cell selectivity of AMPs by investigating the role of peptide aggregation.

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WHAT SHAPES THE PARASITE *ANISAKIS*-HUMAN HOST INTERACTION? INTEGRATING GENETIC, MOLECULAR, AND IMMUNOLOGICAL APPROACHES TO INVESTIGATE THE ZOONOTIC DISEASE, ANISAKIASIS

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The interaction formed by anisakid parasites and their hosts provides fascinating examples of evolutionary adaptation. In the course of evolution, anisakid nematodes have developed numerous strategies to adapt to their hosts, survive, and counteract the host-immune response (Mattiucci & Nascetti, 2008; Mattiucci et al., 2018). However, the genetic and molecular bases that regulates the mechanisms involved in the differential hosts' response to the zoonotic species *A. simplex* (s. s.) and *A. pegreffii* - aetiological agents of human anisakiasis - in both natural and accidental (humans) hosts, still remain largely unknown, as well as the molecules mostly responsible for those mechanisms, at both cellular and tissues level.

Specific objectives of the project are to: 1) investigate the host-parasite genetic variation investigated in the zoonotic species *A. simplex* (s.s.) and *A. pegreffii* responsible agents of human anisakiasis; 2) study the transcript levels of genes encoding for those proteins having antigenic significance; 3) study the modulation of Dendritic Cells functions in response to parasite interaction; 4) assess the presence and role of Microvesicles (MVs) released by the two *Anisakis* spp.; 5) improve the knowledge on the proteins and their transcripts involved in the differential invasiveness of the two *Anisakis* species in different hosts' (natural and accidental) tissues; 6) detect the possible association between human IgE-hypersensitivity *versus Anisakis* and specific HLA Class II loci.

During the year 2019, investigations carried out in the following objectives, have produced the following results:

Objective no.1: Parasite genetic variation was investigated by a multilocus genotyping approach in the zoonotic parasites *A. simplex* (s.s.) and *A. pegreffii*; This is a prerequisite to investigate the genetics of the host-parasite interaction between these parasites and their natural and accidental (human) hosts. Indeed, it has been postulated that a gene-for gene hypothesis is at the base of the genetics of the host-parasite interaction. The investigation about the existence of genes affecting the pathogenic

characteristics of the zoonotic species of *Anisakis* was never carried out. Further, the genetic detection the etiological agents of human anisakiasis, i.e. *A. pegreffii* and *A. simplex* (s.s.), represents the basis also for the characterization of their antigenic characterization.
Therefore the application of NGS approach carried out during the year 2019, has allowed the detection of microsatellite DNA loci in their genomes. This approach allowed the development of SSRs loci allowing to discriminate the two parasite species. Among the SSRs scored in the two species, several of them were resulted to be sex-linked. This finding allowed the possibility to define the sex-determination of the third-stage larvae of the two species, which also represent the zoonotic stage of those parasites species to humans.
Additionally, amongst the genes coding for functional proteins of those zoonotic species, genetic variants (SNPs) fixed for alternative nucleotide positions in the zoonotic species of *Anisakis*, were detected at some gene loci. Finally, a rapid, low cost, specific PCR tool also to was developed based on ARMS-PCR assay, at the metallopeptidase 10 (*nas 10* nDNA), for the genotyping of the sibling species of the *Anisakis simplex* (s. l.) complex. This approach will also allow a rapid identification of those larvae removed from human cases of anisakiasis.
The overall results achieved were the objects of the **Publications no.1, no.2 and no.3**

Objective no. 2: During the infection routes in natural and accidental (humans) hosts, anisakid nematodes generally produce and release a variety of proteins/peptides, excretory/secretory products (ESPs) which have been retained to be key players in the parasite-host adaptation and interaction. It has been also suggested that ESPs production varies, both quantitatively and qualitatively, depending, for instance, by the type of host (intermediate, definitive and accidental), also as a consequence of the different structural and physiological conditions of host groups (Mehrdana & Buchmann, 2017). On the other hand, it has been suggested that parasites ESPs have several functions during infection, e.g. penetration of host tissues and evasion of host immune responses, but also able to elicit immune responses both in fish and mammals (Kuhn et al., 2013; Skrzypczak et al., 2014; Mehrdana & Buchmann, 2017). In general, nematode ESPs have immunomodulatory effects interfering with host immune responses, thus they are key players also in clinical manifestation of the disease in humans. Probably, the L3 of *Anisakis* spp. use mechanical disruption of tissue combined with the release of proteolytic enzymes (peptidases) to penetrate and migrate in fish tissues and into the gastrointestinal mucosa of fish and humans (Bahlool et al., 2013). Proteases, nucleotidases, esterases, glycases, dismutases could be connected to infectivity, immune evasion and pathogenicity. Mehrdana & Buchmann, (2017) and Lee et al (2017) have demonstrated that some metallopeptidases produced by *Anisakis* are possibly involved in the mechanical gastrointestinal penetration of experimentally infected mice. In some patients suffering from anisakiasis, the existence of the multiple, well defined, erosive and/or hemorrhagic lesions usually detected near the main lesion within the gastric

mucosa could be explained by the invasive activity of the larvae, together with the presence of anticoagulant substances in the ESPs.
Besides their role as antigens/allergens, those molecular determinants are thought to be key players in modulating the parasite-host interaction, also in response to temperature, pH, CO_2, oxygen, as well as in response of the host tissues (both invertebrates and vertebrates, including humans). Particularly, for instance, the temperature has been considered as a key factor in modulating both the motility and migration of *A. pegreffii* larvae in the hosts' tissues.
In this regards we have investigated the gene transcripts of proteins having antigenic role among the Excretory Secretory Products (ESPs) (i.e. a Kunitz-type trypsin inhibitor, *A.peg-1*; a glycoprotein, *A.peg-7* and the myoglobin, *A.peg-13*) after 24h, in *A. pegreffii* larvae maintained *in vitro,* under controlled temperature conditions (i.e. 37°C, 20°C resembling respectively homeothermic and ectothermic hosts condition, and 7°C, cold stress condition *post mortem* of the fish host). Primers of genes coding for those ESPs to be used in Quantitative Real-Time PCR were newly designed, and qRT-PCR conditions developed. Expression profiles of the genes *A.peg-1* and *A.peg-13* were found to be significantly up-regulated at 20°C and 37°C, with respect to the control (larvae kept at 2°C for 24h). Conversely, transcript profiles of *A.peg-7* did not significantly change among the chosen temperature conditions. In accordance with the observed transcript profiles, the SDS-PAGE revealed the presence of the three target ESPs at 37°C, while only *A.peg-13* was observed at 7°C. The results obtained seem to indicate that chosen temperature conditions do regulate gene expression profile of *A.peg-1* and *A.peg-13* in *A. pegreffii* larvae. While. because the transcripts of *A.peg-7* in *A. pegreffii* seems to be maintained in all the selected temperature-conditions (i.e. 7°C, 20°C and 37°C), it would indicate that by using this molecule, the parasite would be able to induce and regulate the Th-2 polarizing response associated to *Anisakis* infection in the human accidental host. On the other hand, the capacity of *A. pegreffii* larvae to impair human DC cells biology and functions has been also experimentally demonstrated during the first year of the Project.
The results so far achieved were the object of the **Publication no. 4.**

Objective no. 3: The aim of this objective was to investigate the mechanisms by which *A. pegreffii* influences the human immune response through the modulation of DCs. Generally, the human dendritic cells (DCs) show remarkably phenotypic changes when matured in presence of helminth-derived products. These modifications frequently elicited a polarization towards Th2 cells and regulatory T cells, thus contributing to an immunological tolerance against these pathogens. In *Anisakis* infections, the tissue resident APCs first encounter the parasite, while the larva actively penetrates the gastrointestinal mucosa and migrates into the tissues.
During the first year of the Project we investigated, for the first time, the immunomodulatory effects of *A. pegreffii* on the differentiation and function of DCs. Thus, the interaction between DCs and larvae of *A. pegreffii* was studied: the parasites

were collected from fish hosts and monocyte derived DCs were co-cultured in the presence of the live larvae (L) or its crude extracts (CE). The use of the live larvae allows the direct interaction of differentiating DCs with the molecular determinants of the cuticle layer and the whole molecular repertoire actively released by the larvae (ESPs), similarly to what happens *in vivo*.

We have observed that DCs contribute to the inflammatory chronic response to the parasite by sustaining a strong inflammatory microenvironment and modulating their ability to recruit immune cells at the infection site. In both the experimental conditions, *A. pegreffii* impacted DC viability, hampered DC maturation by reducing the expression of molecules involved in antigen presentation and migration (i.e. HLA-DR, CD86, CD83 and CCR7), increased the phagosomal ROS levels, and modulated the phosphorylation of ERK1,2 pathway.

These biological changes were accompanied by the impairment of DCs to activate a T cell mediated IFNγ. Interestingly, live larvae appeared to differently modulate DC secretion of cytokines and chemokines, with respect to the crude extract of the larvae (CE). Taken all together the results so far obtained, they suggest that DCs might participate to the complex scenario of the immune reaction to *A. pegreffii* infection, according to the infection phase. At the first step, the live larva induces apoptosis, DCs differentiation and maturation and reduces DC ability to migrate to the lymph node. Such DCs contribute to generate an inflammatory microenvironment (IL1α and IL6 increase) to sustain the plasticity for Th differentiation, while blocking Th1 polarization (reduced IFNγ T cell responses), and altering leukocytes recruitment. When the parasite undergoes cell death, the necrotic debris still induces apoptosis and prevents DCs to migrate to the lymph node. The tissue resident DCs upregulates CCL3 chemokine that may favour leukocyte recruitment during the granuloma formation and production of IL4, that, in combination with IL6, might contribute to redirect T cells towards Th2 differentiation. These results demonstrate, for the first time, the immunomodulatory role of *A. pegreffii* on DCs biology and functions. In addition, they suggest a dynamic contribution of DCs to the induction and maintenance of the inflammatory response against *A. pegreffii*. These results were published during the first year of the Project (Napoletano et al., *Parasite Immunology*, 2018).

However, which are the molecules in *A. pegreffii* that are involved in such immunomodulatory effects remained an open question.

Thus, during the second year of the Project, the complete transcriptomic analysis (RNAseq) of *A. pegreffii* larvae maintained *in vitro* culture with and without DC, has been carried out. The comparison of transcripts expression (RNAseq) in *A. pegreffii* larvae co-cultured and not with DCs, was performed**.** The effects of the DCs on transcriptional responses in *A. pegreffii* larvae were investigated by high-throughput RNA-sequencing (RNA-seq) technologies.

In total, 573 and 628 genes were identified as significantly up- or down-regulated, respectively in presence/absence of DCs. Functional analysis based on gene ontology (GO) classification system and the Kyoto encyclopedia of genes and genomes (KEGG)

database revealed that *A. pegreffii* utilize a wide variety of glycosylated molecules to successfully infect the accidental human host.
The results are in preparation for a future Publication, during the 2020**.**

Objective no. 4: assess the presence and role of Microvesicles (MVs) released by the zoonotic species *A. pegreffii*. Extracellular vesicles are bioactive small vesicles (30-1000nm) of endocytic origin, which are released into the extracellular environment and mediate a variety of physiological and pathological conditions. During the second year of the Project, this objective has started. Vesicles were isolated from *A. pegreffii* larvae maintained *in vitro* culture at 37°C. Electron microscopy revealed the presence of round or cup-shaped vesicles with double membranes. The structures ranged 40–450 nm in diameter. Proteomic analyses of vesicles contents unveiled proteins involved in the both invasiveness and modulate the immune resposnse. These findings seem to be promising in the investigation between *A. pegreffii* -derived exosomes and the accidental host (humans).
This objective will be continued during the third year of the Project.

Objective no. 5: The differential gene expression of these ESP and other molecules in the zoonotic *A. simplex* (s. s.), involved in the larval invasiveness in naturally infected hosts' tissues, have been also carried out during the year 2019.
In the intermediate/paratenic fish hosts, *Anisakis* spp. larvae, migrating from the stomach to other fish tissues, tend to encapsulate in different organs; sometimes can reach the muscles. These processes are thought to be involved with both mechanical and biochemical process for host tissue penetration and degrading the extracellular matrix. The aim of this specific objective was to: 1) study the gene expression levels of some proteins in *A. simplex* (s. s.) larvae infecting different host tissues of a naturally infected fish species, i.e. the blue whiting *Micromesistius poutassou*. The following genes encoding for *Anisakis* spp. proteins were studied: a kunitz-type trypsin inhibitor (*TI*), a glycoprotein (*GP*), the hemoglobin (*hb*), the sideroflexin 2 (*SFXN*), the ubiquitin-protein ligase (*hyd*), the trehalase (*TREH*), and the zinc metallopeptidase 13 (*nas 13*). With respect to the control (i.e. larvae *in* recovered in the stomach), significant differences of expression levels of *trypsin inhibitor*, *glycoprotein*, *hemoglobin*, *sideroflexin 2*, *ubiquitin-protein ligase*, *trehalase* and *zinc metallopeptidase 13,* were observed in *A. simplex* (s. s.) larvae located in the different organs of the fish host. The ANOVA analysis performed on comparative values obtained at the *q*PCR of the *TI*, *GP*, *Hb, SFXN*, *hyd, TREH* and *nas 13,* showed that gene expression levels of the parasite species were affected by their infection site in the host. These findings seem to suggest a role played by the larvae in triggering the fish host response of different organs to the parasite species, which has been reported as high in the liver organ, while no immune response was found in the fish host muscle.
The results are part of the **Publication no. 5 (ready to be submitted);**

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UNDERSTANDING AND COMBATING SARCOPENIA: THE ROLE OF METABOLIC DISORDERS AND CYTOKINES-MEDIATED INFLAMM-AGING

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The research project aims to define the pathogenic mechanisms of sarcopenia and the metabolic disorders leading to sarcopenic obesity. Sarcopenia is the age-related loss of muscle mass and function. The causes of sarcopenia are unknown. Current hypotheses indicate that it may be the result of several factors, including hormonal changes, inflammatory pathway activation, fatty infiltration, altered mechanisms regulating the turnover of contractile proteins and organelles, neuro-muscular function as well as altered production and tissue responsiveness of trophic factors. Metabolic disorders, such as obesity, have been suggested as a risk factor for sarcopenia. Sarcopenic obesity, which describes the process of muscle loss combined with increased body fat as people age, is associated with loss of strength and function, reduced quality of life, and early death. Recent findings demonstrate an adverse confluence between sarcopenia and excessive adiposity, as the co-existence of such adverse alterations in body composition may exacerbate systemic inflammation and muscle wasting in the elderly. However, the underlying pathogenic concept of "sarcopenic obesity" is mainly based on phenotypical data derived from clinical observations.
The principal aims of the project are to define whether: i) sarcopenic obesity triggers an inflammatory response; ii) altered inflammatory cytokine expression recapitulate the pathologic phenotype of sarcopenia; iii) disclose the epigenetic signature of sarcopenia. During the first year of financial support we extended preliminary results, studying the potential critical players involved in the pathogenesis of sarcopenia and sarcopenic obesity, namely altered growth factors activity, oxidative stress, and increased inflammatory cytokine expression. In this context, one of the primary objectives was to establish animal models of aging with chronic obesity, using a High-fat diet (HFD). Preliminary results suggested that sarcopenic obesity is associated with increased plasma level of pro-inflammatory markers, including interleukin (IL)-6, and reduced IGF-1 expression. Interestingly, during the first year of financial support we demonstrated that muscle expression of either IGF-1Ea or IGF-1Eb, activating a series of anabolic and compensatory pathways, were able to guarantee muscle homeostasis and to prevent muscle loss, a normal muscle-nerve interaction, counteracting sarcopenia (Ascenzi et al, 2019). Future studies submitting mIGF-1 hyperexpression animals to a

long-term hypercaloric diet (HFD) leading to increased body weight will add new insight into the role of mIGF-1 in muscle homeostasis.
Then, we verified whether the circulating high levels of IL-6, observed in sarcopenic and sarcopenic obesity mice, are causally linked to the activation of aging-associated phenotype. To address this issue, which is part of the aims of the grant proposal, we took the advantage of using a transgenic mouse expressing high circulating levels of IL-6 (6 (De Benedetti et al, 1997). Morphometric analysis revealed that increased levels of circulating IL-6 significantly reduce the cross-sectional area (CSA) of single myofibers, compared to wild type muscle fibers, activating dominant atrophic genes, such as atrogin-1 and MuRF-1, and promoting the establishment of prooxidant conditions, which influence the local redox balance, leading to alteration in endogenous antioxidant response and to accumulation of ROS in muscles of IL-6 transgenic mice (Figure 1).

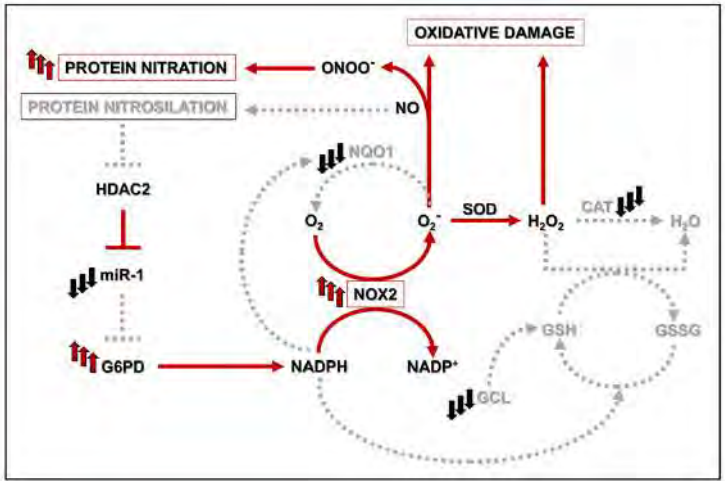

Figure 1. *A proposed model, based on the results recently obtained and published (Forcina et al, 2019) of the impact of elevated levels of circulating IL-6 on skeletal muscle redox balance. The reported scheme represents molecular circuits involved in the generation and neutralization of reactive species in skeletal muscle. Red lines indicate mechanisms that are potentially enhanced by elevated levels of circulating IL-6. Grey dot lines represent processes which might be impaired in NSE/IL-6 muscle. In presence of non-physiologic amounts of serum IL-6 NOX2 expression is enhanced in diaphragm muscle, inducing a sustained generation of superoxide (O_2^-). The down-modulation of NQO1 in muscle tissue exposed to increased levels of serum IL-6 indicates that the NOX2-derived superoxide might be not efficiently neutralized. On the other hand, O_2^- is converted by SOD into hydrogen peroxide (H_2O_2), whilst its further detoxification can be impaired by the reduced expression of CAT. H_2O_2 can also be neutralized through the oxidation of glutathione. The reduced expression of the rate-limiting enzyme to produce glutathione (GSH), GCL, might reflect an impaired activity of the glutathione system. The excess of O_2^- can also interact with nitric oxide (NO) inducing protein modifications. Moreover, the altered regulation of the NO signalling pathway might induce a*

deregulated expression of glucose 6-phosphate dehydrogenase (G6PD), the enzyme responsible for the production of NADPH, further enhancing the activity of the NOX2 complex in a feed-forward circuit. NOX2: NADPH oxidase 2; NQO1: NAD(P)H quinone dehydrogenase 1; SOD: superoxide dismutase; CAT: catalase; GCL: glutamate-cysteine ligase; $ONOO^-$: peroxynitrite; HDAC2: histone deacetylase 2; GSSG: oxidized glutathione.

Interestingly, we also observed that different muscle compartments are differently affected by increased levels of circulating IL-6. In fact, while gastrocnemius, quadriceps and EDL muscle of IL-6 transgenic mice showed a defect at early stage of post-natal life, the soleus muscle of same animals did not display significant and dramatic changes until 6 months of age. These data suggest that increased circulating levels of IL-6 induce a precocious sarcopenic phenotype. Further analysis will be performed to causally link the effect of IL-6 to its activity in different muscle compartments.

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"ANNA TRAMONTANO" RESEARCH PROJETCS - CALL 2019

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CHARACTERIZATION OF THE SIGNALLING MOLECULES AND CELLULAR METABOLIC PROGRAMS REGULATING CD28 PRO-INFLAMMATORY FUNCTIONS

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Several studies have evidenced that specific metabolic pathways control T cell function and differentiation in autoimmune diseases (1). In particular, the growth, survival and inflammatory functions of T lymphocytes depend on a dramatic increase in glucose metabolism (2) and elevated glycolysis has been recently associated with the differentiation and functions of pro-inflammatory Th17 cell subset (3). Thus, the identification of stimulatory molecules and associated signalling pathways coordinating the metabolic processes that maintain/amplify the inflammatory phenotype of peripheral T cells, may be useful for the development of new therapeutic opportunities in autoimmune and inflammatory diseases.
CD28 is a crucial costimulatory receptor necessary for full T cell activation that is able to arise TCR-independent autonomous signals, which induce the expression of pro-inflammatory cytokines and chemokines (4). CD28 also contributes to T cell metabolism by enhancing the uptake of nutrients, aerobic glycolysis and anabolic pathways induced by TCR stimulation (5). Indeed, following stimulation CD28 activates the PI3K/Akt/mTOR signalling pathway that in turn switches metabolism to aerobic glycolysis and promotes Th17 cell differentiation (6). Although CD28 is able itself to recruits and activate PI3K (4), its contribution to T cell metabolism has been always analysed in the contest of TCR stimulation. Thus, the role of CD28 as a TCR-independent signalling unit in activating the metabolic processes regulating T cell differentiation and functions remain still unknown.
In this project, we characterized the role of CD28 and associated signalling pathways in the modulation of the metabolic programs regulating pro-inflammatory T cell responses and Th17 cell subset in primary $CD4^+$ T cells from both healthy donors (HD) and relapsing-remitting multiple sclerosis (RRMS) patients.
Glycolysis and oxidative phosphorylation are the major energy-producing pathways in the cells and most cells possess the ability to shift dynamically between these two processes, adapting metabolically to changes in environment for the purpose of survival. Glycolysis and oxidative phosphorylation were evaluated by using an XF Glycolysis Stress kit and XF Cell Mito Stress kit, respectively, and measuring the acidification of

the medium surrounding the cells or the oxygen consumption rate through a XF Analyzer (Seahorse Bioscience). The results from these analyses evidenced that CD28 stimulation by agonistic Abs induced a significant increase of glycolysis in $CD4^+$ T cells from both HD and RRMS patients, without any significant changes in mitochondrial oxidative phosphorylation. CD28-induced increase of glycolysis was also associated with the up-regulation of the glucose transporter Glut1, specific surface activation markers, such as CD69, CD71 and CD25, whereas the expression of PD-1 was not affected. The analysis of Glut1 expression in different Th cell subsets, revealed that Th17 cells ($CXCR3^-CCR6^+$) expressed higher levels of Glut1 compared to Th1 ($CXCR3^+CCR6^-$) or Th0 cells ($CXCR3^-CCR6^-$) and CD28 stimulation up-regulated Glut1 expression in all T cell subsets, although to a higher extent in Th17 cells.

The analysis of the major enzymes regulating the glycolytic pathway evidenced that CD28-mediated up-regulation of the glycolytic pathway in $CD4^+$ T cells was associated to a strong increase of c-myc expression. By contrast, the expression of the other enzymes regulating glycolysis, such as hexokinase 2 (HK2), enolase 1 (ENO1), pyruvate dehydrogenase kinase 1 (PDK1), glucose 6 phosphate dehydrogenase (G6PD), HIF-1 and lactate dehydrogenase (LDHA) was not significantly affected.

Consistently with our previous data, CD28 autonomous stimulation of $CD4^+$ T cells strongly up-regulated the expression of pro-inflammatory cytokines, most of which were related to the Th17 cell phenotype, such as IL-6, IL-21, IL-22 and IL-17A. Moreover, the strong inhibition of CD28-induced up-regulation of pro-inflammatory cytokines exerted by the glycolysis inhibitor 2-deoxy-D-glucose revealed a pivotal role of glycolysis in CD28 pro-inflammatory functions.

In the attempt to characterize the molecular basis of CD28 pro-inflammatory functions, we also found that CD28 stimulation induced a strong phosphorylation and nuclear translocation of IL-6 receptor (IL-6R)-associated STAT3 as well as NF-κB activation. The inhibitory effects exerted on CD28-induced IL-6 and IL-17A gene expression as well as on STAT3 and NF-κB activation by specific NF-κB inhibitors strongly supported a mutual cooperation of IL-6R-associated STAT3 and NF-κB in regulating CD28-induced IL-17A gene expression. For instance, by performing chromatin immunoprecipitation assays, we found that both STAT3 and RelA were recruited on the human IL-17A proximal promoter inCD28-stimulated $CD4^+$ T cells. Interestingly, we also found that STAT3 and RelA were recruited to the promoter of c-myc and induced its trans-activation with similar kinetics.

Finally, treatment of $CD4^+$ T cells with a class 1A PI3K inhibitor (AS605240) strongly inhibited the glycolytic metabolism, c-myc and Glut1 expressions, as well as the up-regulation of pro-inflammatory cytokines and activation markers induced by CD28 stimulation.

Altogether these data evidence an important contribution of CD28-associated class 1A PI3K in reprogramming the metabolic processes that maintain/amplify the inflammatory phenotype of peripheral T cells and suggest class 1A PI3K as a valid therapeutic candidate for immune-based disease such as MS.

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