ISTITUTO PASTEUR ITALIA
FONDAZIONE CENCI BOLOGNETTI

2018 Annual Report

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CONTENTS

FOREWORD

The **Istituto Pasteur Italia - Fondazione Cenci Bolognetti**, the italian member of the Institute Pasteur International Network (33 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 2018 the Institute has invested a total of 938.000 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 brilliant young researchers with **fellowships** (i.e. to have a work experience abroad and to return to work 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 fourth year of establishment and directed Dr. John Hiscott. It also welcomed a new 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 2018 also saw the beginning of 22 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 rigor and 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** (33 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 2018 is demonstrated by high quality publications in peer-reviewed scientific journals, for a cumulative impact factor: 805,7 (the number is the result of 2018 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 **IV International Course on Persistent Viral Infections and Immune Evasion** organized in collaboration with the Pasteur Institute of Paris. Moreover, the Institute has carried on the well-established **educational project** for secondary schools involving a book series coupling Science with Comics as well as meetings and practical activities with students, so they can learn to appreciate the importance of science.

This Annual Report documents the results obtained during the year 2018 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)**

IMMUNOTHERAPIES FOR CANCER AND INFECTIOUS DISEASES

Director of Research: John Hiscott

IMMUNOTHERAPIES FOR CANCER AND INFECTIOUS DISEASES

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This research program focuses on the early 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. SIRT1 modulates the sensitivity of prostate cancer cells to VSV oncolysis
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 the present study, we demonstrate that the NAD+-dependent histone deacetylase SIRT1 plays a key role in the permissivity of prostate cancer PC-3 cells to VS.VΔ51 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. *(Revised for J. Virology)*

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. In the present study, we further investigated 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. Apoptosis relied on an intrinsic NOXA and PUMA-driven pathway that was largely dependent on IFN-I signaling. Additionally, 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. Moreover, M8 increased the levels of HLA-ABC expression on the tumor cell surface, as well as up-regulation of genes involved in antigen processing and presentation. 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. *(Revised for Cellular Immunology & Immunotherapy).*

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. *(Submitted to J. Virology).*

4. Immunometabolic 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. Markers associated with oxidative stress have been identified in patients with severe DENV infection, suggesting a relationship between oxidative stress and viral pathogenesis. Using genetic, biochemical and pharmacologic approaches, we demonstrated that the antioxidant gene network induced by Nrf2 transcription factor limited antiviral and cell death responses to DENV infection in primary human monocyte-derived dendritic cells (Mo-DC). Recent studies have further demonstrated that Nrf2, activated by the chemical sulforaphane (SFN) or by the Krebs cycle metabolite itaconate, dampened the release of pro-inflammatory cytokines, type I IFNs and IFN-stimulated genes, including the cGAS-STING, in response to DENV infection. Silencing of Nrf2 by RNA interference or CRISPR/Cas knockout increased both DENV infection and the associated antiviral and inflammatory responses. As a viral evasion strategy, *de novo* DENV infection in turn targeted Nrf2 for proteasome-mediated degradation, and also down-regulated metabolic pathways involved in NADPH and glutathione synthesis, resulting in further accumulation of ROS and oxidative stress. 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. *(Manuscript in preparation)*

PUBLICATIONS

De Santis F, Del Vecchio M, Castagnoli L, De Braud F, Di Cosimo S, Franceschini D, Fucà G, **Hiscott J**, Malmberg KJ, McGranahan N, Pietrantonio F, Rivoltini L, Sangaletti S, Tagliabue E, Tripodo C, Vernieri C, Zitvogel L, Pupa SM, Di Nicola M. Innovative therapy, monoclonal antibodies, and beyond. *Cytokine Growth Factor Rev. 44: 1-10 (2018).*
IF 6.4

Olagnier D, **Hiscott J**. Cytokines 2017 in Kanazawa *Cytokine Growth Factor Rev Feb 4. pii: S1359-6101 (2018).*
IF 6.4

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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. At the beginning pictures of tissue sections were sent by satellite telemedicine. However, because of the poor quality of the tissue sections, we asked to receive paraffin blocks. Ten cases each month of pediatric tumors (< 14 years).

In 10 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. The main discrepancies concerned lymphoproliferative disorders and soft tissue tumors since of the poor quality of the technical procedures and the limited use of immunohistochemistry. To improve the quality of the diagnoses, a positive and stimulating discussion on discrepant cases and a continous information concerning the reagents to be used for a correct immunohistochemical analysis.

However, it was clear that the main reason of failure was the cultural isolation in which Iraqi medical staff members lived as a consequence of the long lasting war. Therefore, 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 recent 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. BL is the commonest childhood cancer in tropical Africa and Papua New Guinea, and hence it is considered endemic; endemic BL have a more

frequent head and neck presentation and is EBV-related in 100% of the cases. Elsewhere in Western countries, BL is considered to be sporadic with a prevalent abdominal localization and EBV infection of tumor cells in approximately 20-30% of the cases. In Iraqi children, a high proportion of BL cases (66%) presented with abdominal disease, as expected for sporadic BL. However, 86% of abdominal cases were EBV positive (EBV-encoded RNA) (EBER+) as expected for endemic BL. Our results indicate that BL in Iraqi children represents a distinct form of BL showing intermediate aspects between endemic and sporadic forms of BL. Moreover, it is also of interest that MUM1/IRF4 (multiple myeloma oncogene1/interferon regulatory factor4) is significantly more frequently expressed in cases with head and neck disease as compared with those with abdominal presentation.

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.

2018 PUBLICATIONS

Uccini S, Al-Jadiry MF, Scarpino S, Ferraro D, Alsaadawi AR, Al-Darraji AF, Moleti ML, Testi AM, Al-Hadad SA, Ruco L. Epstein-Barr virus-positive diffuse large B-cell lymphoma in children: a disease reminiscent of Epstein-Barr virus-positive diffuse large B-cell lymphoma of the elderly. Human Pathology 2015, 46: 716-724. doi: 10.1016/j.humpath.2015.01.011 IF:3.125

Uccini S, Al-Jadiry MF, Cippitelli C, Talerico C, Scarpino S, Al-Darraji AF, Al-Badri SAF, Alsaadawi AR, Al-Hadad SA, Ruco L. Burkitt lymphoma in Iraqi children: A distinctive form of sporadic disease with high incidence of EBV+ cases and more frequent expression of MUM1/IRF4 protein in cases with head and neck presentation. Pediatric Blood & Cancer 2018, 65(12):e27399. doi: 10.1002/pbc 27399 IF: 2.646

Anastasiadou E, Stroopinsky D, Alimperti S, Jiao AL, Pyzer AR, Cippitelli C, Pepe G, Severa M, Rosenblatt J, Etna MP, Rieger S, Kempkes B, Coccia EM, Ho Sui SJ, Chen CS, Uccini S, Avigan D, Faggioni A, Trivedi P, Slack FJ. Epstein-Barr virus-encoded EBNA2 alters immune checkpoint PD-L1 expression by downredulating miR-34a in B-cell lymphomas. Leukemia 2019, 33:132-147. doi: 10.1038/s41375-018-0178-x IF: 10.023

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

"ANNA TRAMONTANO" RESEARCH PROJETCS

3 YEARS PROJECTS LED BY UNDER 60 YEAR OLD RESEARCHERS

FIRST YEAR REPORTS

TARGETING MYC TRANSLATION IN COLORECTAL CANCER

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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 preliminary 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 working model is 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 PI3K/mTOR inhibitors, likely via a phosphorylation-dependent stabilization mechanism. The proposed a project aims at elucidating these issues by addressing the following three main tasks: 1) To study the effect of MTR ablation in colorectal cancer mouse models and human cells 2) To elucidate how MTR promotes MYC translation and how PI3K signaling affects this mechanism 3) To study the MTR/MYC axis in human samples of CRCs and its correlation with different stages of the disease. During the first year of the project we have generated mice with heterozygous 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 approach and are performing molecular analyses. Notably, these cells show reduced levels of MYC and impaired in vitro and in vivo tumor growth.
Finally, we have collected and analyzed samples of CRC patients and have observed increased of MTR levels in cancer but not in surrounding normal cells.

Publications

Antonucci L, Di Magno L, D'Amico D, Manni S, Serrao SM, Di Pastena F, Bordone R, Yurtsever ZN, Caimano M, Petroni M, Giorgi A, Schininà ME, Yates Iii JR, Di Marcotullio L, De Smaele E, Checquolo S, Capalbo C, Agostinelli E, Maroder M, Coni S, Canettieri G. Mitogen-activated kinase kinase kinase 1 inhibits hedgehog signaling and medulloblastoma growth through GLI1 phosphorylation. Int J Oncol. 2019 Feb;54(2):505-514. doi: 10.3892/ijo.2018.4638. Epub 2018 Nov 19. IF: 3,333

Ohkubo S, Dalla Via L, Grancara S, Kanamori Y, García-Argáez AN, Canettieri G, Arcari P, Toninello A, Agostinelli E. The antioxidant, aged garlic extract, exerts cytotoxic effects on wild-type and multidrug-resistant human cancer cells by altering mitochondrial permeability. Int J Oncol. 2018 Sep;53(3):1257-1268. doi: 10.3892/ijo.2018.4452. Epub 2018 Jun 22. IF: 3,333

Infante P, Faedda R, Bernardi F, Bufalieri F, Lospinoso Severini L, Alfonsi R, Mazzà D, Siler M, Coni S, Po A, Petroni M, Ferretti E, Mori M, De Smaele E, Canettieri G, Capalbo C, Maroder M, Screpanti I, Kool M, Pfister SM, Guardavaccaro D, Gulino A, Di Marcotullio L. Itch/β-arrestin2-dependent non-proteolytic ubiquitylation of SuFu controls Hedgehog signalling and medulloblastoma tumorigenesis. Nat Commun. 2018 Mar 7;9(1):976. doi: 10.1038/s41467-018-03339-0. IF: 12,353

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Targeting class I histone deacetylases by the novel small molecule inhibitor 4SC-202 blocks oncogenic hedgehog-GLI signaling and overcomes smoothened inhibitor resistance. Int J Cancer. 2018 Mar 1;142(5):968-975. doi: 10.1002/ijc.31117. Epub 2017 Nov 6. IF: 7,360

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

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RESEARCH AREA: GENETICS, BIOLOGY AND PATHOPHYSIOLOGY OF EUKARYOTES

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Defective DNA repair and perturbation of nuclear architecture can threaten genome integrity. The comprehension of molecular mechanisms that underlie these effects can help to understand how cells prevent genome instability, which is a prevalent feature of several human genetic diseases including cancer. Our preliminary genetic and molecular results point towards an unforeseen function of Separase, a conserved endo-peptidase that resolves sister chromatid cohesion through cleavage of cohesins, in maintaining nuclear architecture and chromosome integrity. We found that Separase interacts with Lamins and the Rad50 DNA repair factor. Interestingly both factors accumulate upon depletion of Separase in Drosophila cells indicating that Separase is required to maintain a proper turnover of these proteins. We are exploiting a combination of genetic and molecular approaches in both Drosophila and human cells to study this regulation. Our preliminary results indicated that Drosophila Separase (SSE) is able to precipitate Lamin C (the Drosophila A-type lamin) from neuroblast protein extracts. In addition, GST-pulldown experiments revealed that GST-SSE pulls down bacterially purified 6HIS-Lamin C indicating that both proteins physically interact in a direct manner. Furthermore, immunostaining of salivary gland nuclei revealed that SSE localizes around the nuclear envelope and that this localization is coincident with that of lamin C. We assessed whether a functional relationship occurred also between SSE and the Drosophila B-type lamin, Lamin Dm_0. Our WB analysis revealed that loss of SSE reduces Lamin Dm_0 levels indicating that SSE regulates Drosophila A-type and B-type lamins in an opposite manner. In addition sqRNA analyses showed that this reduction is not due to a general decrease of *lamin Dmo* transcripts, which do not change in *Sse* mutants with respect to control. Consistently, immunostaining on polytene nuclei showed that Lamin Dmo localization on nuclear rim is affected upon depletion of SSE suggesting that the alteration of nuclear membrane morphology previously seen in *Sse* mutants can also depend on a lamin Dmo misbehavior in addition to the accumulation of Lamin C. We also have verified whether a physical interaction occurred between SSE and Lamin Dmo. To this aim we performed a GST pulldown experiment from larval brain extracts using GST-SSE as bait. This assay revealed that SSE could indeed physically interact also with LaminDm0 indicating that SSE-dependent regulation of Drosophila lamins relies on the formation of a well-established SSE-lamin protein complex. In the same experimental set up we have also observed that Pim (the

Drosophila Securin that we have shown to interact with Lam C) does not bind to lamin Dmo. We are currently investigating the molecular bases of these different interactions.

Publications

Graziadio L, Palumbo V, Cipressa F, Williams BC, Cenci G, Gatti M, Goldberg, ML, Bonaccorsi S. Phenotypic characterization of diamond (dind), a Drosophila gene required for multiple aspects of cell division. Chromosoma. 2018 Dec; 127(4):489-504. doi: 10.1007/s00412-018-0680-y (IF 4.021)

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

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We have previously demonstrated that the oncogenic gammaherpesvirus EBV and KSHV dysregulate autophagy during their replication. In particular, they block the last autophagic steps and usurp the autophagic machinery for intracellular transportation of viral particles (Granato et al. J Virol 2014 and Granato et al. Autophagy 2015). This is an important finding as it indicates that autophagy can be manipulated to reduce viral production known to contribute to the maintenance and progression of gammaherpesvirus-associated malignancies. As the immune system play a major role in the control of cancer onset and progression and EBV and KSHV are able to infect immune cells such as monocytes, we then investigated if they could dysregulate autophagy also in these cells to impair their function. Autophagy indeed plays an essential role in the immune response against microbial infections. First, through a selective form of autophagy, the xenophagy, it may directly mediate the lysosomal elimination of viral particles; second, autophagy contributes to viral antigen presentation mainly via class II MHC; third, autophagy is required for GM-CSF and IL-4 driven in vitro monocyte differentiation into dendritic cells (DCs). We previously found that KSHV reduced autophagy in monocytes by decreasing the activation of JNK and altering the balance between calpastin and calpains. The latters were responsible for the cleavage of ATG5, as demonstrated by experiments in which pharmacological inhibitors of their activity was used. ATG5 is an essential autophagic protein whose cleavage, besides reducing autophagy, promotes apoptosis. And indeed, we found that KSHV-infected monocytes displayed a reduced cell survival and differentiation into DCs (Santarelli et al. Autophagy 2016). We have recently found that EBV, the other oncogenic human gammaherpesvirus, can impair DC formation and this goal is also achieved through the inhibition of autophagy. The latter effect is due to the reduction of ROS whose production is induced by GM-CSF and IL-4 and drives monocyte differentiation. Autophagy reduction by EBV in monocytes leads to the accumulation of p62 that, in turn, stabilizes NRF2, the main transcription factors that up-regulate the anti-oxidant response to reduce ROS. Therefore, the virus creates a feed-back positive loop in which the reduction of autophagy reduces ROS and ROS reduction impairs autophagy during monocyte differentiation into DCs (Gilardini Montani et al. Autophagy 2018). Based on our studies, as a general finding, we have shown that gammaherpesviruses may dysregulate autophagy to promote their replication and to reduce immune response, and we are currently investigating if these viruses manipulate

autophagy also to promote tumorigenesis (Cirone M Viruses 2018). Given the strong relationship between autophagy and ROS, we have also investigated whether EBV infection of monocytes could alter ROS production in monocytes. Interestingly, we found that ROS increased upon viral infection, leading to the activation of molecular pathways such as the MAPK, STAT3 and NFkB that were responsible for the up-regulation of PD-L1 on the cell surface (Gilardini Montani et al. J Leuk. Biol. 2018). PD-L1 represents an inhibitory check-point whose expression in tumor cells or in antigen presenting cells hampers the immune recognition mediated by T cells. Interestingly, in the same period it has been demonstrated by a study by our laboratory in collaboration with Harvard University, that EBV increase the expression of PD-L1 also on the in tumor cells, further impairing the immune response (Anastasiadou et al 2018). Focusing on the central role of ROS in the biology of human gammaherpesviruses, we have then shown that their increase by cytotoxic treatments in lymphoma cells harbouring KSHV induce viral reactivation from latency. We also shown that the activation of NRF2 and HSF1 or their target molecule p62/SQSTM1 keep the level of ROS at a moderate level to allow cells to survive and KSHV to replicate (Granato et al, 2018). As viral replication is known to promote KSHV-associated tumor progression, we further focused on the molecular pathways that promote this effect. We found that STAT3 705tyrosine phosphorylation, constitutively active in lymphoma cells harbouring KSHV infection, maintains viral latency, as demonstrated by the use of AG490 STAT3 tyrosine inhibitor that activated KSHV lytic cycle. This study also evidenced that STAT3 phosphorylation in 727serine played an opposite role, as its increase by TPA stimulated viral replication. Interestingly, both 705tyrosine inhibition- and 727 tyrosine phosphorylation-mediated viral reactivation involved the activation of p53-p21 axis (Santarelli et al. Virology 2019), previously shown by our laboratory to be essential for KSHV lytic cycle induction (Gonnella et al. FRBM 2017). The opposite regulation between of STAT3 705tyrosine and p53 serine15 phosphorylation in lymphoma cells KSHV-infected was previously observed following the treatment with the flavonoid Apigenin (Granato et al. JECCR 2017).
We are also focusing on the impact on autophagy of HHV-6A and B on immune cells, as we have recently shown that also this betaherpesvirus manipulates autophagy during its replication in target cells. As consequence of autophagy dysregulation it induced ER stress accompanied by the expression of the pro-apoptotic molecule CHOP and the pro-survival BIP (Romeo et al. Journal of General Virology 2018).

Publications

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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 a high proportion of 'pseudo-diploid' palindromes, designated as P1–P8, which exhibit a strong 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" sequence. 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 X-degenerate region, allows the evolutionary relationships of palindromic sequences to be investigated, and a 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, and the directionality of the events (ancestral to derived or vice-versa).

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 world-wide unrelated males, chosen to represent the most

divergent evolutionary lineages of the MSY. At first, we assessed the phylogenetic relationships existing between our samples, through a bioinformatic analysis of deep-sequencing data of 3.3 Mb of the X-degenerate region (D'Atanasio et al. 2018), from which we obtained a detailed and reliable phylogeny based on 7,264 bi-allelic markers. Then, we used this evolutionary stable phylogeny to temporally frame the mutations and gene conversion events observed in the palindromes.

In the first year of the project, we analysed NGS data from the P7 palindrome, the shortest one among those considered in the present study. P7 is a 30 kb palindrome (each arms extends for about 9 kb, separated by a ~12 kb spacer), for which we obtained sequencing data for 9,352 bp overall (4,676 bp each arm), after the removal of repetitive elements.

Since the MSY palindromes are made up of nearly identical duplicated sequences and 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 'pseudo-hemizygous' states. For this reason, for each sample we evaluated the presence of both P7 arms 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 P7 palindrome for the average depth value of the 3.3 Mb of the MSY non-duplicated region, the same that we used to reconstruct the phylogeny. Both approaches (boundary-specific PCR and NGS depth analysis) did not provide evidence for deletions or duplications.

The genetic diversity of P7 palindrome arms was assessed using standard bioinformatic tools (SAMtools, VCFtools, SNPEff). We obtained a list of putative SNPs which underwent subsequent filtering on the basis of the peculiar 'pseudo-diploid' characteristics of paralogue sequences. We assessed the phase of 'pseudo-heterozygous' variants through a long range arm-specific PCR, followed by nested-PCRs and Sanger sequencing. These experimental and bioinformatic approaches resulted in the identification of 19 variant sites in 16 paralogue positions. These sites show both 'pseudo-heterozygous' and 'pseudo-homozygous' states (Figure 1). We detected 10 gene conversion events involving 4 sites (V638, V640, V648 and V651). We found a higher number of conversion events leading to the derived 'pseudo-homozygous' state than the events leading to the ancestral 'pseudo-homozygous' one (9 vs. 1, $p < 0.05$) suggesting that not necessarily gene conversion acts as a mechanism to retain the ancestral state of the variants. Finally, by mapping gene conversion events within our stable phylogeny, we calculated a gene conversion rate for the P7 palindrome of $3.9\text{-}5.4\times10^{-6}$ events/PSV/year. This figure is much higher than the mutation rate for the same region (7.4×10^{-10} mutations/position/year) indicating that gene conversion is the major driving force in the evolution of MSY palindromes.


Legend
pseudo-homozygous ancestral
pseudo-heterozygous
pseudo-homozygous derived
pseudo-homozygous
which back mutated to the ancestral state

Figure 1: 'Pseudo-diploid' sites identified in the P7 palindrome arms (chrY: 17986738-17995460 and chrY: 18008099-18016824, GRCh37/hg19) by sequencing 157 Y chromosomes. To the left, it is reported the Y chromosome tree showing the phylogenetic relationships between the chromosomes analysed. SNP names are given at the top. Each square is divided into two triangles, representing the paralogous sites of the two arms of the palindrome.

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REGULATION OF VITAMIN B6 METABOLISM AND BIOAVAILABILITY IN EUBACTERIA

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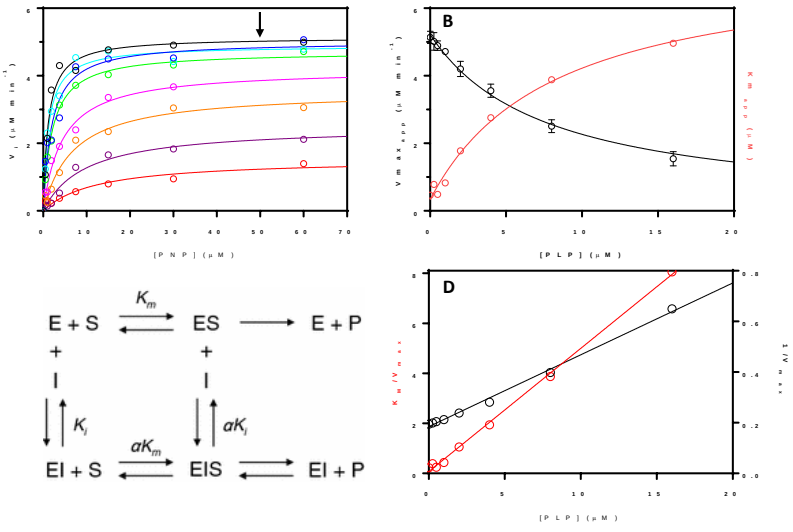
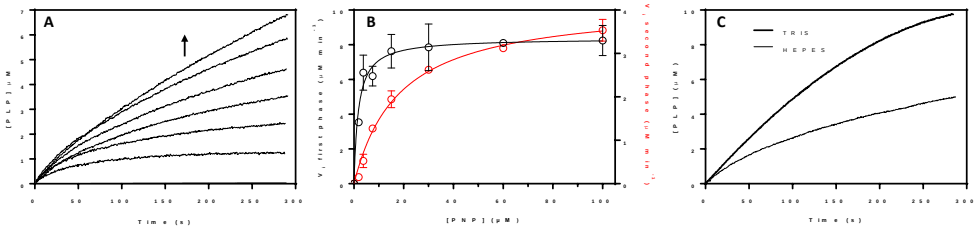
Vitamin B6 plays a fundamental role in physiology and virulence of microorganisms. The aim of our research project is to clarify some obscure aspects of the metabolism of this vitamin in bacteria. The term vitamin B6 refers to six interconvertible vitamers, pyridoxine, pyridoxal, pyridoxamine and their 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. Understanding how these requirements are met, i.e. how PLP availability is regulated and this vitamer is delivered to the apoenzymes that require it as cofactor, is the main target of our project. We intend to investigate several aspects of the regulation of vitamin B6 metabolism and bioavailability, such as the distribution of different biosynthetic pathways in Eubacteria, the regulation of the expression of PLP biosynthetic genes, the transport of B6 vitamers across the membrane and the role of proteins that might serve as PLP carriers involved in PLP homeostasis and delivery to apoenzyme.
The first year of our research activity was mainly focused on the E. coli PLP carrier proteins pyridoxine 5'-phosphate oxidase (PNPO), an FMN-dependent enzyme which converts PNP to PLP, and YggS, a PLP-binding protein with no catalytic activity but with a regulatory function in PLP homeostasis. PLP acts as a product inhibitor of PNPO (Zhao, G., et al. 1995 J. bacteriol. 177, 883-91), however, it also binds with high affinity at a secondary "tight binding site" (PLP-TBS). In vitro experiments showed that this tightly bound PLP is protected by the solvent and at the same time is readily transferred to apo-PLP-dependent enzymes, such as serine hydroxymethyltransferase (SHMT; Yang, E. S., et al. 2000, Arch. Biochem. Biophys. 377, 109-14). Crystallographic studies indicated the possible location of the PLP-TBS on the surface of the enzyme, at a distance from the active site, although apparently connected to it through a tunnel (Safo, M. K., et al. 2001, J. Mol. Biol. 310, 817-26). The actual role of YggS in PLP homeostasis is unknown. In this first year, we also carried out preliminary bioinformatic studies on vitamin B6 metabolism in bacteria.

Results of bioinformatic studies – A homology-based survey on all available bacterial proteomes was carried out to analyse the distribution of DXP-dependent and -independent vitamin B6 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 □-proteobacteria as previously reported (Mittenhuber, G. 2001, J. Mol. Microbiol. Biotechnol. 3, 1-20), but is extended to FCB, PVC and Terrabacteria groups. We are carrying out detailed studies on single species of these groups with the intent to verify whether all genes required for the DXP-dependent pathway are present. Analysis on the association between genes encoding PLP carrier proteins and genes of PLP biosynthesis indicated that 77% of the proteomes containing pdxJ also contain pdxH (encoding PNPO). On the other hand, only 37% of the proteomes containing pdxS (encoding PLP synthase and indicating the presence of the DXP-independent pathway) also contain pdxH. Particularly interesting is the observation that PNPO is present in bacteria that follow the DXP-independent pathway and therefore do not need this enzyme to synthesise PLP. In these bacteria, PNPO may have a role limited to PLP salvage pathway and different functional features.

Results obtained with PNPO – The assumption that PLP binds to E. coli PNPO on a secondary tight binding site (PLP-TBS), spatially distinct from the active site, is based on previous observations that when the enzyme is incubated with PLP and then passed through a size exclusion chromatography column (SEC) it retains PLP with a 1:1 stoichiometry ratio with respect to protein subunits (the enzyme is a homodimer) and maintains unaltered its catalytic activity (Yang, E. S., et al. 2000, Arch. Biochem. Biophys. 377, 109-14). We repeated this experiment and, in contrast, measured a decrease of activity. Product inhibition by PLP had been reported to take place with a Ki of 8 μM (Zhao, G., et al. 1995, J. Bacteriol. 177, 883-91) and attributed to PLP binding at the active site; however, data supporting the competitive nature of product inhibition with respect to the PNP substrate had not been presented. In order to clarify the relation between PLP inhibition and PLP binding to active site and PLP-TBS, we carried out a complete kinetic characterization. Figure 1 shows that increasing concentrations of PLP have the effect to hyperbolically decrease Vmax and increase KM for



PNP (panels A and B). Such a kinetic pattern is against competitive inhibition and is typical of a pure mixed-type inhibition (panel C), indicating that PLP does not compete with PNP for binding at the active site, but it binds to a secondary site, completely abolishing the catalytic activity. Secondary plots of 1/Vmax and Km/Vmax as a function of PLP concentration (panel D) gave inhibition constants of 0.15 ± 0.13 μM (Ki) and 6.5 ± 0.3 μM (αKi) for PLP binding to the free and substrate-bound enzyme, respectively. PLP binding to PNPO was also characterised by means of spectrofluorimetric measurements, taking advantage of the increase in FMN fluorescence observed upon addition of PLP to a PNPO solution. A single binding equilibrium was observed, with an estimated dissociation constant of 0.125 ± 0.035 nM, well in agreement with the Ki for PLP binding to free enzyme. Titration of a concentrated PNPO solution with PLP using the same method gave the stoichiometry of PLP binding to PNPO, which in contrast with previous observations was of one PLP molecule per PNPO dimer. The catalytic activity of PNPO is usually measured in TRIS buffer pH 7.6, in order to remove the produced PLP, which forms a Schiff base with TRIS, and avoid product inhibition (Kwon, O., et al. 1991, J. Biol. Chem. 266, 22136-40). For obvious reasons, we carried out our PLP inhibition kinetics in Na-HEPES buffer pH 7.6. In this occasion, we observed complex kinetics. After mixing substrate and enzyme, an initial phase in which the rate of reaction decelerates is observed, followed by an almost linear phase in which the reaction rate stays constant in the time frame of our observation (Fig. 2, panel A). The initial velocity of the first phase and the velocity of the second linear phase show a hyperbolic dependence on substrate concentration, giving very different saturation curves (Fig. 2, panel B). Estimated values of kcat are 16.7 ± 0.6 and 8.3 ± 0.2 min-1 for the first and second phase, respectively. Values of KM are 1.8 ± 0.4 and 18.2 ± 1.7 μM for the first and second phase, respectively. These KM values are in good agreement with those estimated from the product inhibition analysis in the absence of PLP (0.8 ± 0.3 μM) and in the presence of a saturating concentration of PLP (17.7 ± 1.9 μM). When the PNPO reaction is carried out in TRIS buffer, the first deceleration phase disappears, demonstrating that it is a consequence of PLP accumulation in the solvent (Fig. 2, panel C). These data agree with a mixed-type inhibition by PLP and indicate that as PNP is converted into product, PLP binds to a high affinity secondary site (the PLP-TBS), affecting enzyme activity.



The location of the PLP-TBS site is presently unknown. Published crystallographic data suggested three amino acid residues as responsible for PLP binding at the tight binding site. As a preliminary investigation, we had replaced these residues (N84, K145 and F177) with Ala residues, showing that the triple mutant had a normal catalytic activity

and was still capable to bind PLP tightly. This result indicated that the crystallographic site is not the PLP-TBS. During the first year of our project, we confirmed our preliminary observation by producing and characterising other mutants of the crystallographic site (K145A/F177A and N84W/K145A/F177A) with respect to their catalytic properties and to their FMN and PLP binding properties (Table 1). We carried out docking studies that suggested an alternative location for the PLP-TBS. This is a cleft on the protein surface, mainly delimited by conserved Arg residues and located near the subunit interface. In order to verify the involvement of this docking site in PLP binding, we produced several mutant PNPO forms (R23L, R215L, F202M and R23L/R215L) and characterised them; however, neither mutation resulted in a significant decrease of affinity for PLP (Table 1), indicating that also this hypothetic site is not the PLP-TBS.

Table 1. Kinetic and PLP binding parameters of wild-type and mutant PNPOX forms.

Enzyme form	[1]K_M (μM)	[1]k_{cat} (min^{-1})	[2]K_d FMN (nM)	[3]K_d PLP (nM) ex:280nm
Wild type	1.2 ± 0.1	6.0 ± 1.2	10.6 ± 0.9	125 ± 35
K145A/F177A	2.4 ± 0.4	7.3 ± 0.5	11.5 ± 3.6	391 ± 34
N84A/K145A/F177A	2.7 ± 0.5	2.0 ± 0.3	15.9 ± 2.9	381 ± 13
N84W/K145A/F177A	3.3 ± 0.4	3.9 ± 0.2	24.6 ± 0.5	264 ± 26
R23L	1.3 ± 0.1	4.0 ± 0.8	n.a.	280 ± 18
R215L	2.3 ± 0.9	4.3 ± 1.6	n.a.	486 ± 33
F202M	3.6 ± 0.4	8.2 ±	n.a.	551 ± 25
R23L/R215L	3.9 ± 0.6	5.3 ± 1.9	n.a.	484 ± 34
H199A	[5]70 ± 5.0	[5]8.4 ± 0.6	n.a.	701 ± 34

[1]Kinetic parameters for the PNPOx reaction with PNP as substrate
[2]n.a, data not available
[3]Dissociation constant of the FMN binding equilibrium
[4]Dissociation constant of the FMN binding equilibrium
[5]From di Salvo et al.[6]

The hypothesis that the PNPO active site could coincide with the PLP-TBS was ruled out by the production and characterisation of active site mutants.

Residue H199 is located at the active site and is of fundamental importance for substrate binding, as indicated by its 70-fold higher KM with respect to WT (Gandhi, A. K., et al. 2009, Biochem. Biophys. Res. Commun. 381, 12-5). The H119A mutant, although catalytically impaired, was able to bind PLP, with a Kd which is not drastically higher than that of the wild-type enzyme (Table 1). We also produced a mutant in which the active site R197 was replaced with a Lys residue. This mutant was able to bind PLP through a Schiff base that we reduced with NaBH4 to irreversibly link PLP to the enzyme and fill up the active site. PLP was still capable to bind to the enzyme with a Kd of about 750 ± 50 μM.

All mutant forms also maintained almost unaltered their capability to retain PLP when passed through a size exclusion chromatography column. In these experiments, 300 μM enzyme was incubated with an equimolar amount of PLP in 50 mM Na-HEPES buffer pH 7,6 for 30 minutes and then passed through a Superdex 200 10/300 GL column (GE Healthcare, Little Chalfont, UK) at room temperature and at a flow rate of 0.5 mL/min. The eluted proteins, well separated from any unbound free PLP, were analysed for PLP content. Interestingly, the wild-type protein retained only about 50% of PLP with respect

to protein subunits. Considering that PNPO is a dimer, this percentage corresponds to a molecule of PLP bound per dimer of protein and agrees with the result obtained through fluorometric titration of PLP binding to PNPO. This stoichiometry is against the presence of two symmetric PLP-TBS, such as those indicated by the crystallographic data and the docking studies, and strongly suggests that the PLP-TBS is located along one of the two symmetry axes of the dimer. After a careful analysis of PNPOx crystal structure we have hypothesised that the PLP-TBS may be formed by the two N-terminal ends of the protein and are presently producing a deletion mutant to verify this hypothesis.
Attempts to crystallize WT and mutant PNPO forms, in the absence and presence of PNP, PMP and PLP, are being carried out by our undergraduate student Mihai Sularea, who is visiting Prof. K. Martin Safo (Department of Medicinal Chemistry, Virginia Commonwealth University, Richmond, VA), with the hope that novel structural information on the PLP-TBS will be obtained.
Results obtained with YggS – In collaboration with Prof Safo, we carried out docking studies on E. coli YggS and SHMT that suggested a possible model of interaction between the proteins to form a complex. On the basis of this information, Prof. Valerie de Crecy-Lagard (Department of Microbiology & Cell Science, University of Florida, Gainesville, FL) produced several mutant forms of YggS, concerning residues at the possible protein-protein interface, and found that two of them (E134A and K229A) are not able to complement a □*yggS E. coli* strain. Since E134 and K229 are on the surface of the protein, far from the active site where PLP binds, this result indicates that E134 and K229 may be involved in the interaction of YggS with other proteins. Therefore, we decided to characterize these mutants with respect to their capability to bind PLP and transfer it to apo-SHMT. An E. coli clone expressing YggS as recombinant protein was kindly provided by Prof. de Crecy-Lagard. The preliminary characterization of WT and E134A YggS indicated that for both protein forms Kd of PLP binding equilibrium is about 10 nM, which is a much lower value than that previously published by other authors (0.37 ± 0.49 □M; *Prunetti et al. 2016, Microbiol. 162: 694*-706), confirming that E134 is not involved in PLP binging. We are presently carrying out PLP transfer experiments on WT and mutant forms.

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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 resources provided by the "Anopheles gambiae 1,000 Genome (Ag1000G) project" (www.malariagen.net/projects/ag1000g) to develop and validate novel easy-to-use tools to overcome the limitation of the current approaches in identifying signatures of introgression within the genomes of the two major Afrotropical malaria vectors species, Anopheles gambiae and A. coluzzii (AIM-1). Ag1000G uses high-throughput sequencing of a large number of wild-caught mosquitoes sampled from across Africa (presently 1,142 specimens from 13 African countries) to build a comprehensive catalogue of genetic variation. Based on bioinformatics analysis of data from Miles et al. (2017, NATURE 552, 7683) we developed an Agena iPLEX mass-spectrometry assay to genotype species-specific variants for each chromosomal arm (1) as well as a simple RFLP-PCR to identify SNPs on chromosome-3 (2). Then we applied the assays to shed light on the two case studies of adaptive introgression on a trajectory towards hybrid speciation (3). These 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. In addition, we: (4) are developing molecular karyotyping approaches for main chromosomal inversion polymorphisms believed to have an adaptive value in the 2 species; (5) published 2 papers on the development of a new approach to detect of Plasmodium falciparum sporozoites in mosquitoes and on its application to study malaria transmission patterns in Burkina Faso; and (6) published a paper on the natural female post-mating responses of A. gambiae and A. coluzzii, unravelling similarities and differences in their reproductive ecology. Details are provided below.

1- Development of an Agena iPLEX mass-spectrometry assay to genotype species-specific variants in A. gambiae and A. coluzzii.

We first calculated Allele Frequency Difference (DAF) from Ag1000G phase-1 A. gambiae and A. coluzzii individuals collected from 9 countries in western to east Africa (Miles et al. 2017, NATURE 552, 7683). We then identified species-specific variants with ≥90 DAF with no polymorphism in flanking sequences with using standard IUPAC single-letter notation formatted for MassARRAY® Assay Design (v3.1 Software, Agena Bioscience, San Diego, CA,USA). On a total of 337 SNPs, 216 failed assay design; the remaining 122 valid designs were made split into 4 multiplexes and 104 of them resulted

in a successful mass spectrometry genotyping. Of these, 34 were arranged in a final single multiplex mass-spectrometry assay. Out of the latter 34 variants, 29 were successfully genotyped and 26 showed a >99% concordance between results from Illumina genotyping and from mass-spectrometry assay (N=229 individuals). The 26 most reliable species-specific variants were included in the final mass-spectrometry assay; of these 10 mapped in a 5.5 Mb region on chromosome-X centromere, 6 in a 2.2 Mb region on chromosome-2L centromere, 3 in a 9.5 Mb region on chromosome-2R, 3 in a 251 Kb region on chromosome-3L centromere and 4 in a 37 Kb region on chromosome-3R telomere. The Agena iPLEX mass-spectrometry array was validated by genotyping 343 individual mosquitoes from the Ag1000G Phase-1 samples, with result consistent with those obtained by Illumina sequencing,

2- Development of a PCR-assay to genotype species-specific variants in A. gambiae and A. coluzzii.

While the above Agena iPLEX mass-spectrometry approach is very useful to investigate fine-scale levels of recombination and introgression along the genome, it is not affordable for large-scale studies. We thus 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 two autosomal SNPs situated on chromosome-3 (3R: 42848; 3L:129051) as species-specific SNPs on chromosome-2 are known to be affected by adaptive introgression of kdr mutation associated to insecticide resistance and were more interested in neutral markers of introgression. We validated the PCR-approach on 106 specimens from the High-Hybridization-Zone in Guinea Bissau already genotyped by Agena iPLEX mass-spectrometry approach. The concordance between the results obtained by PCR of one of the two loci with those from the multilocus genotype (loci=6) was ~80% and raised to 96% when considering PCR-results of both loci, showing that a high sensibility of the PCR approach in detecting hybridization events on chromosome-3. A manuscript summarizing these data and those obtained by Agena iPLEX mass-spectrometry array is in preparation.

3- Analysis of adaptive introgression on a trajectory towards hybrid speciation in Guinea Bissau and The Gambia.

A total of 564 indoor-resting An. coluzzii and An. gambiae individuals collected in the High-Hybridization-Zone along a west to east transect from coastal The Gambia to Senegal (N=188; Caputo et al. 2008; 2011) and from coastal to inland Guinea-Bissau (N=376; Vicente et al., 2017) were genotyped by the Agena iPLEX mass-spectrometry array. More than 50% of all the species-specific variants, as well as >50% of loci/chromosome, were successfully genotyped. Three species-specific variants (X-22164043, 2R-49438586 and 3L-380974) had a pass-rate <75% in samples from Gambia/Senegal (likely due to lower DNA quality/quantity) and were excluded from the following analyses. The results showed that hybridization is high in coastal areas and is

almost absent in inland sahelian regions. Also, hybridization is highest in Guinea Bissau, where also the sexual chromosome show evidence of extensive admixture, to the point that the two species are no more recognizable, confirming previous hypothesis of ongoing hybrid speciation (Vicente et al. Sci Rep 2017). Further population genetics and phylogeographic analyses are in progress (manuscript in preparation).

4- Development of a PCR- assay to molecular karyotypization of 2Rb chromosomal inversion in A. gambiae and A. coluzzii.

In collaboration with the group of prof. Nora Besansky (Notre-Dame University, IN, USA), we exploited the results of a bioinformatic analysis to identify SNPs specific for chromosomal paracentric inversions widespread in A. coluzzii and A. gambiae to produce a PCR/RFLP approach to molecularly karyotype 2Rb inversion. We identified and tested 3 candidate tag SNPs with 2Rb karyotype concordance rates with microscopic karytyping >96%, whose allelic state results in the gain/loss of a restriction site cleavable by commercially available restriction enzymes. We designed flanking PCR primers, and tuned conditions for PCR, restriction digestion and agarose gel electrophoresis, using A. gambiae laboratory colonies with defined karyotype. Working in tandem between Rome and Notre Dame, we then validated each of these assays using ~500 cytologically karyotyped A. gambiae and A. coluzzii from 9 African countries. The DraIII assay showed the lowest genotype - karyotype discordance rate in both species and could be used singly if 5% error is within acceptable experimental limits. The other two assays worked well in one but not both species, due to population structure. If a lower error rate is required, use of two assays per mosquito would reduce the error at least two-fold, if specimens with discrepant molecular karyotypes for the two assays are discarded. A manuscript reporting these results is in preparation. Characterization of molecular breakpoints of 2Rc and 2Rd is ongoing and results will be exploited to produce molecular karyotyping tools also for these two inversions. Finally, since we recognise the need for whole-genome karyotyping (as different inversions act cooperatively on phenotypes) we are developing a high throughput approach for molecularly karyotyping multiple mosquitoes at multiple inversions that relies on custom TaqMan assay manufactured onto "OpenArray" genotyping plates, taking advantage of the DNA extracted from the carcasses of ~1800 A. gambiae mosquitoes collected in 2006 from Burkina Faso and cytologically karyotyped under the auspices of a former NIH project.

5- Analysis of sporozoite rates and human-blood index in a LLIN-protected village in Burkina Faso.

Despite the effectiveness of mass distribution of long-lasting insecticidal nets (LLINs) in reducing malaria transmission in Africa, in hyperendemic areas such as Burkina Faso the burden of malaria remains high. We carried out a 4-month survey on the feeding habits and Plasmodium infection in malaria vectors from a village in Burkina Faso one year following a national LLIN distribution programme (Pombi et al. Sci Rep 2018). Low values of human blood index (HBI) observed in the major malaria vectors in the area (A. coluzzii: N=263, 20%; A. arabiensis: 5.8%, N=103) are consistent with the hypothesis that LLINs reduced the availability of human hosts to mosquitoes. A regression meta-

analysis of data from a systematic review of published studies reporting HBI and sporozoite rates (SR) for A. gambiae complex revealed that the SR values (A. coluzzii: 7.6%, N=503; A. arabiensis: 5.3%, N=225, assessed by the method developed by Calzetta et al., Med Vet Entomol 2018) are out of the ranges expected based on the low HBI observed. We hypothesize that a small fraction of inhabitants unprotected by bednets acts as a "core group" repeatedly exposed to mosquito bites, representing the major Plasmodium reservoir for the vectors, able to maintain a high risk of transmission even in a village protected by LLINs. Further studies measuring HBI and sporozoite rates in areas of high levels of pyrethroid resistance, accompanied by information on net usage and the prevalence of malaria in bednet users versus non-bednet users, are ongoing to establish whether the pattern reported reflects contemporary patterns of malaria transmission in areas with high mosquito densities and high (but not complete) LLIN use.

6- Analysis of natural female post-mating responses of A. gambiae and A. coluzzii from Burkina Faso.

Anopheles gambiae and A. coluzzii are known to have radiated only recently and to be reproductively isolated even in areas of sympatry, despite being fertile under laboratory conditions. In females from these species, sexual transfer of male accessory gland products, including the steroid hormone 20-hydroxyecdysone (20E), induces vast behavioral, physiological, and transcriptional changes that profoundly shape their post-mating ecology, and that may have contributed to the insurgence of postmating prezygotic reproductive barriers. As these barriers can be detected by studying transcriptional changes induced by mating, we analyzed the post-mating response of wild females captured in natural mating swarms in Burkina Faso. While the molecular pathways shaping short- and long-term mating-induced changes are largely conserved in females from the two species, we unravelled significant inter-specific differences that suggest divergent regulation of key reproductive processes such as egg development, processing of seminal secretion, and mating behavior, that may play a role in reproductive isolation. Interestingly, a number of these changes occur in genes previously shown to be regulated by the sexual transfer of 20E and may be due to divergent utilization of this steroid hormone in the two species (Thailayil et al. Sci Rep 2018).

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Research Group	**Collaborations**
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HEDGEHOG/GLI SIGNALING REGULATORY NETWORKS IN COLORECTAL CANCER STEM CELLS

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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 first year of the project, we carried out experiments as planned in Task 1 and Task 2 of the research plan.

TASK1: Characterization of the HH-GLI signalling pathway activation in CR-CSCs. The aim of this Task was to characterize the HH-GLI signalling pathway in CR-CSCs derived from human colorectal cancer specimens.
We first analysed patient derived CR-CSCs, already available at the beginning of the study. In particular, we evaluated the expression of some components of the HH-GLI signalling pathway in a series of fourteen CR-CSC lines derived from primary tumours (n=14) (pCSCs). pCSCs were classified according to their KRAS and BRAF mutational status. In detail, 6 were KRAS mutant, 3 were BRAF mutant and 5 were both KRAS and BRAF wild type, recapitulating the genetic variability of colorectal cancer.

We investigated mRNA and protein expression levels of the upstream receptor of the HH-GLI signalling, Smoothened (Smo), and the downstream transcription factors Gli1 and Nanog. Smo resulted expressed at very low levels in 2 out of 14 pCSCs. The transcription factors Gli1 and Nanog resulted to be expressed in 12 out of 14 pCSCs. These data show that pCSC are characterized by the expression of molecular components of the HH-GLI signalling pathway with a heterogeneous pattern.
In addition, we had the opportunity to derive CR-CSCs from metastasis (mCSCs). In detail, we derived and used for the study five mCSCs from liver metastasis of patients

affected by CR cancer. mCSCs were classified according to their KRAS and BRAF mutational status. Specifically, 1 was KRAS mutant, 1 was BRAF mutant and 3 were wild type for KRAS and BRAF. We investigated mRNA and protein expression levels of the downstream transcription factors of the HH-GLI signaling, Gli1 and Nanog, that were expressed in 4 out of 5 mCSCs.
These data show that almost all mCSCs analysed are characterized by the expression of molecular components of the HH-GLI signalling pathway.

TASK 2: Evaluation of the role of HH-GLI signalling pathway in CR-CSCs
The aim of this Task was the analysis of the functional role of the HH-GLI signalling pathway in CR-CSCs.

We first proceeded with the genetic inhibition of the transcription factor Gli1.
We performed experiments to investigate the short hairpin mediated silencing of Gli1 (as planned). For these experiments we used a lentiviral vector already available in our laboratory that was previously used successfully in CSCs derived from lung adenocarcinoma (Po et al Oncogene 2017). However, because of technical reasons, we were not able to obtain a satisfactory down-modulation of Gli1 levels in CR-CSCs. Indeed, efficiency of lentiviral infection was not very high in CR-CSCs (only 15-20% of cells resulted infected) and those infected cells rapidly disappeared from the cell culture, presumably due to the death of Gli1 silenced CR-CSCs or to the rapid outgrowth of non-silenced cells. To overcome this issue, we are now cloning a new lentiviral vector carrying an inducible shGLI1. This new tool will allow the stabilization and selection of infected cells and the evaluation of GLI1 silencing in a doxicyclin dependent way.

We then proceeded with the pharmacological inhibition of Gli1.
We selected CR-CSCs, from both primary and metastatic disease, and we performed drug treatment using two GLI1 inhibitors: Arsenic Trioxide (ATO) (Beauchamp EM et al J Clin Invest 2011) and GANT61 (Agyeman A et al Oncotarget 2014).
Both ATO and GANT61 were able to inhibit Gli1 mRNA levels as well as mRNA levels of its target genes. For this reason, we proceeded with the use of ATO since it is already FDA and EMA approved and currently used in the therapy of adult patients affected by acute promyelocyte leukemia (Kerl K et al Int J Can 2014).

Drug-mediated Gli1 targeting with ATO was performed and cell viability was evaluated by measuring endogenous ATP levels (CellTiter-Glo® Luminescent Cell Viability Assay, Promega). Cells were treated with 1 uM, 2,5 uM, 5 uM and 10 uM ATO for 24, 48 and 72 hours. Concentrations were chosen on the basis of the clinical use of ATO. Indeed, the conventional dose of ATO in acute promyelocytic leukemia patients approved for the treatment is 0.16 mg/kg, that allows a peak concentration of 6–8 uM in the plasma.
This set of experiments was performed on eight CR-CSCs, four of which were from primary colorectal cancer (pCSCs) and four were from liver metastasis (mCSCs). In

detail, of primary CSCs, two were BRAF and KRAs wild type and two KRAS mutated; while of metastatic CSCs, two were BRAF and KRAS wild type, one KRAS mutated and one BRAF mutated. The impairment on CR-CSC after ATO treatments was heterogeneous. All CR-CSCs analysed resulted sensitive to ATO at different concentrations and/or time points. Of note, all eight CSCs tested showed a significant reduction of cell viability after 72 hours of ATO treatment at 5 uM.

The results of this set of experiments show that all pCSCs and mCSCs analysed are sensitive to ATO treatment with a significant inhibition of the expression of molecular components of the HH-GLI signalling pathway and an impairment of cell viability.

Research Group	Collaborations
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HARNESSING REPLICATION STRESS TO UNDERSTAND AND TACKLE MYCN-DEPENDENT TUMORS

GIUSEPPE GIANNINI
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As all protoncogenes of the MYC family, MYCN is known to induce replication stress (RS) and DNA damage by multiple mechanisms, including, but not limited to replications-transcription collisions, shortage of nucleotide pools and increase in the number of firing replication origins. Upon MYCN expression, both acute and transcriptional responses occur to limit the deleterious effect of RS and DNA damage. In example, we recently demonstrated that the MRE11/RAD50/NBS1 (MRN) complex, an important component of the DNA damage response (DDR), is a transcriptional target of the MYCN and is essential to control RS associated with MYCN-driven proliferation. Dysfunctions in this axis impair the fast expansion of neural progenitors occurring during CNS development, as we have shown for cerebellar granule cell progenitors (GCPs) (Petroni et al., Mol Cel Oncol, 2015, Petroni et al. Cell Death & Diff, 2016). Following up on this research line, we have now published that: i) MRE11 is overexpressed in MYCN-dependent tumors, such as neuroblastoma, in cellular models in vitro and in ex vivo samples; ii) its genetic and/or pharmacological inhibition raises the level of RS and DNA damage and causes cell death. By using PLGA-PEG nanoparticles to incapsulate an otherwise insoluble MRE11 inhibitor called mirin, we inoculated this drug in xenograft models of MYCN-amplified neuroblastoma. Mirin induced the occurrence of DNA damage and cell death leading to tumor shrinkage. By this mean, we provided a proof of principle that inhibition of the MRN complex is a potential strategy to target MYCN-dependent tumors (Petroni et al., Cell death & Dis, 2018).
Among the DNA repair proteins involved in the response to MYCN-dependent RS and DNA damage, we have recently demonstrated the relevance of PARPs and the deleterious effects of their inhibition by pharmacological inhibitors, such as olaparib (Colicchia et al. Oncogene, 2017). Indeed, by trapping PARPs onto damaged DNA, olaparib further raise MYCN-dependent RS, evoking a CHK1-driven S-phase checkpoint which prevents mitotic entry with broken DNA. Nevertheless, a small, but consistent amount of MYCN overexpressing cells enter mitosis and undergo mitotic catastrophe, after olaparib treatment. Inhibition of the CHK1-driven S-phase checkpoint anticipate and increase the rate of mitotic catastrophe induced by olaparib (Colicchia et al. Oncogene, 2017). Interestingly, CHK1 inhibitors are being tested in clinical trials. It Is known that CHK1 belongs to the transcriptional response to MYC genes required to limit the deleterious effects of RS. Significant work suggested the use of CHK1 inhibitors

to treat MYCN amplified neuroblastoma. Based on all these evidences and on our own data, we speculated that the association between CHK1 and PARP inhibitors might increase the level of RS and induce cell death in MYCN-dependent tumors. We have now investigated this issue systematically using a panel of MYCN- amplified and MYCN single copy neuroblastoma cell lines. Indeed, we showed that this drug combination synergistically act to kill MYCN-dependent tumors by rising RS and DNA damage. We are working to address whether this approach might be efficiently transferred also in animal models.

A second line of research focuses on the role of the MRN complex in cerebellar development and tumorigenesis. It is known that inherited mutations in the NBS1 (or NBN) gene cause the Nijmegen breakage syndrome, an autosomal recessive DDR-syndrome. In mice, CNS-restricted inactivation of the Nbn gene results in several abnormalities including microcephaly, growth retardation, cerebellar defects and ataxia. Loss of Nbn causes proliferation arrest of cerebellar GCPs, whose postnatal expansion is typically due to Sonic Hedgehog (Shh)-Nmyc pathway. To test whether a constitutive activation of Shh-Nmyc signalling may compensate for the defective proliferation of GCPs observed in NBN-KO mice, we crossed a Shh-constitutive and neural specific mouse (ND2-SmoA1 mouse) with the NBN KO model. Surprisingly, we obtained evidence for a dual role for NBS1, depending on its genetic state in the animal models. Indeed, NBN biallelic KO completely suppresses SmoA1 phenotype. While this might be partially due to loss of NBN function in controlling MYCN-dependent RS, our preliminary evidences also suggest that NBN defect is epistatic on SHH pathway. Indeed, SmoA1-NBN-KO GCPs show impaired Shh-pathway activation in vivo and in vitro.

On the other side, monoallelic loss of NBS1 facilitate SmoA1 induce medulloblastoma development. In order to understand this dual role of NBS1 in cerebellar development and tumorigenesis, we are developing mice and primary cellular models to better investigate on the molecular and cellular origins of this phenotype.

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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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Microbial infections represent one of the most serious life threats leading to almost 50,000 deaths/year in Europe and USA. Remarkably, this number is expected to grow up to tenfold by 2050, killing more than cancer. This is mainly due to the rising emergence of microbial strains that are resistant to the available antibiotics whose future seems to be quite bleak, in the face of an urgent need for alternative strategies to address the vital problems of infectious diseases.
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 form sessile communities (i.e. biofilms) trapped in a matrix protecting them from chemicals, antibiotics and host cells phagocytosis. They can easily colonize host tissues which are most exposed to the external environment, such as the respiratory tracts or the ocular surface. 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 contrast with traditional antibiotics that are active only against the planktonic form of bacteria and generally recognize specific targets or biochemical pathways, the majority of these AMPs display multiple activities, of which membrane damage is of major importance.
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. Remarkably, one of these AMPs, i.e. Esc(1-21) and particularly its diastereomer Esc(1-21)-1c carrying two D-amino acids, i.e. DLeu14 and DSer17 (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. Furthermore, a promising strategy to circumvent AMPs' short half-life and cytotoxicity is given by their immobilization on biomaterials surface.
In line with the above, the major objectives of the present proposal are (i) to develop our frog skin AMPs/optimized analogs for new antimicrobial formulations to treat

lung/ocular surface microbial infections, and (ii) to develop peptide-coated contact lenses (CLs) capable of preventing microbial colonization of lenses and therefore the emergence of keratitis.

An attractive approach to achieve the first goal is shown by the usage of nano-systems capable to shield the peptide and to lead it up to the target site. In this context, we encapsulated Esc peptides inside biodegradable polymeric NPs made of poly(lactic-co-glycolic) acid and engineered with the hydrophilic polymer poly-vinyl alcohol. They represent an excellent strategy to overcome lung barriers (i.e. the sticky mucus lying the airways epithelia, mostly in cystic fibrosis sufferers) that usually interfere with the antibiotic treatment; and to provide a sustained drug release, limiting the number of drug administrations and side-effects. The in vitro and in vivo characterization of these Esc peptide-loaded NPs are in progress.

With reference to the second aim, we covalently bound Esc peptides to hydrogel soft CLs. CL wear is a risk factor for development of microbial keratitis, a vision threatening infection of the eye. Adverse events associated with colonization of lenses, especially by P. aeruginosa remain a major safety issue. We initially analyzed the activity of the Esc peptides on P. aeruginosa biofilm formed on soft CLs. Microbiological assays and scanning electron microscopy (SEM) analysis indicated that the peptides were able to disrupt the bacterial biofilm, with the diastereomer having the greater efficacy (up to 85% killing. Fig. 1)

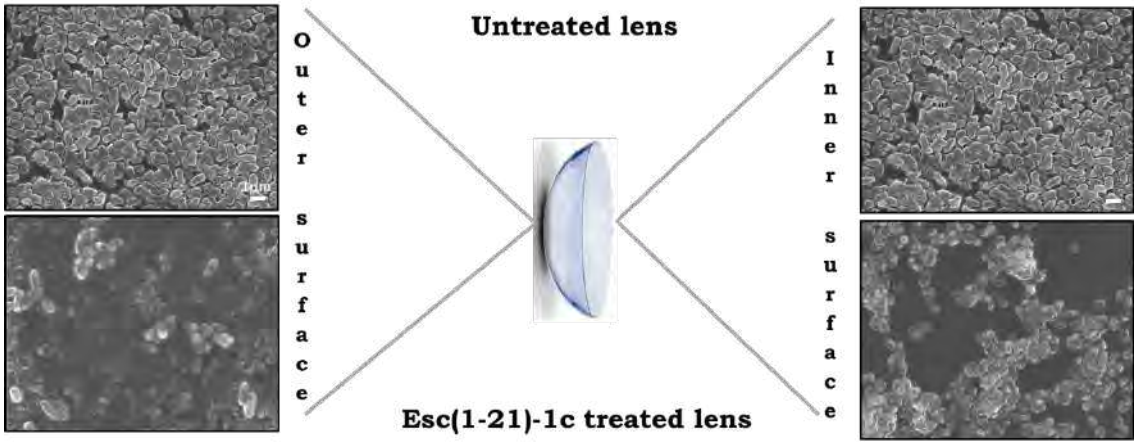

Fig. 1. SEM of P. aeruginosa ATCC 27853 biofilm formed on both (inner and outer) sides of soft CLs and after 2 hours treatment with Esc(1-21)-1c at 16 □M

Importantly, upon covalent immobilization to etafilcon A CLs which contain methacrylic acid (MAA) in their matrix, the two peptides were found to cause more than four log reduction in the number of bacterial cells within 20 minutes and to reduce bacterial adhesion to the CL surface (77%-97% reduction) in 24 h (Fig. 2). This indicated that covalent attachment of the peptides to CLs via carbodiimide mediated coupling

represents an effective strategy to achieve an antimicrobial surface which is bactericidal against Pseudomonas and with the ability to inhibit the establishment of an infection, once the antimicrobial CL is placed on the eye. Remarkably, peptide immobilization did not make CLs toxic to mammalian cells and did not affect the lens surface parameters (i.e. diameter, central curvature, hydrophobicity). Overall, our data have suggested that both peptides have great potential to be developed as novel pharmaceuticals for prevention and treatment of CL-associated P. aeruginosa keratitis.

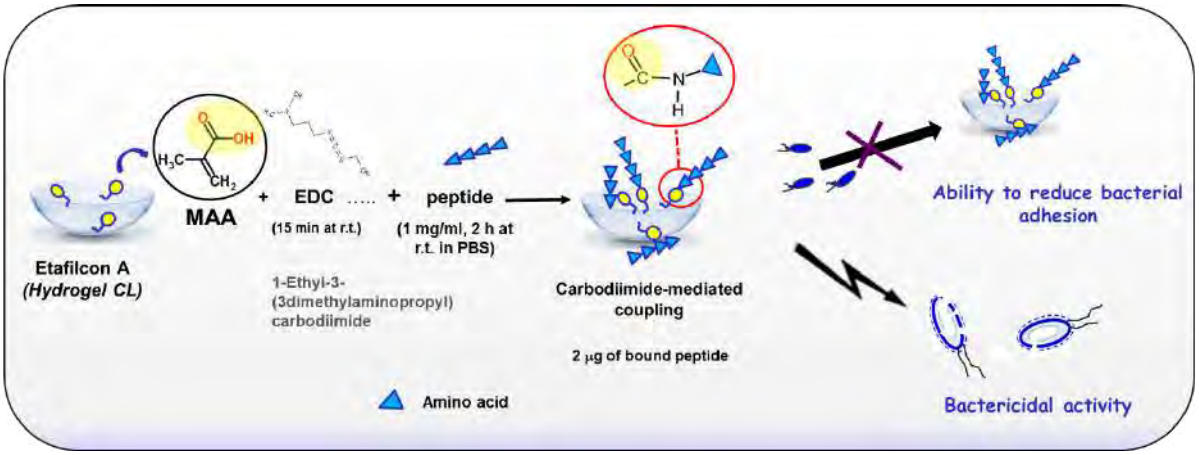

Fig. 2. Schematic representation of peptide-coated CLs and their properties

In parallel, besides displaying direct killing activity against a large spectrum of pathogens, these frog skin-derived AMPs have also been found to show insulinotropic and ß-cell protective activities. Esc(1-21) produced concentration-dependent stimulations of insulin release from BRIN-BD11 rat clonal ß-cells, 1.1B4 human-derived pancreatic ß-cells and isolated mouse islets with no cytotoxicity at concentrations of up to 3 µM. The analogue Esc(1-21)-1c was less potent in vitro. However, its intraperitoneal injection in mice together with a glucose load improved glucose tolerance with a concomitant increase in insulin secretion, whereas the all-L peptide was without significant effect on plasma glucose levels. This suggests that the multifunctional peptide Esc(1-21)-1c constitutes a template also for development of compounds to treat Type 2 diabetes. Similar results were found for the temporins family.
Furthermore, another goal of this Project was to investigate the antiviral activity of the selected frog skin AMPs. Among enveloped viruses, the herpes simplex virus 1 (HSV-1) is widespread in the population, and in most cases its infection is asymptomatic. The currently available anti-HSV-1 drugs are acyclovir and its derivatives, but long-term therapy with these agents can lead to drug resistance. Thus, the discovery of novel anti-herpetic compounds deserves additional effort. We demonstrated the in vitro anti-HSV-1 activity of temporin B (TB). In particular, when HSV-1 was pre-incubated with 20 µg/ml TB, significant antiviral activity was observed (5-log reduction of the virus titer). Such

an effect was due to the disruption of the viral envelope, as demonstrated by transmission electron microscopy (Fig. 3). Moreover, TB partially affected different stages of the HSV-1 life cycle, including the attachment and the entry of the virus into the host cell, as well as the subsequent post-infection phase. Furthermore, its efficacy was confirmed on human epithelial cells, suggesting TB as a novel approach for the prevention and/or treatment of HSV-1 infections.

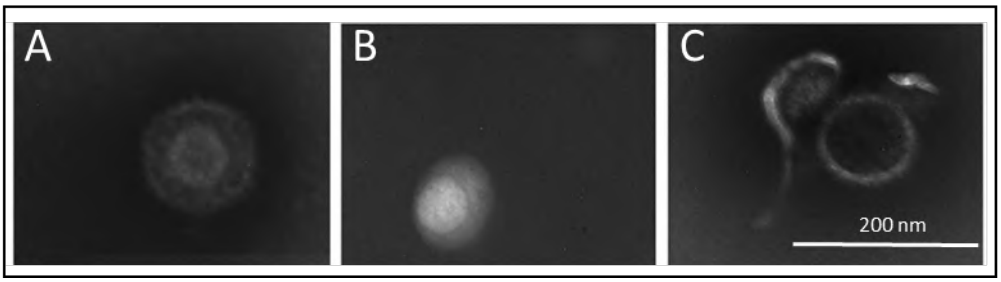

Fig. 3. Morphology of HSV-1 viral particles analyzed by transmission electron microscopy. Untreated particles with intact envelope, tegument and nucleocapsid (A) and TB-pretreated virions (B,C) where envelope integrity is evident, confirming the virucidal activity of TB

Finally, peptidomic analysis of norepinephrine-stimulated skin secretions from the Trinidadian leaf frog Phyllomedusa trinitatis led to the identification and structural characterization of new 26 host-defense peptides. On the basis of amino acid sequence similarity, the peptides were divided into the followings groups: dermaseptins, plasticins and phylloseptins, supporting the notion that P. trinitatis and P. tarsius are very closely related phylogenetically but are probably not conspecific.

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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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Anisakis is considered by the EFSA as the most important Biological Hazard in the "seafood". It is a heteroxenous parasite, involving marine organisms (crustaceans, fish, squids, cetaceans) in its life-cycle. Humans may act as accidental host, acquiring infection through consumptions of raw, smoked, marinated salted, or undercooked fish and squids infected by larval stages. Worldwide, there is an increasing recognition that the fish-borne zoonosis, known as Anisakiasis, is an important emerging human disease. So far, *A. pegreffii* and *A. simplex* (s. s.) are known as the agents of gastric, intestinal and allergic anisakiasis. Main scope of the Project is to improve the knowledge on the molecular and immunological mechanisms involved in the disease due to those parasites by a multidisciplinary and multi-methodological approach. Specific objectives of the project are to: 1) characterize the variety of proteins/antigens of *Anisakis pegreffii* and *A. simplex s.s.*, that are involved in the human IgE-hypersensitivity; 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 (DCs have a key role in orchestrating the immune response); 4) assess the presence and role of Microvesicles (MVs) and miRNA 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 2018, the investigations carried out in the following objectives, have produced the following results:

Objective 1: During all the stages of their life cycle, the nematode parasites produce and release a series of excretory and secretory products (ESPs), which may be key players in the parasite-host interaction. ESPs have several functions during the parasites' infection, e.g. penetration of host tissues and evasion of host immune responses, but are, at the same time, known to elicit immune responses (including antibody production) both in natural and accidental hosts. The ESPs released by parasites are also assumed to be key players in clinical manifestation of the disease in humans. Those molecules in *Anisakis* spp. are retained to play a general biological role in both invertebrates and

vertebrates. In general, *A. simplex* (s. l.) ES products/antigens are known to be more potent, which could be a result of their higher affinity to specific IgE, compared to the somatic ones (SA). Studies carried out in the present project with the aim to characterize those proteins/antigens in the species *A. pegreffi*, the parasite responsible for the human cases of anisakiasis so far reported in Italy, have allowed to discover that, among the 14 antigens/allergens so far detected, *Apeg-1* (24 kDa), *Apeg-7* (139 kDa) and *Apeg-13* (37 kDa) were recognized by IgE response in immunoblotting (WB), in a large number of sensitized patients, and in up to 100% of patients with gastro-allergic anisakiasis. They have been detected as the most important ESP antigens/allergens involved in the human immune response. Whereas, the allergens *Apeg-2* (a paramyosin; 97 kDa) and *Apeg-3* (a tropomyosin; 41 kDa), which are somatic antigens (SA), thus generally considered as panallergens of *Anisakis* spp., were found to be primarily responsible for cross-reactivity in patients showing chronic urticaria. Those proteins may indeed cross-react with homologous antigens of other parasites, i.e. ascarid nematodes (e.g. *Ascaris* spp., *Toxocara* spp.), or arthropods (German cockroach, chironomids, prawns and house dust mite). These studies have thus permitted not only to characterize those antigens in the species *A. pegreffii,* but also to perform molecular epidemiological studies on human anisakiasis, and gather information about the occurrence of human IgE-hypersensitivity to the parasite species.

Objective 2: Besides their role as antigens/allergens (objective 1), 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 the present objective, we aimed to evaluate the impact of thermal conditions on the gene expression patterns of those target molecules, which are putatively involved in the triggering of IgE mediated immune response in humans. Thus, as first, those molecules proteins, i.e. *A.peg1* (24 kDa), *A.peg-7* (139 kDa) and *A.peg-13* (37 kDa) of the species *A. pegreffii*, having a major role in human IgE-immune response, were sequenced. cDNA sequences of the *A.peg1*, *A.peg-7* and *A.peg-13* genes were first obtained, and species-specific primers to be used in qReal-time PCR, were designed. qRT-PCR was used to determine the transcript levels of those proteins in *A. pegreffii* larvae maintained *in vitro*, under the effect of different temperature. It was found that a significant high level of *A.peg-1* transcripts have been produced at 20°C and at 37°C after 24h with respect to the control (0°C); this finding suggests a possible role of *A.peg-1* in tissue invasion in both intermediate/paratenic (fish), as well as in the accidental (humans) hosts. On the other hand, in support of those experimental findings, *A.peg-1* was also recognised, in our studies, by the IgE immune response in the human serum after 24h from the larval detection invading the human gastric mucosa in Gastro-Allergic-Anisakiasis (GAA) due to *A. pegreffii*. Whereas, the results so far obtained, showed that the transcripts of *A.peg-1* do not significantly

increase at the temperature of 7°C, with respect to the control larval sample maintained at the 0°C temperature. This result seems to be in accordance with the findings obtained in our previous experiments, showing that *A. pegreffii* larval motility and invasive capacity in the tissues of naturally infected hosts (fish) increases at temperature >7°C, with the significant high level of larvae found infecting the fish host musculature, at the temperature of 20°C after 24h of the fish storage. Conversely, other molecules seem to be upregulated under cold temperature; indeed this study suggests that transcripts of the mioglobin *A.peg-13,* a protein which has an adaptive role in parasites, in *A. pegreffii* larvae is higher at low temperature (7°C). Finally, in our experimental study, the transcripts of *A.peg-7* did not change significantly at the chosen temperature conditions. *A.peg-7* is a glycoprotein which has been considered as one of the major excretory/secretory (ES) antigens, being recognized in WB by IgE human response in invasive anisakiasis. On the other hand, it is known that some products among the ESPs of the parasites can down-modulate the host immune response. For instance, in the case of *Schistosoma mansoni* eggs, an ES omega-1 glycoprotein was found to promote Th-2 skewing of dendritic cells (DCs) and T cells during infection. DCs are the APcells performing an essential role in the regulation of adaptive immune response. DCs are equipped with a wide range of receptors (PRRs-pathogen recognition receptors) for the recognition of parasites. Among the PRRs, the C-type lectins recognize carbohydrate structures on self and non-self glycoproteins and glycolipids. In particular, Macrophage C-type lectin (MGL) has been identified as selective binder of the carbohydrate residue GalNAc-O-S/T (Tn) carried by several parasites. Thus, 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 (see objective 3).

This study represents the first experimental work aimed to evaluate the transcripts of some ES products *(i.e A.peg-1*, *A.peg-7* and *A.peg-13*) by the species *A. pegreffii*, *in vitr*o cultured, under different thermal conditions. Particularly, the expression levels of *A.peg-1* and *A.peg-13* were found to be sensitive to the temperature variation, and it is suspected to enhance larval parasite tolerance and adaptive response to the host microhabitats and immune response. This finding would further suggest that transcripts of some enzymatic proteins (i.e. *A.peg-1*) could be used as a biomarker of the infection with *A. pegreffii* larvae, in homeothermic hosts. The data so far acquired in this part of the study will be object of a manuscript in preparation, to be submitted for its publication. Further, the differential expression of these and others molecules by the two zoonotic parasites (i.e. *A. pegreffii* and *A. simplex* (s. s.), involved in the larval invasiveness in hosts' tissues, are also in progress.

Objective 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. Which are the ligands in *A. pegreffii* that are involved in such immunomodulatory effects remains an open question. Recently, the C-type lectin receptors, recognizing carbohydrate ligands, seem to play a key role in triggering immunosuppressive functions of DCs by helminths. Interestingly, *A.peg-7*, among the major antigens/allergens of *A. pegreffii*, is indeed a glycoprotein.

The post-translational modification that decors the protein array of the anisakid species *A. pegreffii* may be extremely relevant to understand the mechanisms underlying the impact of the parasite on innate immunity and DCs, in particular in order to manage and prevent the immune-mediated pathological responses caused by this zoonotic parasite.
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.*

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

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The aims of the project are to define the pathogenic mechanisms of sarcopenia and the metabolic disorders leading to sarcopenic obesity, to disclose muscle and systemic/serological factors that can be used as biomarkers of muscle frailty, and to verify potential epigenetic alterations that impinge muscle stem cells activity and muscle function in sarcopenic conditions.
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 also 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.
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. To this purpose, we established the best experimental conditions to induce evident obesity in adult wild type mice. We used a High-fat diet (HFD) approach to induce obesity in both 4 and 24-week-old mice. Four and 24-week-old C57BL/6 mice were divided into two groups and were fed either a high-fat diet (HFD) (19% protein, 36% fat and 35% carbohydrate [g/weight, Diet F3282] or received continuous feeding of a normal diet (ND) (18.9% protein, 5.7% fat and 57.3% g/weight carbohydrate) for up to 3 months. Animals had ad libitum access to food and water. Food intake and body weight were measured once a week. Body weight was higher in mice fed the HFD already after the first week. The mice were sacrificed after 3 months of treatment and muscles and blood samples were collected. Preliminary results

suggest that sarcopenic obesity is associated with increased plasma level of pro-inflammatory markers, including interleukin (IL)-6, and reduced IGF-1 expression. Among pathways that regulate protein turnover, insulin-like growth factor 1 (IGF-1), together with cascade of intracellular effectors that mediate its effects, has been implicated in many anabolic pathways in skeletal muscle, where it plays a central role in cell growth and survival, proliferation, differentiation, metabolism and muscle regeneration. In light of its role, the age-dependent decline could be responsible to the loss of muscle mass and strength associated to sarcopenia. IGF-1 exists in different isoforms, that might exert different role in skeletal muscle. At first, we studied the effects of two full propeptides IGF-1Ea and IGF-1Eb selectively expressed in skeletal muscle, with the aim to define whether and through which mechanisms their overexpression impacts muscle aging. We observed that only IGF-1Ea expression promotes a pronounced hypertrophic phenotype in young mice, which is maintained in aged mice. Nevertheless, beside the promotion of muscle growth, both IGF-1Ea and IGF-1Eb are able to counteract sarcopenia, activating pathways normally affected during aging, namely autophagy and PGC-1-mediated signaling, which control two important destabilizing factors associated with sarcopenia: the removal of dysfunctional mitochondria, that could produce an excess of ROS, and the maintenance of NMJ integrity that guarantees muscle function and muscle-nerve interplay. Interestingly, we observed a significant up-regulation of relevant molecular markers of the autophagic pathway in aged muscles of both IGF-1Ea and IGF-1Eb mice, compared to wild type mice. Our data also revealed that the activation of autophagic pathway underlies the ability of both isoforms of IGF-1 to preserve the integrity and the morphology of NMJ during aging, protecting muscle fibers by denervation. In addition, the modulation of PGC-1α, by IGF-1Ea and IGF-1Eb isoforms, emerges as a key aspect of the ability of the two IGF-1 isoforms to counter sarcopenia. There is growing recognition of the central roles of inflammatory cytokines, such as IL-6, in aging-induced sarcopenic phenotypes. We observed the negative modulation of IL-1ß and IL-6 levels by both IGF-1Ea and IGF-1Eb overexpression, counteracting the inflame-aging process.

One of the mechanisms attributed to the loss of muscle mass during aging is a preceding myofiber denervation. We aimed to determine if IGF-1 expression would counteract neuromuscular junction (NMJ) degeneration during aging, based on the evidence that age-related autophagy inhibition, affects neuromuscular synaptic morphology and function and that, under conditions of denervation, IGF-1 is able to preserve the NMJ functionality and prevent the consequent muscle atrophy. We examined the morphology of postsynaptic plaques in IGF-1Ea and IGF-1Eb mice during aging, comparing them with those of wild type mice. Histological analysis revealed marked alterations in the NMJ of aged wild type mice, compared to NMJ of age-matched IGF-1Ea and IGF-1Eb mice. In particular, while adult wild type endplates displayed the classical pretzel-like shape, aged wild type endplates were dispersed and extensively fragmented. Quantitative analysis of the maximum projection images of NMJ revealed that the postsynaptic primary gutters and the morphological integrity were more maintained and

preserved in both IGF-1Ea and IGF-1Eb during aging, compared to wild type mice, suggesting that expression of IGF-1 isoforms counteracts NMJ fragmentation. To support this observation, we performed gene expression analysis for the gamma subunit of AChR (AChRγ), which is closely related to the innervation status. AChRγ is normally expressed at high levels in muscle during embryonic development and perinatally, whereas its expression is low or undetectable in a normal active or disused adult muscle. Conversely, AChRγ expression increases in denervated muscle or under conditions that alter the NMJ functionality. Real time PCR analysis revealed that AChRγ expression was dramatically up-regulated during aging in the muscle of wild type mice and was significantly reduced in the muscle of both IGF-1Ea and IGF-1Eb aged mice. Our data are consistent with a model (Figure 1) in which muscle expression of either IGF-1Ea or IGF-1Eb, activating a series of anabolic and compensatory pathways, are able to guarantee muscle homeostasis and to prevent muscle loss, a normal muscle-nerve interaction, counteracting sarcopenia.

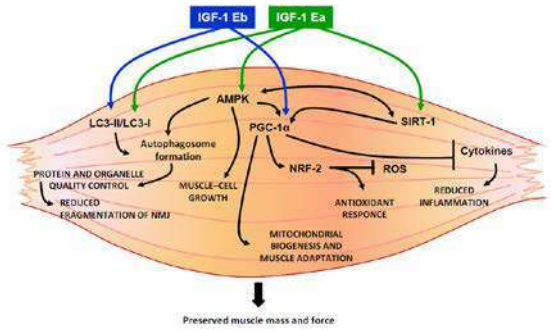

Fig. 1 *A summary of the molecular pathways responsible for the protective role of IGF-1 isoforms against sarcopenia.*

Based on these data and preliminary results, we also defined whether a selective muscle perturbation in redox signaling promotes sign of sarcopenia. Interestingly, we demonstrated a causal link between localized increased oxidative stress, mitochondria morphology and function, and destabilization of NMJ. In our study, we also disclosed the molecular mechanisms by which muscle perturbation in redox signaling induces NMJ dismantlement. Based also on preliminary data on HFD mice, we analysed the transgenic mice that express increased plasma levels of IL-6 for sign of sarcopenic phenotype. The initial finding of this study is that increased circulating levels of IL-6 modulates muscle growth and size. In particular, we observed that different muscle compartments are differently affected by increased levels of circulating IL-6.

Further studies will define the mechanisms by which increased plasma levels of IL-6 contribute to sarcopenia and whether IGF-1 expression is also able to counteract sarcopenic obesity

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"UNDER 45" RESEARCH PROJETCS

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SEARCH FOR IMMUNE-RELATED PATHOGENETIC MECHANISMS OF AORTIC ANEURYSM, INDUCED BY DISRUPTED TGFB SIGNALING IN SMOOTH MUSCLE CELLS, TO FIND NOVEL THERAPEUTIC TARGETS.

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Aneurysms are focal dilatations in the wall of an artery that are often asymptomatic until dissection or rupture occurs (Milewicz & Ramirez 2019). Thoracic aortic aneurysms (TAA) have a strong genetic basis, and often develop in individuals affected by hereditary connective tissue disorders (Milewicz & Ramirez 2019). Very few pharmacological therapies exist, and the only proven treatment option to prevent rupture is surgical repair.
Studies in the last decade have considered TGF-ß activation as the mechanism responsible for aneurysm development in different diseases (Gillis et al, 2013). Involvement of TGF-ß signaling was initially established in patients with Marfan syndrome and in a mouse model carrying a mutation frequently found in this disease (Habashi et al, 2006). The role of TGF-ß in the development of TAA was also investigated in patients with mutations of various genes of the TGF-ß pathway (TGFBR1 and TGFBR2) in the context of different variants of Loeys-Dietz syndrome, a disorder with significant phenotype overlap with MFS. However, the signaling of TGF-□ is complex, requiring a multifaceted approach to be investigated. Binding of TGF-ß to tetrameric receptor complexes composed of 2 type I (TßRI) and 2 type II (TßRII) subunits, induces receptor-mediated phosphorylation of intracellular signaling mediators Smad2 and Smad3 (mothers against decapentaplegic homolog 2 and 3) at the C-terminal Ser-X-Ser motif; binding of phosphorylated Smad2 and Smad3 (p-Smad2/3) to Smad4 induces translocation of this complex to the nucleus, and transcription of TGF-ß target genes in concert with other transcription and chromatin remodeling factors. In addition, binding of TGF-ß to its receptors can also activate Smad- independent pathways, with temporal dynamics that may vary depending on cell type.
In order to gain insights in the complex role played by TGF-ß signaling pathway in TAA, we have developed a mouse model with selective postnatal inactivation of Smad4 in SMCs (generating *Smad4*-SMC^{iko} mice), allowing to reproduce a condition of defective TGF-ß signaling in adulthood. We have reported that *Smad4*-SMC^{iko} mice develop a spontaneous aortic pathology after 45 days of TGF-ß signaling inactivation in SMC, manifested as aortic dilation and aneurysm formation (Da Ros et al, 2017). During 16 weeks, mice showed severe TAA and increased mortality for aortic ruptures. At the examination of aortic tissue, a significant infiltration of immune cells was found,

suggesting that requirement of TGF-ß in SMC to balance homeostasis and inflammation formation (Da Ros et al, 2017). Interestingly, histological analysis revealed progressive fragmentation of elastic lamellae with increased cellularity at sites of breaks in *Smad4*-SMC^{iko} mice as compared to control mice. In addition, mutant mice also showed alterations at the media-adventitia border of the aorta, manifested as proliferation of adventitial fibroblasts and infiltration of CD45^{+} cells, localized especially in regions of severe fragmentation of elastic lamellae. Inflammatory infiltrates at sites of disarrangement of aortic wall structure were rich in macrophages positive for CD11b and CD68; on the contrary, the presence of CD3^{+} lymphocytes was negligible. The infiltrate of immune cells in the aortic walls was better characterized by flow cytometric analysis of aorta single cells suspension. In particular, we have found a significant infiltrate of CD11b^{+} pro-inflammatory monocytes (marked as CD11b^{hi}Ly6C^{hi}F4/80^{low} cells) and macrophages (marked as CD11b^{hi}Ly6C^{low}F4/80^{hi} cells).
Although several cytokines were found to be altered by the inactivation of Smad4, IL1-ß and Ccl2 were the dominant pathways implicated. Interestingly we found that the inactivation of Smad4 in SMCs led to an early up-regulation of IL-1ß in a cell-autonomous way. Then in turn, the production of IL-1ß activated the Ccl2-CCR2 chemokine axis for later recruitment of innate immune response, thus suggesting that a more complex non-cell autonomous immune mechanism is required to make the pathology progress over time.

Research aims

The project proposed pursues the specific aim to unravel the role of resident versus recruited innate immune system in the aortic pathology of *Smad4*-SMC^{iko} and to characterize novel molecular mechanisms targetable for innovative therapies. The project has been conceived with two major work packages, where **WP1** will dissect the role of the immune system and **WP2** will assess potential therapeutic implications of the resulting mechanistic findings.
Specific aims of this project are:

- To investigate the non-cell autonomous determinants involved in the TAA provoked by inactivation of TGF-ß signaling in SMC, by characterizing the contribution of circulating immune system vs secondary lymphoid organs and determining the role of resident immune cells in the aorta.
- To investigate novel therapeutic implications of the vascular/immune mechanisms involved.

Results

As described above, we observed the presence of key features of inflammation and innate immune cells activation in our model of Smad4 dependent aortic aneurysms. Although the existing paradigm of macrophage origins in vascular disease has always emphasized recruitment of bone marrow or splenic derived monocytes via the circulation (Swirski et al, 2009), more recent data suggest that in some conditions, like the atheroma,

macrophage activation and burden may be maintained predominantly by local proliferation (Robbins et al, 2013). Thus, we have performed a set of experiments aimed at investigating various routes of innate immune cells recruitment/activation in the aortic walls.

At the flow cytometry analysis of innate immune cells infiltrating the aortic walls of mice with Smad4 inactivation in SMC, as compared to control mice, we found a significant increase of macrophages and monocytes. The increased number of infiltrating macrophages could be due to proliferation of cells in the aortic walls or to continuous recruitment from circulation or immune reservoirs.

We have previously found that IL-1ß is upregulated before infiltration of monocytes and macrophages, suggesting a role of this cytokine in transferring signals from the aortic walls to the immune system, which is activated in a second stage. By generating a further murine model where the ablation of *Smad4* in SMCs, we have already shown that mice with *Ccr2* deficiency (*Smad4*-SMC^{iko};*Ccr2*$^{-/-}$) were partially protected from the progression of aortic aneurysm severity, although infiltrating macrophages were still present in the aortic walls. Thus, even though a role of recruited immunity was highlighted, we could not rule out a further role of resident macrophages.

Here we have evaluated the potential contribution of various reservoirs of innate immune cells, able to respond to the Ccl2/CCR2 axis. The current paradigm of immune system activation in vascular diseases strongly points to a contribution of splenic-derived monocytes that would account for the macrophage burden in vascular tissues upon pathological conditions (Swirski et al, 2009). So far, there is no evidence of a role of this reservoir in aortic aneurysm. To evaluate the contribution of spleen versus circulating and resident macrophages to the process of immune system activation in the aortic walls of aneurysm, we have splenectomized mice before inducing *Smad4* deletion with tamoxifen (or vehicle in the control group). Then, by serial echographic analysis, we monitored the development and the progression of aneurysm, finding that no difference was determined by the absence of the spleen. In fact, both splenectomized and sham mice, after inactivation of Smad4 in SMC, showed the same timeline of aortic dilation and aneurysm progression, thus ruling out the role of this immune reservoir in the innate immune activation infiltrating the aortic walls.

The pool of monocyte/macrophages recruited by the Ccl2/CCR2 axis could also be dependent from the circulating monocytes. Thus we performed an additional experiment by intravenously administrating clodronate liposomes or pbs liposomes as control to mice with Smad4 inactivation in SMC. At the early histological analysis, we found that although blood monocytes were depleted, aortic macrophages were still present in the aorta, thus indicating that arterial macrophages residing within the vessel may be directly activated by the loss of TGF-ß signaling. Moreover, we also found that, despite the depletion of circulating monocytes and macrophages, IL-1ß activation in SMC was still present after Smad4 inactivation. Currently, the long term evaluation of aneurysm progression after depletion of macrophages with clodronate liposomes is still ongoing. Definitive results will be delivered in the next months of the projects. However, taken

together these partial data were suggestive of the existence of further mechanisms of immune activation in the aortic walls of *Smad4*-SMC^{iko} mice.
It has been typically recognized that macrophages in tissues like the aorta arise from transformation of Ly6C^{hi} recruited monocytes or direct recruitment of Ly6C^{low} monocytes. While the recruitment of the first population usually relies on the Ccl2/Ccr2 axis that we have already tested, the second wave of Ly6C^{low} monocytes depends on a mechanisms activated by CX3CR1, which is a receptor for fractalkine (Nahrendorf et al, 2007). In order to test the contribution of the latter one, the model of Smad4 deletion in SMCs was backcrossed on a background null for CX3CR1 receptor (*Smad4*-SMC^{iko};*CX3CR1*$^{-/-}$ mice).
Thus far, we have generated the colony of mice homozygous for the CX3CR1 KO, homozygous for the Smad4 floxed allele and carrying the Cre recombinase under SMC promoter in hemizygosis. Currently we are phenotyping the aortic pathology and molecular/immune mechanisms induced by Smad4 inactivation in the absence of CX3CR1 pathway. Definitive results will be delivered in the next months of the projects.

Research Group	Collaborations
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DEVELOPMENT OF A COMBINATION STRATEGY BASED ON ER AND OXIDATIVE STRESS IN ACUTE MYELOID LEUKEMIA

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Up to now, the only effective molecular targeted therapy for acute myeloid leukemia (AML) is based on retinoic acid (RA) and/or arsenic trioxide (ATO), and exclusively (APL) responds fully to this therapy. The aim of this project is to design a strategy to target other types of AML, developed on the idea that some categories of leukemic cells could be particularly sensitive to endoplasmic reticulum (ER) stress. The ER is responsible for proper folding of secretory proteins and alterations in its capacity, due for example to the expression of mutant proteins that cannot be properly folded or to oxidative stress determine accumulation of misfolded proteins, a condition defined ER stress. The response to severe ER stress leads to apoptosis. We hypothesized that AML cells expressing oncogenic proteins that, because of their mutant nature, can be easily misfolded causing low levels of chronic ER stress, could be more sensitive than normal ones to exogenous ER or oxidative stress.
We set up a combination of RA, ER stress inducing drugs and ATO, that generates oxidative stress, that resulted highly toxic on AML cell lines bearing the proteins MLL-AF6 or MLL-AF4 with FLT3-ITD and on primary leukemic blasts expressing FLT3-ITD, in which each drug is used at low doses not detrimental if used alone nor for normal human hematopoietic progenitors even in combination.

The combination of RA, ER and oxidative stress inducers leads to AML cell lines death

We began by screening a panel of different AML cell lines, bearing mutant or fusion proteins, for sensitivity to ER and oxidative stress by using the N-glycosilation inhibitor Tunicamycin (Tm) and arsenic trioxide (ATO) in combination with Retinoic Acid (RA).
From the screening, we selected two cell lines, one expressing the fusion MLL-AF6, and the other expressing MLL-AF4 and the mutation FLT3-ITD. In order to assess whether these cells, in the presence or not of RA, result sensitive to a combination of ER and oxidative stress, we treated them with low doses of RA, of the ER-stress inducer N-glycosylation inhibitor Tm and of the oxidative stress inducer ATO. After 72h of treatment, we observed effects which were slightly different in the two cell lines but not for Tm alone that exhibited low toxicity in both cell lines. In particular, in MLL-AF6 expressing cell line, we observed cytotoxicity of the combination of Tm and ATO that was significantly increased by the addition of RA. Importantly here, RA alone or in

combination with ER- or oxidative stress, had only a slight impact on cell viability. On the contrary, RA slightly affected the MLL-AF4+FLT3-ITD expressing cells when used alone and significantly in combination with Tm and ATO. This indicates that RA activates pathways, leading to cell death, independent from those triggered by ER- and oxidative stress. Nevertheless, also in these cells as in the MLL-AF6 expressing cells, is the combination of the three drugs together that maximally increased the cell death rate.

Attenuation of global translation protects from ER and oxidative stress-related cell death

In the cells undergoing ER and oxidative stress in combination, we found prolonged activation of the antioxidant response and of the unfolded protein response (UPR), activated by ER stress.
The UPR consists of three parallel branches, arising from three transmembrane proteins: inositol requiring enzyme 1 (IRE1), activating transcription factor-6 (ATF6) and protein kinase RNA (PKR)-like ER kinase (PERK) (Figure 1). The main function of the PERK pathway is to attenuate translation initiation, through the phosphorylation of the translation initiation factor eIF2α. This leads to the preferential translation of the transcription factor ATF4 which finally induces CHOP expression. This in turn promote the expression of the phosphatase GADD34, whose function is to dephosphorylate eIF2α restoring translation (Figure 1). In our previous work (Masciarelli et al. Leukemia 2018), we described that inhibition of PERK by the GSK2606414 (GSK) inhibitor dramatically enhanced ER stress-related toxicity whereas prolonged activation of PERK downstream signaling, by the GADD34 inhibitor Guanabenz (guana), blocked apoptosis of APL cells following treatment with RA and/or ATO and Tm.
In MLL-AF6 expressing cells we observed that restoration of translation, following treatment with GSK, further sensitized cells to ER and oxidative stress as well. In particular, GSK in combination with Tm was already very toxic, but it had a much stronger effect on the cells treated with Tm+ATO and RA+Tm+ATO. On the contrary, the maintenance of translational attenuation with guanabenz, led to a strong reduction of apoptosis in the samples treated with Tm and/or RA and ATO.
Instead, the treatment with the same inhibitors on MLL-AF4+FLT3-ITD expressing cells moved in a different direction, supporting the idea that in these cells other pathways besides the UPR play an important role. In fact, we found that guanabenz protected from death only the cells treated with RA+Tm and Tm+ATO and that resulted even slightly more toxic than the respective control with RA and RA+ATO. We observed a similar behavior for GSK that, despite a basal low toxicity as showed in the control, enhanced cell death only in RA and RA+ATO samples. These indications suggest that in MLL-AF4+FLT3-ITD expressing cells there is not a clear-cut role of ER- or of oxidative stress but rather a complex interplay between them and with the signaling induced by Retinoic Acid to generate the effects we observed on cell viability.

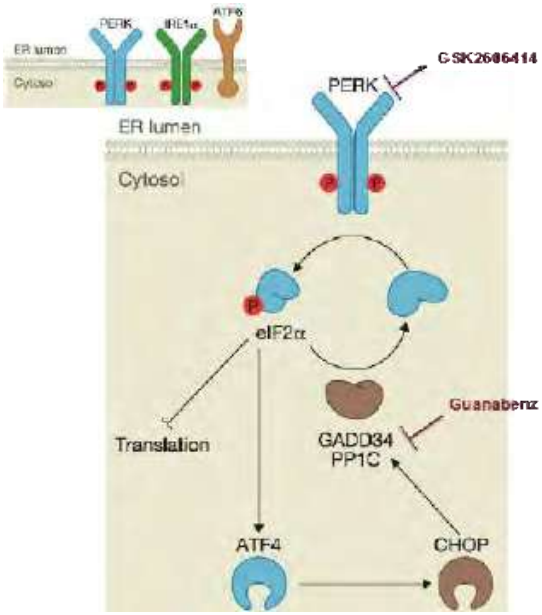

Figure 1. Attenuation of global translation protects from ER and oxidative stress-related cell death. a) Schematic representation of the three UPR transmembrane proteins and the PERK pathway inhibitors function.

FLT3-ITD positive primary blasts are affected by the combination of RA, Tm and ATO

Subtypes of leukemia characterized by FLT3-ITD mutation are among the most aggressive and with the poorer prognosis. Chemotherapy, as for other subtypes, is the only therapeutic choice up to now. Even though many steps forward have been made, the prognosis for FLT3-ITD leukemias is still bad so far and new strategies to fight them are required.
In order to verify the efficacy of the combination of RA, Tm and ATO on primary leukemic cells, we performed colony forming unit (CFU) assays that unravels their potential to form colony. The count of the total number of cells forming each colony revealed that leukemic cells isolated from 4 different FLT3-ITD positive patients and treated with RA, Tm and ATO, alone or in combination, exhibited a general lower colony forming potential compared to control cells. Nevertheless, data obtained from samples treated with all the three drugs were of noteworthy importance, since here the number of cells forming colonies was the lowest compared to all the other samples. Most importantly, the same assays performed on bone marrow progenitors isolated from

healthy subjects reported that no one of the treatments affected normal cells. These data, together, are encouraging in the perspective of the future use of stress inducers and modulators to fight FLT3-ITD leukemia.

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Research Group	Collaborations
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FUNCTIONAL CHARACTERIZATION AND PHARMACOLOGICAL INHIBITION OF COLISTIN RESISTANCE IN *PSEUDOMONAS AERUGINOSA*

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The world is currently facing a worrying threat due to the emergence of multidrug resistance in opportunistic Gram-negative bacterial pathogens. The lack of new antimicrobials active against these bacteria has prompted to re-evaluate the use of the old polymyxin antibiotic colistin (polymyxin E) in clinical practice, which is currently considered a last-resort treatment option to combat recalcitrant Gram-negative infections.
Colistin is a cationic polypeptide, and its antibacterial activity mainly relies on its interaction with the negatively-charged lipid A moiety of lipopolysaccharide (LPS) in the outer membrane of Gram-negative bacteria. This leads to the displacement of Ca^{2+} and Mg^{2+} cations which stabilize the LPS layer, therefore causing derangement of the outer membrane, increased membrane permeability, leakage of cell contents, and ultimately cell death (Nation & Li 2009. doi:10.1097/QCO.0b013e328332e672).
Reintroduction of colistin in clinical practice has inevitably led to the emergence of colistin-resistant isolates (Jeannot *et al.* 2017. doi: 10.1016/j.ijantimicag.2016.11.029). Gram-negative bacteria acquire resistance to colistin primarily through genomic mutations in regulatory genes causing transcriptional activation of genes responsible for remodeling of LPS, through the covalent addition of 4-amino-4-deoxy-L-arabinose (L-Ara4N) or phosphoethanolamine (PEtN) to the lipid A moiety. These modifications reduce the negative charge of LPS and, therefore, its affinity for colistin, ultimately leading to resistance (Olaitan *et al.* 2014. doi: 10.3389/fmicb.2014.00643).
The present project was aimed at assessing the relevance of the L-Ara4N modification for colistin resistance, as well as at investigating its suitability as molecular target for the development of colistin resistance inhibitors, using the opportunistic human pathogen *Pseudomonas aeruginosa* as model organism. *P. aeruginosa* is responsible for severe infections in immunocompromised patients and represents a major threat to patients suffering from cystic fibrosis (CF), in whom *P. aeruginosa* frequently causes life-threatening chronic lung infections. Colistin is extensively used for treating multidrug-resistant *P. aeruginosa* infections, especially in CF patients. Unavoidably, colistin-resistant *P. aeruginosa* strains are not infrequently isolated in CF and non-CF centers (Falegas *et al.* 2010. doi: 10.1016/j.drup.2010.05.002).
In *P. aeruginosa* colistin resistance is generally associated with overexpression of the *arn*

operon, encoding the enzymes for L-Ara4N modification of lipid A. The expression of this operon is controlled by a complex regulatory network involving at least five two-component systems (TCSs), namely PhoP/PhoQ, PmrA/PmrB, ParR/ParS, ColR/ColS and CprR/CprS. Accordingly, mutations within these TCSs that result in the constitutive activation of the *arn* operon are typically identified in colistin-resistant *P. aeruginosa* (Jeannot *et al.* 2017. doi: 10.1016/j.ijantimicag.2016.11.029; Olaitan *et al.* 2014. doi: 10.3389/fmicb.2014.00643; Falegas *et al.* 2010. doi: 10.1016/j.drup.2010.05.002). However, some *in vitro* studies provided evidence that individual TCSs are not essential for the acquisition of colistin resistance in *P. aeruginosa*, leading to the hypothesis that alternative or compensatory mechanisms may exist (Lee *et al.* 2014. doi.org/10.1093/jac/dku238). This is in line with the results of independent random transposon mutagenesis projects or genome sequencing of colistin-resistant clinical isolates which identified genes unrelated to L-Ara4N likely involved in polymyxin resistance in *P. aeruginosa* (Gutu *et al.* 2013. doi.org/10.1128/AAC.02353-12; Fernández *et al.* 2013. doi.org/10.1128/AAC.01583-12; Lee *et al.* 2014. doi.org/10.1093/jac/dkt531).

In the attempt to investigate whether lipid A aminoarabinosylation is actually necessary and sufficient for colistin resistance acquisition in *P. aeruginosa*, we applied genetic engineering to construct (i) mutant strains unable to synthesis L-Ara4N (Δ*arnBCA* mutants, with a deletion in the first three genes of the *arn* operon) or recombinant strains which constitutively express the *arn* genes (P*rpsA*::*arn* strains, in which the *arn* promoter has been replaced with the promoter of the housekeeping gene *rpsA*). These mutant or recombinant strains were generated both in reference laboratory strains (PAO1 and PA14), which are commonly used worldwide for *in vitro* studies on *P. aeruginosa*, and in a small collection of clinical isolates from CF and non-CF patients.

To assess whether *P. aeruginosa* can develop colistin resistance even in the absence of L-Ara4N-modified lipid A and, thus, to verify whether L-Ara4N-independent colistin resistance mechanisms exist in this bacterium, Δ*arnBCA* mutants were compared to parental strains in long term experimental evolution assays, in which the strains were sequentially cultured in the presence of increasing concentrations of colistin in the attempt to select for mutants which acquire successive mutations leading to high-level colistin resistance. In several independent assays, the Δ*arnBCA* mutants of both reference and clinical *P. aeruginosa* strains never grew with colistin concentrations ≥ 4 μg/ml, which corresponds to the epidemiological cutoff (ECOFF) of colistin for *P. aeruginosa*, *i.e.* the highest MIC for isolates devoid of any detectable acquired resistance mechanisms (Ellington *et al.* 2017. doi.org/10:1016/j.cmi.2016.11.012). In contrast, the parental strains always acquired the ability to grow in the presence of very high colistin concentrations, and MIC assays on a representative number of spontaneous mutants confirmed that these isolates developed stable and high-level colistin resistance (MIC ≥ 64 μg/ml). Reintroduction of the *arnBCA* genes *in trans* restored the ability of the Δ*arnBCA* mutants to acquire colistin resistance at wild type levels and rates. Overall, these data directly confirmed that lipid A aminoarabinosylation is an essential prerequisite for the development of colistin resistance in *P. aeruginosa* (Lo Sciuto &

Imperi 2018. doi: 10.1128/AAC.01820-17), indicating that pharmacological inhibition of L-Ara4N biosynthetic enzymes may represent a suitable approach to counteract colistin resistance in *P. aeruginosa*.

Concurrently, we also investigated whether lipid A aminoarabinosylation is sufficient to confer high-level colistin resistance to *P. aeruginosa.* To this aim, we compared *arn* gene expression, lipid A aminoarabinosylation levels and colistin MIC between parental and recombinant P*rpsA*::*arn* strains (see above). qRT-PCR and mass spectrometry assays confirmed that the replacement of the *arn* promoter with the constitutive *rpsA* promoter leads to *arn* gene overexpression and modification of lipid A with L-Ara4N, at levels comparable to those observed in the previously-described *in vitro* evolved colistin-resistant spontaneous mutants. However, lipid A aminoarabinosylation increased colistin resistance in most but not all strains, as some recombinant P*rpsA*::*arn* strains showed colistin MIC comparable to parental strains. Moreover, we observed that the increase in colistin resistance in recombinant strains is culture condition-dependent, being much more relevant in media containing high concentrations of divalent cations. Conversely, divalent cations did not affect colistin resistance in the parental strains, suggesting that they might specifically stabilize the L-Ara4N-coated outer membrane, making it more impervious to colistin. High resistance levels were also observed under conditions mimicking *P. aeruginosa* infections, such as growth in human serum and, to a lesser extent, in artificial CF sputum. These analyses revealed that the degree of colistin resistance conferred by L-Ara4N is strain- and culture condition-dependent, and that the evolution of high colistin resistance levels in *P. aeruginosa* likely requires some genetic and/or phenotypic adaptation(s) in addition to lipid A aminoarabinosylation, as also recently suggested by other groups (Jochumsen *et al.* 2016. doi: 10.1038/ncomms13002).

We finally aimed at examining whether L-Ara4N-modified lipid A could somehow affect *P. aeruginosa* fitness; this issue is important to predict and evaluate the evolution rates and the spread of colistin resistance in this bacterium. Notably, we did not observed significant differences in *in vitro* growth (in different media), biofilm formation, cell wall stability and functionality of the outer membrane permeability barrier between parental and recombinant (P*rpsA*::*arn*) strains. Nevertheless, preliminary experiments in a simple infection model based on the larvae of the insect *Galleria mellonella* revealed some detrimental effect of lipid A aminoarabinosylation on *P. aeruginosa* infectivity which, again, was highly variable among different strains. Experiments are in progress to confirm this initial evidence and to further characterize the effect of lipid A aminoarabinosylation during *P. aeruginosa* infection.

The second aim of the project was to validate lipid A aminoarabinosylation as a pharmacological target for compounds able to restore colistin sensitivity in resistant isolates and/or to hinder the emergence of colistin resistance. Through a docking-based virtual screening for inhibitors of ArnT, *i.e.* the integral membrane enzyme responsible for L-Ara4N attachment to lipid A (Petrou *et al.* 2016. doi: 10.1126/science.aad1172), we recently identified a natural compound (hereafter named BBN149) that partially reverted colistin susceptibility in a colistin-resistant *P. aeruginosa* isolate. This

compound is currently under consideration by the Sapienza University for a patent application. Here we demonstrated that BBN149 has a specific and wide range activity, being active against all colistin-resistant *P. aeruginosa* isolates tested so far, without influencing colistin resistance levels in susceptible strains (which do not express the *arn* operon). This result strongly supports the hypothesis that BBN149 might specifically target colistin-resistance mechanism(s), likely ArnT-mediated lipid A aminoarabinosylation. To verify this hypothesis, we are attempting to co-crystallize ArnT with BBN149, in collaboration with Prof. Filippo Mancia (Columbia University), and to assess the effect of BBN149 on lipid A aminoarabinosylation levels by mass spectrometry. Lipid A extraction and mass spectrometry assays revealed that BBN149 slightly reduces but does not completely hamper lipid A aminoarabinosylation in colistin-resistant isolates, although additional experiments are required to confirm the statistical significance of the observed reduction. In parallel, we demonstrated that BBN149 is not toxic to lung epithelial cells, at least at the concentrations tested in our assays, and that it is also active against a small collection of recently-characterized colistin-resistant isolates of a different Gram-negative bacterium (*Klebsiella pneumoniae*) harbouring mutations causing *arn* gene overexpression (Esposito *et al.* 2018. doi: 10.3389/fmicb.2018.01463). Finally, in collaboration with Bruno Botta (Sapienza University), a number of BBN149 derivatives have been generated and compared to BBN149 for the capability to counteract colistin resistance in *P. aeruginosa*. Although none of the tested compounds were more effective than BBN149 against colistin resistance, this analysis allowed us to characterize the backbone structure and (some) functional groups potentially important for activity. This information will be used to design and synthesize a second generation of BBN149 analogues, with the final goal to obtain compounds with higher activity and/or specificity and improved water solubility, which represents an important issue for the *in vivo* efficacy tests.

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CELLULAR PATHWAYS INVOLVED IN THE TOXICITY OF NEUROSERPIN POLYMERS THAT CAUSE THE DEMENTIA FENIB

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Serpins are the largest and most broadly distributed superfamily of protease inhibitors (Irving et al., 2000, *Genome Res*). Members of the serpin superfamily are found in all major branches of life including viruses, prokaryotes and eukaryotes, and are characterised by more than 30% amino acid sequence homology with the archetypal serpin, alpha-1 antitrypsin, a secretory serpin produced in the liver and delivered to the blood, through which it reaches the lungs to inhibit the protease neutrophil elastase (Gooptu and Lomas, 2009, *Ann Rev Biochem*). Amino acid substitutions in serpins result in aberrant conformational transitions that cause a spectrum of pathologies known as serpinopathies. These are protein conformational diseases characterised by the polymerisation and intracellular deposition of mutant variants of the serpins within the endoplasmic reticulum (ER) of the cells that synthesise the protein. This mechanism underlies the neurodegenerative dementia FENIB (familial encephalopathy with neuroserpin inclusion bodies), an autosomal dominant condition caused by mutations in neuroserpin (NS) that cause polymer formation (Davis et al., 1999, *Nature*). To date, six different mutations have been described in NS that promote its polymerisation in people affected by FENIB: Ser49Pro (*Syracuse*), Ser52Arg (*Portland*), His338Arg, Gly392Glu, Gly392Arg and Leu47Pro. Mutant NS was found to accumulate within affected neurons forming periodic acid-Schiff (PAS)-positive inclusion bodies known as Collins bodies, most abundant in the cerebral cortex but also present in other regions of the central nervous system (Davis et al., 1999, *Am J Pathol*). Clinically, this accumulation translates in a spectrum of phenotypes from dementia to epilepsy (Roussel BD et al., 2016, *Epileptic Disord*). The phenotypical and biochemical features of FENIB have been deduced from its clinical manifestations and from the postmortem analysis of affected brains, and have been confirmed through the expression of mutant NS in diverse cellular systems (Miranda et al., 2004, *J Biol Chem*; Miranda et al., 2008, *Hum Mol Genet*; Roussel et al., 2013, *Hum Mol Genet*; Moriconi et al., 2015, *FEBS J*), in *Drosophila melanogaster* (Miranda et al., 2008, *Hum Mol Genet*) and in mice (Madani et al., 2003,

Mol Cell Neurosci; Galliciotti et al., 2007, *Am J Pathol*). Transgenic mice overexpressing S49P and S52R NS showed the formation of abundant intraneuronal Collins bodies, neuronal loss in the cerebral cortex and hippocampus, and pathological phenotypes reminiscent of FENIB during late adulthood, while overexpression of human S49P, S52R, H338R and G392E NS in *Drosophila melanogaster* led to a decrease in locomotor activity, with decreasing mobility correlating to increased polymer content in the brain. In cellular models, polymer formation and its correlation with the disease phenotype was confirmed in transiently transfected COS-7 and stable inducible PC12 cell models of FENIB, where overexpression of each mutant variant lead to intracellular accumulation of polymeric NS within the ER to a degree that was proportional to the severity of FENIB seen in patients. Despite these results, the mechanism of toxicity of NS polymers has been elusive so far, since these cellular systems failed to show clear signs of cell malfunction and death upon NS polymer accumulation, precluding a detailed investigation of the mechanisms underlying NS polymer toxicity.

The lack of a toxic phenotype in cellular models of FENIB could be related to the proliferative nature of these cell lines used so far. To overcome this issue, we have recently developed a neuronal model with stable overexpression of wild type, G392E and delta NS. The last is a mutant form with a premature stop codon causing the synthesis of a truncated version of NS, which does not polymerise and, contrary to polymers, activates a classical unfolded protein response (UPR) (Davies et al., 2009, *J Biol Chem*). Mouse neural progenitor cells (NPCs) were isolated from several regions of the mouse foetal brain, stably transfected with the NS variants described above and propagated *in vitro* under proliferative conditions or differentiated to mature, non-dividing neurons. We found that in differentiated NPCs, expression of polymerogenic NS led to the upregulation of several genes involved in the defence against oxidative stress (Guadagno et al., 2017, *Neurobiol Dis*). Oxidative stress, the imbalance between generation and disposal of reactive oxygen species (ROS), is an important factor in several neurodegenerative disorders including Alzheimer's disease, Parkinson's disease, Huntington's disease and amyotrophic lateral sclerosis (Cobb and Cole, 2015, *Neurobiol Dis*). Neurons are particularly vulnerable to oxidative stress due to their high energy requirements, to a decrease in antioxidant defences with age and to their terminally differentiated nature (Gandhi and Abramov, 2012, *Oxid Med Cell Longev*). The ER, where NS polymer formation takes place, provides an oxidizing environment for correct formation of disulfide bonds during protein folding. ROS can be generated as a by-product of protein oxidation during normal ER function and also upon ER stress due to the accumulation of misfolded proteins. All these evidences support that the

accumulation of serpin polymers within the ER may upset the redox balance in this organelle, and this perturbation can affect the redox state of mitochondria through mitochondrial-ER contact sites. Mitochondria are important organelles in all cell types, but they are particularly important in the nervous system. Their function is essential to neuronal processes such as energy production, Ca^{2+} regulation, maintenance of plasma membrane potential, protein folding by chaperones, axonal and dendritic transport and the release and re-uptake of neurotransmitters at synapses, and they present a highly dynamic behaviour comprising mitochondrial fusion and fission events (Hoppins et al., 2007, *Annu Rev Biochem*; Zhang et al., 2007, *FEBS Lett*).

We thus decided to look at the mitochondrial morphology in our cell lines by staining the cells with Mitotracker Red CMXRos ®, which is a red fluorescent dye that labels mitochondria in living cells using the mitochondrial membrane potential. Based on the literature and our analysis of the cellular phenotypes, we defined three categories of mitochondrial morphology and intracellular distribution: i) network-like: healthy cells showed mitochondria homogeneously distributed throughout the cytoplasm, forming a tubular network within the neurons; ii) perinuclear: mitochondria that appeared clustered in the neuronal soma and generally on one side of the nucleus; and iii) fragmented: mitochondria appeared as small, rounded and located close to the nucleus. In our analysis, we grouped perinuclear and fragmented mitochondria as 'altered' morphology. Neuronal exposure to the oxidative insult caused by H_2O_2 resulted in a significant change in mitochondrial morphology of control cells overexpressing GFP (green fluorescent protein): healthy mitochondria were organised in a tubular network; however, after exposure to H_2O_2 their network transformed into a clustered distribution and in some cells into a fragmented morphology. These changes resulted in a significant shift of mitochondria from category (i) into categories (ii) or (iii). In basal conditions, wild type and delta NS expressing cells mostly showed the network-like mitochondrial morphology, indicative of a healthy mitochondrial morphology. Cells expressing G392E NS showed a higher proportion of altered mitochondria (nearly 40%), which were often found clustered close to the nucleus with no mitochondrial fragmentation, indicative of the underlying toxicity caused by NS polymers. We also found that the alterations described in mitochondrial distribution correlated with modifications of the normal phenotype of differentiated neurons. By staining the actin cytoskeleton with fluorescent phalloidin, we observed that neurons with perinuclear or fragmented mitochondria lacked the long neurites typical of healthy neurons.

To better understand the mechanism underlying the toxicity of NS polymers related to mitochondrial distribution we performed pharmacological inhibition and enhancement of the cellular defences against oxidative stress. Glutathione (GSH) is an important antioxidant metabolite that prevents cell damage caused by the presence of ROS, lipid oxidases and free radicals, and is involved in the mechanism of action of many enzymes that were overexpressed in our G392E NS expressing cells. Cellular levels of GSH can be depleted by treating the cells with DEM (diethyl maleate). We stained the cells with Mitotracker Red CMXRos ® after treating them with the GSH chelator DEM at a final concentration of 50 µM. Cells overexpressing GFP, wild type and delta NS showed limited alteration in mitochondrial morphology after DEM treatment, while cells expressing G392E NS showed a stronger response to the pro-oxidant treatment with DEM, presenting a significant increase in the number of cells with fragmented mitochondrial morphology, thereby supporting the oxidative nature of the response caused by the presence of G392E NS polymers in the ER.

We next decided to verify if, in the opposite way, an anti-oxidant treatment could recover the perinuclear mitochondrial morphology seen in the G392E NS cells. Melatonin is usually known as a pineal gland neurohormone that mediates photoperiodicity in mammals, but it is also a well characterised antioxidant. We stained wild type and G392E NS cells with Mitotracker Red CMXRos ® after treating them with increasing concentrations of melatonin for 24 and 48 h. After determining the optimal concentration, we treated all cell lines with 10 µM during the last 48 h of the differentiation period. All cell lines showed some improvement in mitochondrial distribution in response to this antioxidant, but for cells expressing GFP, wild type and delta NS the effect was only mild, while cells expressing G392E NS showed a clear recovery and reduction of the perinuclear phenotype to values similar to control cells. To confirm our results, we also analysed the effect of alpha tocopherol, a vitamin that functions as a lipid-soluble antioxidant protecting cell membranes from oxidative damage. As observed for melatonin, the treatment with this antioxidant slightly improved the mitochondrial phenotype in all cell lines, but the most pronounced effect was obtained for the G392E NS line. Currently, we are advancing our research in two directions: i) we are further assessing the state of mitochondria in cells expressing G392E NS by using the fluorescent probe JC-1, which reports on the state of the mitochondrial membrane potential, and should be an earlier marker of mitochondrial dysfunction when confronted with cellular distribution; ii) we are optimising the transfection of reporter plasmids in our cell lines with the scope of investigating the activation of the UPR. It is well stablished that misfolded protein accumulation within

the ER leads to activation of a stress signalling pathway from the ER to the nucleus, the UPR, and that NS polymers, contrary to most forms of protein aggregation, do not activate this pathway in the cell models studied so far. Since our system represents the first neuronal cell culture model for FENIB, we will use a luminescent reporter of UPR activation to determine if NS polymers do elicit the UPR in neuronal cells, or on the contrary the ER stress toxicity caused by polymer accumulation is due to a mechanism independent from the classical UPR.As part of our research on serpin polymers, and using approaches that are complementary to our NS studies, we also investigate the polymerisation of the archetypical serpin alpha-1 antitrypsin (AAT), in collaboration with Prof. David Lomas and his group at UCL (London, UK). Our contribution in these studies includes the production of conformation-specific and functional monoclonal antibodies, which has recently led to the characterisation of two novel reagents: an antibody able to interfere with the inhibitory activity of AAT against several cognate proteases (elastase, chymotrypsin and proteinase 3), and an antibody with strong preference for the non-polymerised, monomeric state of AAT, currently being applied to the investigation of heteropolymers formed by wild type and mutant AAT in the liver of heterozygous patients of AAT deficiency.

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MOLECULAR BIOMARKERS PREDICTIVE OF HEMATOLOGICAL RESPONSE TO DIRECT ANTIVIRAL THERAPY IN HEPATITIS C VIRUS ASSOCIATED LYMPHOPROLIFERATIVE DISORDERS

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Hepatitis C virus associated lymphoproliferative disorders (LPDs) range from benign diseases, as mixed cryoglobulinemia (MC) to malignant lymphomas, usually indolent B-cell non-Hodgkin lymphomas (NHL). Dependence of the LPDs from the viral chronic infection provided by HCV has been postulated based on the regression of these diseases after virus eradication, but the inciting viral antigen, responsible for B cell stimulation, has not yet been identified. The advent of direct acting antivirals (DAA) able to eradicate HCV in less than one month has completely changed the natural history of HCV-associated LPDs and it is likely that in a not too far future this infection will be eliminated. Now we assist to a widespread virus eradication associated with high efficacy in the cure of extrahepatic manifestations as MC and NHLs. Nonetheless both situation seem not to be completely defeated as relapses of MC vasculitis and partial or non-response of NHL have been described by other and our group (Visentini M. Rheumatology 2018; Arcaini L. et al. Blood 2016).
This project has the aim to identify molecular and cellular characteristics of B cells clonally expanded in MC and in NHL able to predict the response to virus eradication and/or to shed light in the pathogenic mechanisms involved in disease relapse and non-response.
During this first year we have analyzed the phenotypical and functional characteristics of rheumatoid factor (RF)-producing clonal B cells expanded in MC and NHL and followed their fate after eradication of the virus. Our group already published preliminary observations regarding clonal B cells in HCV-LPDs after DAA (Del Padre M. et al. Blood 2016). New recent data were extended to a larger group of patients and, more relevant, we analysed the clonal B cells for a longer follow-up, correlating our findings with MC vasculits response to DAA. More in detail 45 HCV-cured MCV patients were followed-up for a median of 18.5 (range 9-38) months after the clearance of HCV and circulating B-cell clones were detected using flow cytometry either by the skewing of kappa/lambda ratio or by the expression of a VH1-69-encoded idiotype, commonly expressed in expanded clonal B cells. We found that circulating B-cell clones were detected in 18/45 patients, and in 17 of them persisted through the follow-up; nine of the latter patients cleared cryoglobulins and had complete response of vasculitis, but several months later, two of these patients had relapse of MCV. We have defined these cells as

"dormant" cells that may be reactivated by events, as infections or onset of cancer that perturb B-cell homeostasis and can give rise to the relapse of cryoglobulinemic vasculitis (Visentini M. et al Liver International 2019). How this is explainable is still a matter of debate and we have now preliminary data, suggesting that *in vitro* co-stimulation with immune complexes and with the TLR9 ligand CpG may induce the proliferation of RF+ clonal B cells from HCV-cured patients with MCV. These observations, if confirmed in a large cohort of patients, could strongly suggest that relapses of MCV in HCV-cured patients might occur during conditions characterized by high amounts of circulating IgG immune complexes that induce B cell clones to proliferate owing to the rheumatoid factor specificity of their BCRs.

Beside investigating the cellular features of clonal B cells in LPDs, big effort has been made for the molecular analysis of the B cell receptor in these cells, usually characterized by stereotypy and rheumatoid factor activity. We investigated IGHV-D-J rearrangements and IGKV-J/IGLV-J rearrangements of patients with HCV-related LPDs in an attempt to untangle the relative role of immunoglobulin heavy and light chains in generating potentially autoreactive or virus-specific BCRs. So far, we sequenced heavy chain V-D-J and light chain V-J rearrangements in 21 patients with HCV-related LPDs. The sequences obtained are submitted to the IMGT V-QUEST tool (http://www.imgt.org), and information on IGHV-D-J, IGKV-J and IGLV-J gene and allele usage and on mutational status are extracted.
Analyzing 21 patients we obtained a biased usage of genes involved in V(D)J rearrangements, 70% of cases express VH1 (VH1-69 in 20% of cases) or VH3 family genes, and 71% express Vκ3 family genes (Vκ3D-20 in 58% and Vκ3-15 in 42%). These Vκ sequences showed two subsets of 9 and 10 amino acids length: one subset had a strong similarity in the κCDR3 sequences and the other one displayed a unique stereotyped sequence identical to other RF-producing LPDs. Moreover κCDR3 were highly enriched in sequences homologous to RFs (82%), but also to HCVE2 antibodies (47%), while among HCDR3 37% of the cases had homology with RF antibodies and only 1 with anti-HCVE2 antibodies.

During the next second year of project we plan to analyze a larger group of patients, possibly through NGS technology, both for the sequencing of the Immunoglobulin genes and for the mutational analysis of a specific panel of gene involved in lymphomagenesis.

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"TERESA ARIAUDO" RESEARCH PROJETCS

2 YEARS PROJECTS LED BY UNDER 35 YEAR OLD RESEARCHERS

FIRST YEAR REPORTS

UNMASKING THE ENCRYPTED ROLE OF TIP60 CHROMATIN REMODELLING COMPLEX IN CELL DIVISION

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Cells utilize ATP-dependent nucleosome-remodelling complexes to carry out histone sliding, eviction or incorporation of histone variants. The Tip60 remodelling complex in *Drosophila* (dTip60) includes proteins with high homology to the subunits of two distinct chromatin-modifying complexes, the human P400/Tip60 complex harbouring HAT (Histone Acetyl-Transferase) activity and the SRCAP (SNF2-related CBP activator protein) ATP-dependent chromatin remodelling complex which catalyze the replacement of canonical H2A by H2A.Z histone variant; and it is involved in many processes, including DNA repair and both transcriptional activation and repression.

Mitosis consists of a series of tightly controlled events leading a cell to segregate its genetic material into two daughter cells. Its failure and the resulting generation of genetic instability, which includes both numerical and structural chromosomal abnormalities, lead to an unstable state, hence activating tumorigenic transformation.

In the last few years, sparse evidence shows that subunits of human SRCAP and P400/Tip60 remodelling complexes have a dynamic subcellular localization during cell cycle; therefore, independently on their role in modulating chromatin organization and gene expression, they may take part in cell division.

In past works, mutations in the *Drosophila yeti* gene cause chromosome organization defects and impair cytokinesis. *Yeti* encodes a chromatin protein belonging to or interacting with the *Drosophila* dTip60 chromatin remodelling complex.

Worth of mentioning, a conserved role in cell division has been highlighted by preliminary experiments in cultured S2 *Drosophila melanogaster* cell line. Four subunits of the dTip60 complex in *Drosophila*, DOM-A, YETI, Tip60 and MRG15 which are orthologous to human SRCAP, CFDP1, Tip60 and MRG15 proteins, respectively, are recruited to the mitotic apparatus during mitosis in *Drosophila* S2 cells. Interestingly, DOM-A RNAi-mediated knock-down leads to unfocused and aberrant microtubule arrays, resulting in kinetochores attachment defects and chromosome misalignment.

There is also evidence for a role in cytokinesis of BAP55, another member of dTip60 chromatin remodelling complex which is functionally related to SRCAP and P400/Tip60 complexes and shares with them several conserved subunits.

This project addresses the function of dTip60 chromatin remodelling complex during cell division (mitosis and meiosis) in *Drosophila melanogaster*.

We plan to use CRISPR/Cas9 technology to substitute each gene of interest (*dom*, *Tip60*, *yeti*, and *Mrg15*) with its AidDP::EGFP-tagged version in order to exploit an experimental strategy quickly degrading the target protein rather than its mRNA (AiD system).

For these purposes, a substantial Working Package (WP1) of this work is to clone gRNAs targeting the Cas9 endonuclease to the genes of interest, and their corresponding donor vectors to generate fly strains expressing the related tagged-protein under the control of its own promoter. These stocks will be used to perform follow-up living Drosophila embryos and/or ex vivo testes after Auxin-induced protein degradation by using time-lapse microscopy to monitor chromosome segregation and mitotic defects during cell division.

We have already obtained plasmids carrying out each gRNA (*dom*, *tip60*, *yeti* and *mrg15*) and we are currently working to clone right-and-left flanking sequences into a suitable vector that will be used as 'donor plasmids' for homologous recombination (HR) repair.

In parallel, we are performing experiments to understand whether depletion of DOM, Tip60, Yeti and MRG15 proteins by RNAi may show meiotic defects in Drosophila testes.

To date, our preliminary study on male meiosis show that remodellers depletion due to aberrant chromosome segregation and impair meiotic division (Fig.1).

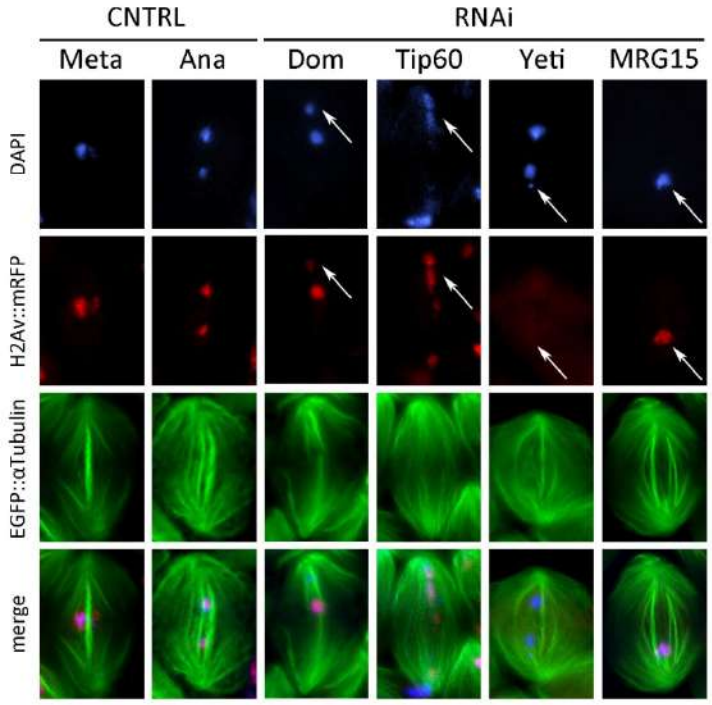

Fig.1 Remodellers RNAi effects in Drosophila male meiosis

Virgin females *H2Av::mRFP, Tubulin::EGFP; Bam>Gal4* have been crossed with remodeller RNAi males (Dom, Tip60, Yeti and Mrg15) to specifically drive interference of protein of interest in testes.

Taken together, these results support our hypothesis about the existence of an unexpected phenomenon, whereby ATP-dependent chromatin remodelling factors, in addition to their role in chromatin organization, have a functional relevance in cell division and emphasize a surprising scenario in which chromatin remodelling, cell cycle and tumorigenesis are closely interlinked.

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[1] *Co-corresponding Author*

ANALYSING THE N_{TAIL} INTERACTION WITH THE P_{XD} DOMAIN TO DEVELOP ANTIVIRAL STRATEGIES AGAINST PARAMYXOVIRIDAE

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The interaction between the C-terminal, intrinsically disordered domain of the nucleoprotein (N_{TAIL}) and the X domain of the phosphoprotein (P_{XD}) has a key role in the replication and transcription of the genome of three human pathogens, Measles (MeV), Hendra (HeV) and Nipah (NiV) viruses. In this project I'm focusing in understanding the molecular mechanism of the interaction between the MeV HeV and NiV variants of N_{TAIL} and P_{XD} proteins.
The genes encoding P_{XD} wild-type and N_{TAIL} wild-type variants from HeV and NiV, were cloned into pDEST14 plasmids, that were used to transform the E. coli strain Rosetta [DE3] pLysS (Novagen). Site-directed mutagenesis was performed in order to obtain Y682W variant of HeV P_{XD} and L483W, A487W, I488W variants of HeV N_{TAIL}, as well as Y684W variant of NiV P_{XD} and L483W, A487W, A488W variants of NiV N_{TAIL}.
Cultures were grown in Luria–Bertani (LB) medium containing 100 µg/mL of ampicillin and 34 µg/mL of chloramphenicol at 37 °C. When the optical density at 600 nm (OD600) reached 0.6–0.8, isopropyl ß-D-thiogalactopyranoside was added to a final concentration of 0.2 mM, and the cells were grown at 37 °C for 4 additional hours. The induced cells are collected by centrifugation (5000g, 10 min). The resulting pellets were frozen at −20 °C. Proteins are purified from the soluble fraction of bacterial lysates through a first step of Hi-Trap Chelating Ni^{2+} GE Healthcare column followed by SEC Superdex 200 16/60, GE Healthcare.
In order to study the molecular details of the interaction between P_{XD} and N_{TAIL}, equilibrium and kinetic binding experiments were performed. Equilibrium binding experiments were performed to calculate the equilibrium dissociation constants of the binding between the two proteins and to detect which position of the tryptophan, whether in P_{XD} or N_{TAIL} proteins, shows the better signal-to-noise ratio upon binding reaction. Equilibrium binding experiments were carried out on Fluoromax single photon counting spectrofluorometer (Jobin-Yvon, Edison, NJ). Tryptophan fluorescence emission spectra were recorded in a cuvette (1-cm light path) between 300 and 400 nm. The excitation wavelength was 280 nm. Protein concentration was keeped fixed at 3 µM for P_{XD} Y682W /N_{TAIL} L483W, A487W, I488W (in the case of HeV variants) and P_{XD} Y684W /N_{TAIL} L483W, A487W, A488W (in the case of NiV variants) and mixed with increasing concentration of wt Hev/NiV N_{TAIL}/P_{XD} ranging from 3 to 60µM. The buffer used was 50

mM sodium phosphate pH 7.2 and the temperature was 25°C. The binding between HeV P_{XD} Y682W/N_{TAIL} wt, and NiV P_{XD} Y684W/N_{TAIL} wt showed the best signal to noise ratio upon binding and these variants were chosen for kinetic experiments. To explore the kinetics of the binding reaction between the two proteins, stopped-flow kinetic experiments were performed. The binding experiments were carried out, for both HeV and NiV variants, by keeping the concentration of the P_{XD} protein fixed (3μM or 6μM) and rapidly mixing it with increasing concentrations of N_{TAIL}, ranging from 10μM to 60μM. The experiments were conducted in several different experimental conditions, changing pH, ionic strenght of the solutions and temperature, however I was not able to record any reliable change in fluorescence emission upon binding reaction, the reaction being too fast for the stopped-flow apparatus. To solve this issue I am currently carrying out temperature jump kinetic binding experiments, the T-jump apparatus being able to resolve kinetics on a much faster time scale compared to the stopped flow. Preliminary results suggest that kinetics of the binding reaction for both HeV and NiV proteins do not fit with a simple two-state binding mechanism, the reaction being slowed down by a limitating step that may correspond to the folding of the MoRE region of NTAIL. At the same time, in order to perform Φ-value analysis and obtain structural information of the transition state and contribution of single residues in the binding reaction between N_{TAIL} and P_{XD} extensive site directed mutagenesis is being carried out, systematically and strategically mutating residues in the region of the MoRE of N_{TAIL}. All the variants obtained are being expressed and purified to be employed in temperature jump kinetic binding experiments.

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Research Group

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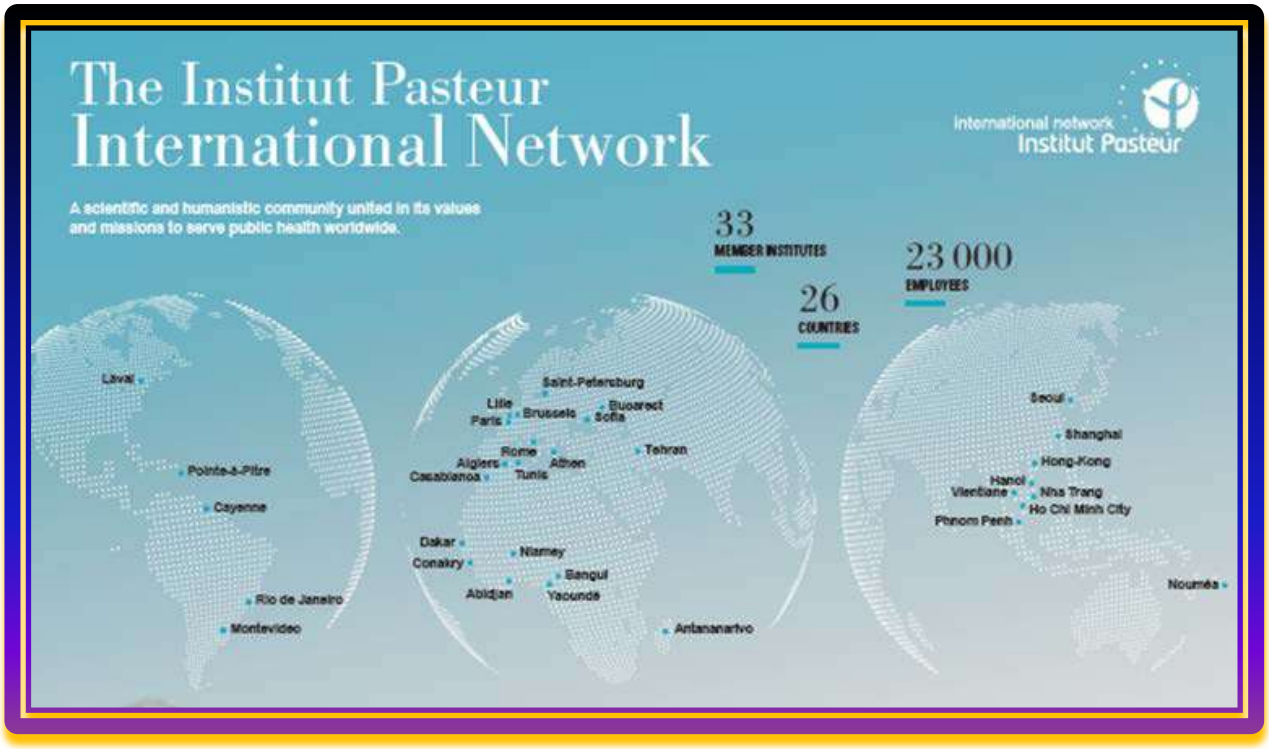
COLLABORATIONS WITHIN THE INTERNATIONAL NETWORK OF INSTITUTES PASTEUR



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PTR - PROJETS TRASVERSAUX DE RECHERCE
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GPF – GRAND PROGRAMME FÉDÉRATEUR

Seed International Research Project (First year report)
NK CELL-BASED REGULATION OF MESENCHYMAL STEM CELL DIFFERENTIATION IN EXPERIMENTAL MODELS OF BONE FORMATION AND CARTILAGE DEGENERATION

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RESEARCH AREA: INFLAMMATION AND IMMUNITY

Our research activity is focused on the analysis of the ability of NK cells to alter MSCs chondrogenic/osteogenic capacity. We are also investigating the role of CXCR3/CXCL10 axis in NK cell accumulation and activation in the joints during osteoarthritis and how CXCR3 regulates the process of bone/cartilage regeneration.
We previously showed that NK cells and neutrophils are among the first cells that infiltrate the inflamed joint and exert a pathogenic role in a mouse model of collagenase-induced osteoarthritis (CIOA) promoted by intra-articular injection of collagenase (Benigni et al. J. Immunol. Vol 198, year 2017). We also showed that CXCL10/CXCR3 chemokine/chemokine receptor axis is crucial for disease establishment and progression, being CXCL10 increased in synovial fluid of CIOA mice and $CXCR3^{-/-}$ mice protected from disease development. We found that $CXCR3^{-/-}$ mice maintains intact cartilage and bone despite the injection of degradative collagenase at the knee joint, suggesting that intrinsic abnormalities of cartilage/bone tissue formation and/or that impaired recruitment/activation of immune cells have a protective effect in $CXCR3^{-/-}$ mice.
Regarding the latter hypothesis, our current observations indicate a direct role of CXCR3 ligands on NK cell migration to synovium during osteoarthritis as *in vitro* chemotaxis of purified mouse NK cells to synovial fluids collected from CIOA mice joints was completely abrogated by CXCL10 blocking antibodies

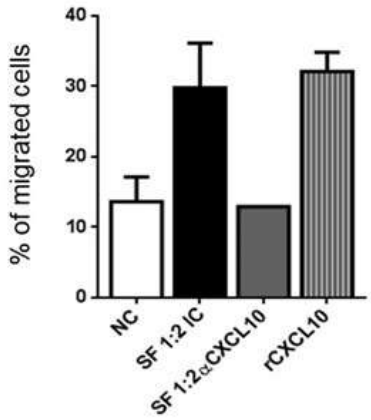

Figure 1. CXCL10 blockade inhibits NK cell migration in response to synovial fluids from CIOA mice. Mouse NK cells were isolated from spleen using NK cell isolation kit (Miltenyi biotech, Germany). NK cell migration was assessed against a pool of synovial fluids from 2-3 mice per group diluted in migration medium. Cells were plated over the membrane of transwell 96-well plate 5µm pore size and allowed to migrate for 2 h in response to factors added to the lower wells in triplicate. Synovial fluid was either left untreated or pre-incubated with anti-CXCL10 blocking mAb (αCXCL10, RnD systems) for 30 min before the assay. Migration medium alone (NC) or containing recombinant CXCL10 (rCXCL10) were used as negative and positive controls respectively.

(Figure 1).

A role of CXCR3 in NK cell accumulation in synovium is also supported by the reduced number of macrophages and NK cells in the inflamed CIOA joint of CXCR3-/- mice (Benigni et al. J. Immunol. Vol 198:2115, year 2017). To confirm this conclusion *in vivo*, NK cells were purified from spleen of CD45.1 WT and CD45.2 $CXCR3^{-/-}$ mice by negative depletion using magnetic bead-based kit. The two groups of cells were mixed in equal amount and were labelled with the cell staining fluorescence dye Carboxyfluorescein succinimidyl ester (CFSE) for their identification after transfer. Afterwards, competitive adoptive transfer experiments were performed by i.v. transfer of the donor cells into CIOA WT mice at day 2 after collagenase injection to analyze their differential recruitment into the recipient joint and their distribution in spleen, blood and synovial fluids and extracts at 1 day after transfer. Unlike what was expected, we found comparable number of WT and KO NK cells in the synovium. Nevertheless, the interpretation of this result is overshadowed by the higher accumulation of KO than WT donor NK cells in blood, which may better supply the entry of KO cells in the synovium. In order to exclude the influence of intrinsic differences associated to CD45 allelic variants or of other cell population (i.e. macrophages) that may be affected by CXCR3 deficiency, we will perform analysis of donor WT and KO NK cells expressing the same CD45.2 allelic variant and transferred separately into distinct mice groups. These experiments will also be instrumental to determine the direct contribution of $CXCR3^{+}$ NK cells to CIOA development by analysis of the extent of damage after transfer WT and $CXCR3^{-/-}$ mice.

Since we previously showed that CXCR3 deficient NK cells display reduced expression of activation markers in the inflammed joint from CIOA mice, we have hypothesized that CXCR3 ligands promote NK cell activation in the synovium, which in turn cause cartilage degeneration. Thus, we investigated the effect of CXCR3 ligand treatment on the killing capacity of NK cells against chondrocytes. In these set of experiments, we have used the human chondrosarcoma cell line HTB-94 as this is a well characterized cell model that can be genetically manipulated to analyse mechanisms of killing. Our analysis evidenced that incubation of NK cells from human peripheral blood mononuclear cells (PBMCs) with HTB-94 cells promotes low but significant capacity to degranulate as assessed by measuring the plasma membrane expression of lysosomal associated membrane protein-1 (or CD107a), that is indicative of lytic granule exocytosis (data not shown). Incubation of HTB-94 and NK cells with the CXCR3 ligand CXCL10 markedly increased the killing capacity of NK cells as shown by elevated HTB-94 cell death upon incubation with the higher CXCL10 dose (Figure 2).

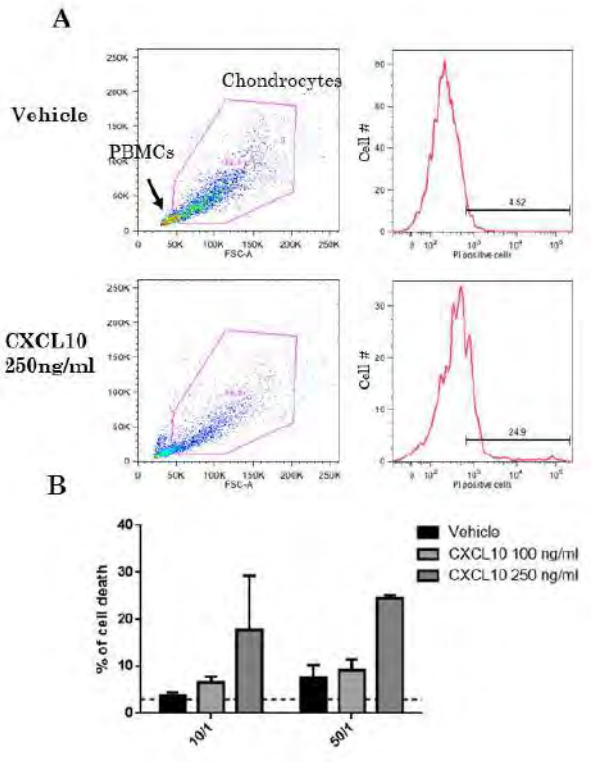

Figure 2. CXCL10 increases condrocytes susceptibility to NK cell killing. Ten thousand HTB-94 cells were plated on 96-well plates the day before the experiment and maintained in growth medium with or without addition of CXCL10 (100 and 250ng/ml) for 18 h. The day of the experiment PBMCs were isolated from PB of heathy donors by stratification on Lympholyte (Cedarlane) and were co-incubated with HTB-94 cells at 10/1 and 50/1 effector/target (E/T) ratios. CXCL10 was added were required to mantain the stimulation conditions. After 4 ½ h PBMCs were collected to analyze the level of NK cell degranulation and adherent HTB-94 cells were detached by trypsinization to anayze the levels of target cell death. NK cell (CD56+CD3-) degranulation was determined by FACS analysis of PBMCs stained with anti-CD56 and -CD3 antibodies in combination with mAb specific for the degranulation marker CD107a (not shown). HTB-94 cell death was measured by FACS analysis of cells stained with propidium iodine (PI). PBMCs incubated for the same time without target were used to assess the basal levels of degranulation. HTB-94 incubated without PBMCs were used to assess the basal levels of cell death. Panel A, Representative histogram plots of PI staining of HTB-94 cells. Panel B, bar graph shows mean +/- SD of % of death HTB-94 cells upon incubation with NK cells in three independent experiments. Dotted line represents % of spontaneous HTB-94 cell death

We attributed the effect of CXCL10 to increased susceptibility of chondrocytes to apoptosis and not to increased NK cell activation as we could not show an effect of CXCL10 on NK cell degranulation (data not shown). This hypothesis will be investigated by knocking down the expression of CXCR3 in HTB-94 cells using a siRNA approach.

Thus, NK cell functions can be regulated by CXCL10 increased expression during osteoarthritis, which can promote NK cell chemotaxis and NK cell-mediated chondrocyte killing. We are currently performing similar experiments on primary mouse chondrocytes isolated from articular cartilage of WT and CXCR3$^{-/-}$ mice.

To better determine the contribution of NK cells and CXCR3 in cartilage turnover, we have established a protocol for the isolation of pure populations of mouse bone marrow mesenchymal stromal cells (MSC), which are known to be critically involved in the process of regenerative repair. Experiments are ongoing to evaluate osteogenic/chondrogenic differentiation *in vitro* of MSC cultures supplemented with escalating doses of NK cells and/or CXCL10. To support data obtained *in vitro*, in collaboration with the group of Prof. Riminucci, we are going to assess the influence of CXCR3 and NK cells on the degree of bone formation *in vivo* on a subcutaneous implant of hydroxyapatite/tricalcium phosphate (HA/TCP) carrier loaded with MSC.

In order to evaluate a direct role of NK cells in bone turnover and their contribution to the effect of CXCR3 in the promotion of joint damage, we are performing analyses of bone and cartilage structure in WT and CXCR3$^{-/-}$ mice after 4-week depletion of NK cells by repeated intraperitoneal injection of anti-NK1.1 antibody. Femurs and tibias from untreated and NK cell depleted mice has been sent to the Co-PI of the program that will perform micro-CT analysis of bone structural and cartilage thickness differences.

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Research Group	Collaborations
Fabrizio Antonangeli Researcher (RTD type A); **Valentina Bonanni** PhD student (*7 months of the program*)	**Prof. Mara Riminucci** *(MSC differentiation in vitro and in vivo)* Department of Molecular Medicine Sapienza University of Rome **Prof. Scotto D'abusco** *(purification and characterization of mouse chondrocytes)* Department of Biochemical Sciences Sapienza University of Rome

Seed International Research Project (First year report)
WHOLE TRANSCRIPTOME (RNA-SEQ) COMBINED WITH BIOCHEMICAL STUDIES TO EVALUATE THE CONTRIBUTION OF THE METABOLISM OF GLUTAMATE AND GABA IN *MYCOBACTERIUM TUBERCULOSIS* STRAINS CIRCULATING IN SALVADOR, BAHIA, BRAZIL.

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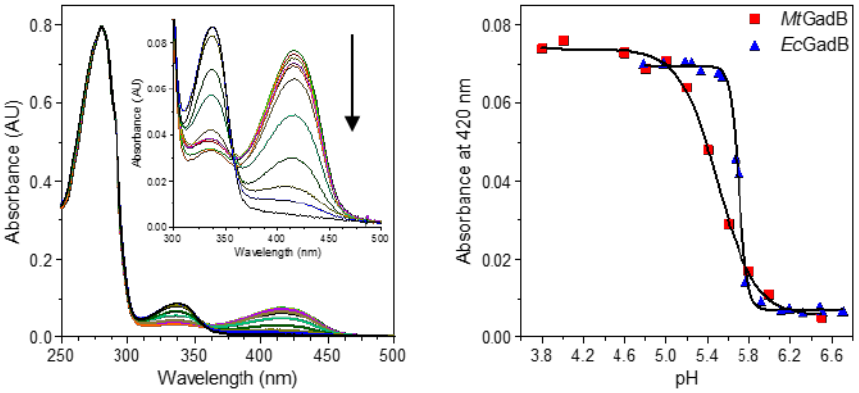
RESEARCH AREA: GENETICS AND BIOLOGY OF MICROORGANISMS

Mycobacterium tuberculosis is the world most deadly bacterial pathogen. In 2016, the World Health Organization reported that 10.4 million people were newly diagnosed with Tuberculosis, and that 1.7 million people died from it. The emergence of *M. tuberculosis* strains multi(MDR), extensively(XDR) and totally (TDR) drug resistant is disappointingly accompanied by little knowledge about *i)* the bacterial mechanisms of resistance and *ii)* the host factors that provide a selective pressure driving the evolution of these strains. What is known is that the stress caused by the antibiotics (oxidative stress) or in the macrophage (oxidative and acid stress) can trigger a bacterial response that leads to the "stepwise" building-up of mutations that eventually lead to MDR, XDR and TDR. A better knowledge on the points above is highly desirable.
As part of this "Seed International Research Project", the research team is evaluating the correlation between specific metabolic pathways and stress resistance in *M. tuberculosis* strains representatives of two contemporaneous bacterial collections obtained in the city of Salvador (Bahia, Brazil) and available at the Fiocruz Institute. One collection contains drug-sensitive strains obtained from a survey conducted evenly in the city health facilities that are responsible for diagnosing and treating tuberculosis cases, while the other collection regards the bacterial strains from all patients diagnosed with MDR tuberculosis in the city, that has only one service to diagnose and treat drug-resistant tuberculosis cases. This study involves the whole transcriptome analysis (RNA-seq) of MDR strains isolated from patients for which a history and follow-up are available, as well as epidemiological data. By combining these information we aim to contribute to the understanding of how changes in gene expression correlate to drug resistance. Our preliminary data indicate that particular strains are increased in

frequency when comparing drug sensitive with MDR tuberculosis, while other strains are less represented among MDR cases. Strain cultures have recently been started and no preliminary data are available regarding the evaluation of gene expression from these strains. As an additional goal of this project, the enzyme glutamate decarboxylase from *M. tuberculosis* (*Mt*GadB) was expressed in a recombinant form to perform a characterization at the biochemical level and help to uncover its physiological role(s). Alike the already characterised *Escherichia coli* and *Brucella microti* GadB, *Mt*GadB may in fact be required in acidic environments, such as that of the phagosome of macrophages. Alternatively *Mt*GadB may provide a path to compensate for the lack of the α-ketoglutarate dehydrogenase activity in *M. tuberculosis*.

During this year we have optimised the expression conditions and the purification of *Mt*GadB. Two different pET vectors were used: pET28 was eventually chosen for the large-scale purification and His_6-*Mt*GadB was purified via ion metal affinity chromatography (IMAC) and size-exclusion chromatography (SEC). According to the elution profile on SEC, *Mt*GadB eluted as a mixture of different quaternary structures (hexamer, tetramer and dimer), probably because of the His_6-tag. Experiments aimed at determining the influence of the His_6-tag on the oligomeric assembly of *Mt*GadB are in progress.

The purification strategy described above yielded a highly pure enzyme which was characterized spectroscopically and for activity. The figure below shows the changes of the UV-visible absorption spectrum of *Mt*GadB as a function of pH (on the left) and how these changes compare with those in the *E. coli* homologue, *Ec*GadB (on the right.



Moreover, fluorescence studies allowed to show that *Mt*GadB undergoes pH-dependent conformational changes and that in its inactive form (pH>6.0, absorbing maximally at

340 nm) *Mt*GadB has a substituted aldamine in the active site, a chemical species responsible for the enzyme auto-inhibition.

Preliminary data were reported at the EFB Meeting "Microbial Stress: from Systems to Molecules and Back" (23rd-25th April 2018, Kinsale Ireland) and at the 9th BeMM Symposium (13th November 2018).

Research Group	Collaborations
Fabio Giovannercole - PhD student **Marsel Koci** – Medical student	**John Blanchard -** Albert Einstein College of Medicine, New York, USA; **Johan Van Weyenbergh** - KU Leuven, Belgium

Seed International Research Project (First year report)

CELL MECHANICAL PROPERTIES OF INFECTED ERYTHROCYTES IN THE MATURATION OF *PLASMODIUM FALCIPARUM* TRANSMISSION STAGES IN THE BONE MARROW NICHE INVESTIGATED WITH A HUMANIZED MOUSE MODEL

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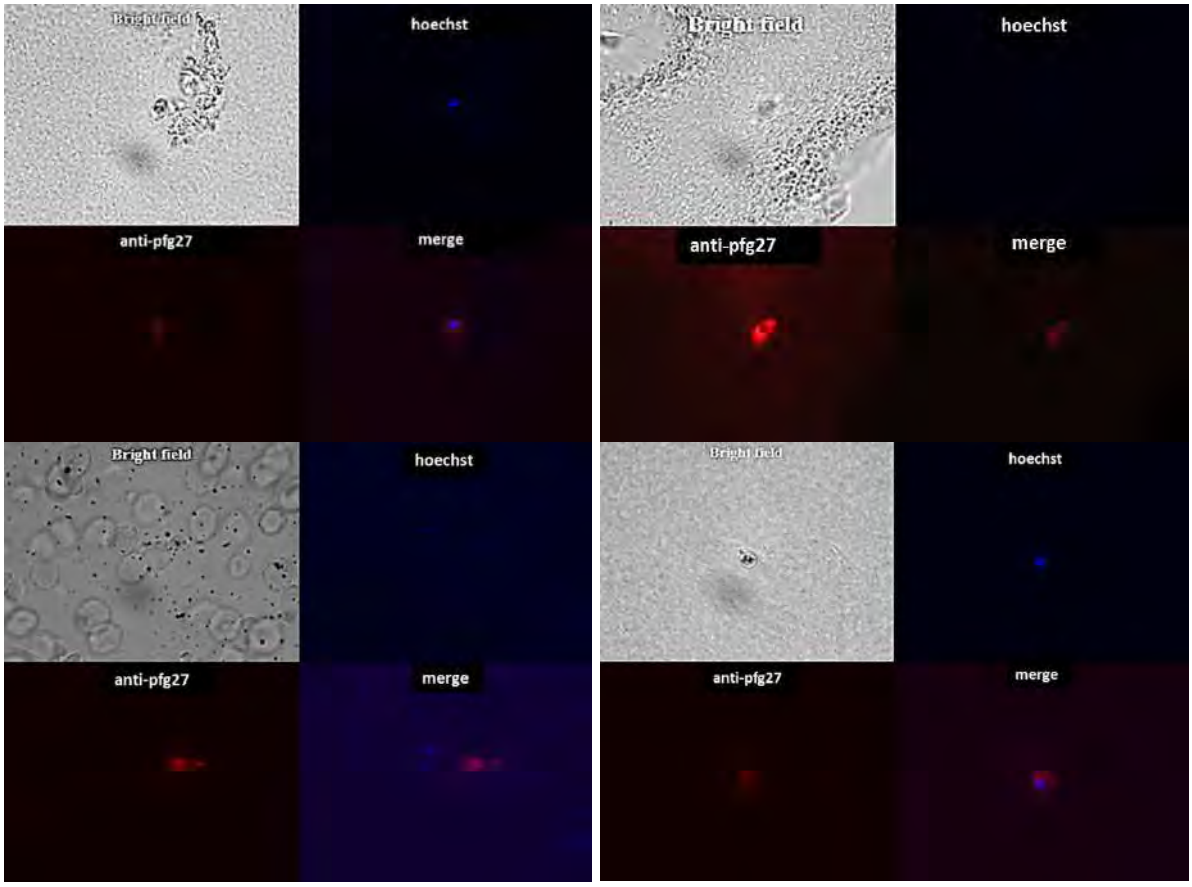
RESEARCH AREA: INFECTIOUS AGENTS AND ASSOCIATED DISEASES

In this project, we use a Bone Marrow Humanized Mouse model based on osteoprogenitor cell transplantation to investigate the role of the human bone marrow extravascular compartment in the maturation of *Plasmodium falciparum* gametocytes.
The first activities of the project aimed at a) refining the experimental protocols for the analysis and quantitative evaluation of the tissue distribution of immature and mature gametocytes of *Plasmodium falciparum* in the ectopic bone marrow organoid and in relevant organs of the humanized mouse (bone marrow, spleen, liver, lung, brain, kidney) and b) determining how the tissue/organ distribution and the extra- vs intra-vascular localization of gametocytes are affected by their maturation stage.
We first developed an in vitro experimental system based on human bone marrow derived skeletal progenitors (Bone Marrow Stromal Cells, BMSCs) to optimized the identification of immature and mature gametocytes of *Plasmodium falciparum* by using an antibody against the gametocyte-specific protein 27 (Pfg27). A scaffold of BMSCs including immature gametocytes was generated in Matrigel® at 37°C, fixed in formaldehyde and embedded in paraffin. Three-micron sections were then cut and different protocols for immunolocalization of Pfg27 were tested. We tested a) two methods for antigen retrieval, boiling of slides in citrate buffer and trypsin digestion and b) two anti-Pfg27 primary antibodies, one developed in rabbit and one developed in rat. Only the combination of trypsin-based antigen retrieval with the rabbit anti-pfg27 antibody (overnight incubation) allowed us to clearly identify *Plasmodium falciparum* gametocytes in the scaffold, as shown in the figure below.



Immunolocalization of *Plasmodium falciparum* gametocytes in the Matrigel-BMSC scaffold. The gametocytes (red) were highlighted by using the rabbit anti-pfg27 antibody, the nucleus (blue) was stained with the DNA specific Hoechst dye.

In collaboration with the Istituto Italiano di Tecnologia (IIT), based in Rome, we then developed a protocol to acquire a 3D image of the bone marrow organoid (and mouse bone) by two-photon confocal fluorescent microscopy. This fluorescence imaging technique allows the imaging of tissues up to about one millimetre in depth, thus allowing a detailed analysis of the ectopic human bone marrow organoid used in this project. Using a rabbit anti-laminin antibody, able to recognize both the human and mouse molecule, a protocol to acquire a 3D image of an entire human bone marrow organoid (and of segments of a mouse bone) was established. We optimized the methods for bone fixation, decalcification and "clarification" in order to clearly detect the immunofluorescence signal while preserving the organ architecture (data not shown). Although both the experimental protocols (immunolocalization of the *Plasmodium*

falciparum gametocytes and 3D image of the bone marrow organoid vascularity) were successfully established, the subsequent step of simultaneous detection of the organoid blood vessels and *Plasmodium falciparum* gametocytes was delayed by the fact that the anti anti-pfg27 antibody and the anti-laminin antibody are both developed in rabbit. As alternative strategies, we plan a) to test different anti-laminin antibodies generated in different animal species and b) to use transgenic gametocytes expressing a fluorescent reporter such as GFP or Tomato. For the latter approach, we are developing an alternative protocol for bone fixation, decalcification and "clarification" that will allow the detection of the endogenous fluorescence from the reporter protein.

Publications

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Research Group	Collaborations
Alessandro Corsi, researcher; **Samantha Donsante,** post-doc fellow; **Marianna Melito**, student.	**Pietro Alano and collaborators**, Istituto Superiore di Sanità, Roma. **Valeria de Turris,** Centre for Life Nano Science, Istituto Italiano di Tecnologia, Roma.

PTR 2017 – 2018 (Second Year Report)
"SELF B-LIVER"
COMPLEXITY OF IMMUNE INTERACTIONS IN CHRONIC HEPATITIS B VIRUS INFECTION: HOW THE EXACERBATED INFLAMMATORY RESPONSE BY SELF ANTIGEN-SPECIFIC CD8 T CELLS AND REGULATORY T CELLS DICTATE THE FATE OF HBV- SPECIFIC RESPONSES

SILVIA PICONESE AND VINCENZO BARNABA[1], MARYLINE BOURGINE[2], YU WEI[3]
1. IP Rome, 2. IP Paris, 3. IP Shanghai

Hepatitis B virus (HBV) chronic infection is associated with hepatic lesions of variable severity, ranging from a practically normal liver to severe lesions of active chronic hepatitis that can develop into cirrhosis and hepatocellular carcinoma. Inflammation plays a critical role in tissue repair by eliminating injured cells through the action of immune cells, which are controlled by cytokines and chemokines. Highly coordinated interactions between immune cells and secreted mediators guarantee efficient development and resolution of the inflammatory response and restoration of tissue homeostasis. In contrast, uncontrolled and excessive inflammation produces pathologic effects. It has been shown that, during chronic disease, virus-specific T-cell responses are weak and impaired in HBV patients. Exhausted T cells are characterized by a high susceptibility to apoptosis. Apoptotic cells are engulfed by dendritic cells (DCs) that process, in a caspase-dependent way, the structural cellular proteins (such as myosin, vimentin, actin, etc.) and cross-present apoptosis-derived epitopes (AE) to autoreactive CD8 T cells. These cells, when activated, perform their effector functions, undergo apoptosis and are phagocytosed by DCs, maintaining a vicious circle responsible for the maintenance of chronic immune activation. This mechanism has been demonstrated in viral infections such as hepatitis C virus (HCV) or human immunodeficiency virus (HIV). Based on these observations, we hypothesize that the autoreactive response against apoptotic T cells may play a role in the state of chronic inflammation in HBV infection.

Our project aimed to characterize the CD8 cells specific for self epitopes derived from apoptotic T cells and their role in maintaining the state of chronic inflammation associated to HBV infection, and to study the effects of the regulatory T cells (Tregs) in balancing the activity of autoreactive CD8 T cells. To achieve this goal, our unit has studied the AE-specific CD8 response in patients (HLA A*0201-positive) with HBV infection at different stages of the chronic disease. In parallel, our partners at IP Paris and IP Shnaghai have studied AE-specific T cells in mouse model for chronic hepatitis B, i.e. wild-type mice transduced with an adeno-associated vector carrying a replication-competent HBV DNA genome (AAV-HBV) vector: AAV-mediated HBV transfer in the liver generates intra-hepatic stable viral replication without eliciting apparent immune-mediated damage against transduced cells, thus mimicking chronic HBV infection in

humans during the so-called “immunotolerant phase”.

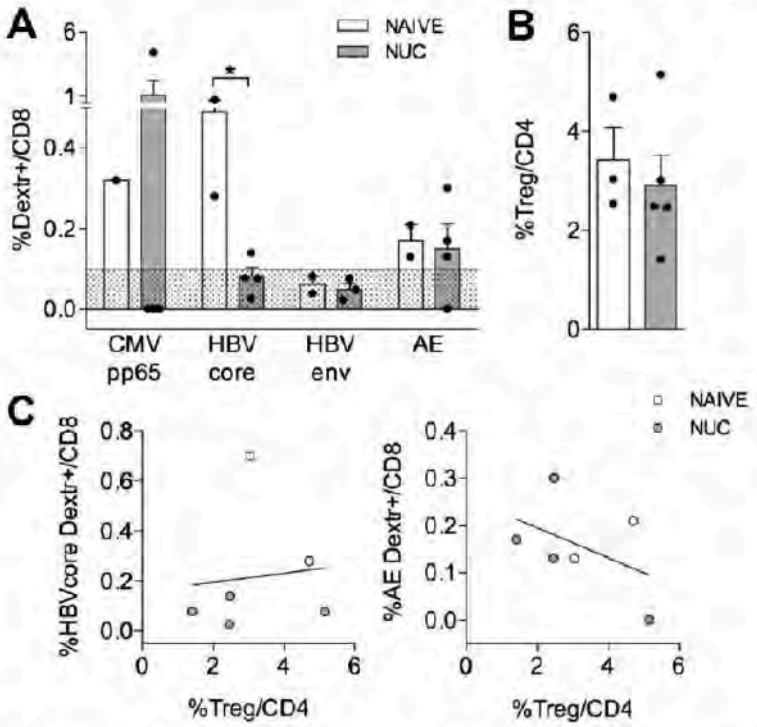
In order to study antiviral and autoreactive responses in chronic hepatitis B (CHB) patients, first we have analyzed, by flow cytometry, the frequency and the phenotype of AE-specific and HBV-specific CD8 T cells and Tregs in correlation with clinical parameters. In collaboration with Prof. G. B. Gaeta (Second University of Naples), we have collected the peripheral blood from 21 CHB patients with were positive for HLA-A*0201. Among these, 3 were treatment-naive (with HBV-DNA levels all below 104), while 18 were under nucleos(t)ide analogue (NUC) treatment. All of the 18 treated patients had ALT <60, and only 1/18 showed detectable HBV DNA levels (47 IU/ml). Ten out of 18 treated patients also showed hepatic cirrhosis (with fibrosis score f4). We have started our analysis from the 3 treatment-naive patients and from 5 NUC-treated (and notcirrhotic) patients. Flow cytometry analysis was performed on thawed PBMCs using the following antibodies: for Treg analysis, CD4, FOXP3, CD127, CD25, CD45RA (after exclusion of dead, CD14+ or CD8+ cells); for CD8 analysis, CD8, CD45RA, CCR7, PD1, Eomes, Tbet, CD127 (after exclusion of dead, CD14+ or CD4+ cells), and dextramer. The following HLA-A2*0201-dextramers were used: HBV core 18-27 (FLPSDFFPSV), HBV envelope 335-343 (WLSLLVPFV), pooled AE including actin-B 266-274 (FLGMESCGI), myosin-9 478-486 (QLFNHTMFI), myosin-9 741-749 (VLMIKALEL), vimentin 78-87 (LLQDSVDFSL), vimentin 225-233 (SLQEEIAFL), and CMV pp65 as control (NLVPMVATV); the two HBV specificities were selected based on published data in NUC-treated CHB patients (Boni C et al, Gastroenterology 2012). In line with those data, we could not detect HBVenv-specific CD8 in naïve or NUC patients, while HBVcore-specific were detected in 2/2 naïve and 1/4 NUC-treated patients, at significantly different frequencies; conversely, AE-specific T cells were detected in most patients irrespective of treatment **(Figure 1A)**. Tregs were also found in normal frequency in CHB patients, showing a trend for a negative correlation with AE- but not HBV-specific CD8 T cell frequency **(Figure 1B-C)**. A deeper analysis of HBVcore- and AE-specific CD8 T cells revealed that, while the former included only Tem and Temra, the latter also contained T naive cells, paralleling the polyclonal (dextramer-negative) CD8 T cell distribution. Moreover, while HBVcore-specific Tem/Temra were mostly Tbet+Eomes+/-PD1-, AE-specific Tem/Temra also contained Tbet+/-Eomes+PD1+ cells. These results suggest that AE-specific T cells are present in CHB patients, are not affected by NUC therapy, do not show an activated phenotype, and may be indeed controlled by Tregs.

We have assessed the effects of Treg towards antigen-specific CD8 T cells by performing an in vitro suppression assay. Due to the low yield of cells isolated from CHB patients, we performed our analysis on CMV-specific CD8 T cells obtained from healthy donors. Tem/Temra CD8 T cells and Tregs were obtained from CMV-positive healthy donors (in which most CMV-specific CD8 T cells display a Tem/Temra phenotype), and were cocultured in vitro in the presence of CMV pp65 peptide-pulsed autologous APCs, or aCD3/aCD28 as positive control. After 7 days, we only detected a very poor suppression,

if any, against both peptide- and polyclonally-stimulated Tem/Temra cells. This result may suggests that Tem/Temra cells, including CMV-specific CD8, may be relatively resistant to Treg suppression. In the context of HBV, our data indicate that, while most HBV-specific CD8 are Tem/Temra, AE-specific CD8 comprise both Tn and Tem/Temra: thus, we hypothesize that Tregs may exert differential suppression against different specificities.

In conclusion, our results suggest that an autoreactive immune response, directed to apoptosis-associated antigens, may persist in CHB patients after NUC therapy-induced control of viremia, and may thus contribute to chronic immunopathology in this context.

Figure 1
Frequencies of AE- and HBV-specific CD8 T cells and Tregs in CHB patients.



(A) Frequency of CMVpp65-, HBVcore-, HBVenv-, or AE-specific CD8 T cells was evaluated by flow cytometry in PBMCs of naïve-to-treatment (n=2) and NUC-treated (n=4) CHB patients. Shadowed area indicates the background for dextramer staining (0.098). *p<0.05 by unpaired Student's t test, two-tailed.
(B) Treg frequency was assessed as the percentage of FOXP3+ CD127low/CD4 T cells in PBMCs of naïve-to-treatment (n=3) and NUC-treated (n=5) CHB patients.
(C) Tendencies for correlations between Treg frequency and HBVcore-specific (left) or AE-specific (right) CD8 T cell percentages in each patient (n=6).

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Research Group	Collaborations
Ilenia Pacella PostDoc Researcher **Ilenia Cammarata** PhD student	**Prof. G. B. Gaeta** Second University of Naples

PTR 2017 – 2018 (Second Year Report)
A MULTIDISCIPLINARY INVESTIGATION OF NEGATIVICUTES: ATYPICAL FIRMICUTES WITH LPS-OUTER MEMBRANES THAT INHABIT THE HUMAN GUT.

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2 IP Paris

The aim of this project was to combine bioinformatics and proteomics and immunological approaches to provide novel information on the surface exposed fraction of *Veillonella parvula.* The bioinformatic approach was finalized to the identification of the OM components that are common to the whole clade, of their similarities with those of classical diderm bacteria, as well as to the study of their unique characteristics. All these information are needed to clarify evolutionary origin of the whole clade, and discover novel pathways and structures. Finally, during the project, current genomic data from uncultured lineages has led to increase significantly the sequence coverage for the Negativicutes, with the acquisition of 50 new genomes, one of which corresponds to a new and deep branching lineage of this class. Moreover, this study has allowed to identify a third and distinct clade of diderm Firmicutes, indicating that the presence of outer membranes is even more widespread than currently known in this phylum. Genetic tools have been successfully developed in *V. parvula* following the identification of naturally competent strains. These tools definitely establish *V.parvula* as a new model for the outer envelope.

The role of the unit at the Istituto Pasteur Italia – Fondazione Cenci Bolognetti was focused on the analysis of the immunological impact of the OM components of *V. parvula* with particular attention to the immunopotential of lipopolysaccharide (LPS).

The LPS purification and analysis of *V.parvula* grown under laboratory condition (planktonic LPS) has been carried out during the project. The lipid A of *V.parvula* shows an *Escherichia coli*-like structure. It mainly contains a species with six acyl chains (ranging from C11 to C15) and two phosphates in positions 1 and 4', in agreement with the *E.coli* lipid A structure. However, in contrast to *E.coli* lipid A the MALDI-TOF MS analysis of *V.parvula* lipid A has revealed an heterogeneous mixture of lipid A species including tetra- to hexa-acylated forms containing one or two phosphates that are poorly present or absent in the *E.coli* lipid A blend.

The *V.parvula* LPS has been evaluated for its immunologic impact in several cell models. First, various concentrations of *V.parvula* LPS have been assessed in the model of HEK293 cells expressing the complex of the human TLR4 (hTLR4/MD2/CD14). A fully immunocompetent hexa-acylated *E.coli* LPS and the synthetic tetra-acylated TLR4 antagonist, Lipid IVa, were the positive and the

negative controls, respectively in all the experiments. *V.parvula* LPS activated the nuclear factor NF-kappaB and induced the release of the cytokine CXCL-8 at significantly higher extent with respect to *E.coli* LPS, especially at lower concentrations. In accordance with these results, in human peripheral blood monocyte-derived macrophages (MoMs) and in human peripheral blood monocyte-derived dendritic cells (MoDCs) the release of the inflammatory cytokines, TNF-alfa and IL-6 was higher than that induced by the *E.coli* LPS. In MoMs, *V.parvula* LPS induced the production of the anti inflammatory cytokine IL-10 as *E.coli* LPS while in MoDCs the IL-10 amount was higher with *V.parvula* LPS. In these cell populations, the cytokine Interferon-gamma Induced protein 10 kDa (IP-10) was stimulated by *V.parvula* LPS as by *E.coli* LPS. This result indicates that *V.parvula* LPS activates the two signaling pathways, the MYD-88 and the TRF-mediated pathway, upon the recognition of the LPS by the TLR4 complex. In agreement with this finding, in murine Bone Marrow Derived Macrophages (BMDMs) the mRNA for IFN-beta, which is under the control of the TRIF pathway, was highly stimulated by *V.parvula* LPS and the level of IP-10 was higher with *V.parvula* LPS than with *E.coli* LPS. Likewise, in BMDMs the cytokines TNF-alfa and IL-10 were stimulated as with *E.coli*, while IL-6 was more abundant with *V.parvula* LPS than with *E.coli* LPS.
V.parvula has been isolated from periodontitis ad other inflammatory disorders in the gut. The high inflammatory potential of its LPS is therefore not surprising. It was important to analyze the immunologic impact of this LPS in association with other LPSs that could be simultaneously present in the same environment. The hexa-acylated *E.coli* LPS when combined with a tetra-acylated TLR4-antagonist LPS (like that of Lipid IVa) is unable to activate the TLR4-downstream signaling as the tetra-acylated non-stimulatory LPS competes with the fully immunostimulatory haxa-acylated LPS for the binding in the MD2 hydrophobic pocket. Consequently, the downstream signaling pathways are blocked. We therefore combined the *V.parvula* LPS with the tetra-acylated Lipid IVa at different concentrations, in the cell model of HEK2913 cells expressing hTLR4/MD2/CD14. The presence of Lipid IVa decreased ($P= 0.03$) the NF-kappa signaling only when *V.parvula* LPS was tested at a ratio of 1 ng/mL (*V.parvula* LPS)/ 100 ng/mL (Lipid IVa). At higher *V.parvula* and lower Lipid IVa LPS concentration the antagonistic effect was lost. However, when the *E.coli* hexa-acylated LPS was used at the place of the Lipid IVa in this test the NF-kappaB signaling was not significantly potentiated. These experiments seem to suggest that *V.parvula* LPS could act as a balance in the gut by maintaining high levels of immune system stimulation, despite the presence of other fully immunostimulatory or antagonist LPS.

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PTR 2017 – 2018 (Second Year Report)
UNDERSTANDING THE SELECTIVE BENEFIT OF THE SHIGELLA GENOME SPECIFIC ARCHITECTURE.

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Bacteria have long been thought to have a simple genome architecture based on a unique circular chromosome, occasionally accompanied by plasmids, and it is only in the late 1980s that the first prokaryote with multiple chromosomes was characterized. Since this initial acquisition, considerable information has accumulated on how varied the organization of the bacterial genome can be. The increase in available sequence data has uncovered genome complements that include from one to several chromosomes, some of which even endowed with a linear structure, and has paved the way for the debate on the elusive difference between "chromosome" and "megaplasmid". While providing a number of clues on the evolution of bacteria and on the distribution of genes and operons, the knowledge derived from genomic sequencing still leaves open some fascinating questions: Why do some bacteria have a single chromosome, others more than one, and still others maintain relevant genes on mobile genetic elements? Why are virulence genes in some bacteria always confined on a plasmid?
In *Shigella*, the etiological agent of bacillary dysentery, the pathogenicity process depends on the presence of a 220-260 kb, low copy number plasmid (pINV) functionally interchangeable between species and serotypes. The plasmid contains a 32 kb pathogenicity island (PAI) –like structure coding for invasins, molecular chaperones, intracellular motility proteins, regulatory factors, and for a specialized Type III secretion apparatus. The regulator gene *virF* and other genes, like *icsA*, *virA* or *apy*, required for optimal invasion of culture cells, are located outside this cluster. pINVs share with the IncFIIA plasmids a high homology in the regions involved in replication (*rep*) and conjugation (*tra*). Due to large deletions in the *tra* region, pINVs are not capable of self-transfer by conjugation, but they can be mobilized by other conjugative plasmids. Stable inheritance of pINVs is ensured by the presence of several systems of plasmid segregation and maintenance distributed on the plasmid genome. Loss of the pINV plasmid is a very rare event and determines an avirulent phenotype. All over the plasmid, a very high number of insertion sequences (IS) is found as a mixture of complete and incomplete IS elements repeated several times. These elements represent up to one-third of the pINV and are often organized in mosaic structures which, in many cases, flank virulence associated genes.

Harbouring a plasmid often imposes a fitness cost on the bacterial host because at a minimum its maintenance requires its replication and partitioning during each bacterial generation. Nevertheless, comparative analyses of *Shigella* genomes confirm that in no case the 32 kb PAI-like structure has moved from the pINV to the chromosome. The

pINV location of the virulence genes in *Shigella* is in contrast with the chromosomal localization of the genes responsible for the virulence in many pathogenic *E.coli*, as UPEC, UTEC and EHEC.

Like *Shigella*, also a group of intestinal pathogenic *E. coli*, collectively known as enteroinvasive *E.coli* (EIEC), has stably acquired the pINV plasmid and has become able to induce the same pathological processes. However, EIEC retain the ability to catabolize substrates widely used by *E. coli* and have been traditionally considered as an evolutionary intermediate between *Shigella* and *E. coli*.

Previously our research group have shown that that the pINV plasmid of EIEC strain HN280 is able to integrate into the host chromosome and that integration results in the silencing of all the pINV virulence genes. While these observations have led to the intriguing hypothesis that pINV integration might constitute a further strategy to ensure plasmid maintenance, relevant steps of the integration process and of the mechanisms responsible for the modification of the regulatory pathways are still undefined.

Therefore, we evaluated the evolutionary significance of the preferential localization of virulence genes on an extrachromosomal element in the *Shigella flexneri* 5a strain M90T, despite a greater cost for the maintenance of the virulence plasmid. To this aim we decided to direct the integration of pINV into the chromosome of M90T in different loci, adapting a procedure previously set up in laboratory of Prof. Didier Mazel at the the Institut Pasteur (Paris) to study the *Vibrio cholerae* chromosomes, in order to evaluate if integration gives rise to silencing of the vir genes.

We first developed a simple strategy to unidirectionally and specifically direct the integration of pINV into the chromosome of the *Shigella flexneri* M90T strain, a strain widely used in *Shigella*-host interaction studies. Our technique takes advantage of the λ Red recombination system to introduce attP sites for λ and HK022 on the *Shigella* virulence plasmid (pINV). This step is essential to allow site-directed recombination between the attB site, naturally present on the *Shigella* chromosome and the attP site introduced into the virulence plasmid. The related phage HK022 forms lysogens in the same way but uses a different pair of att sites. The Int proteins of the two phages are similar in primary structure, but neither promotes efficient recombination of the heterologous pair of att sites. The integration event results in the formation of two hybrid sites, attL and attR, each consisting of half attP and half attB sequence. Results obtained after the integration of the pINV plasmid into the *Shigella* chromosome mediated by HK att sites, clearly indicate that *virB* expression isseverely affected upon pINV integration, while *virF* and *icsA* expression only partially decreases. From these data we have hypothesized that repression of *virB* transcription upon pINV integration could be due to changes in DNA topology which would favor a more stable binding of the H-NS repressor to the *virB* promoter.

We are now creating other *S. flexneri* derivatives harboring the pINV integrated into the different loci of the *Shigella* chromosome in order to clarify whether the position

and the distance from the replication origin might influence the expression level of the *vir* genes. This will be done using the above mentioned □/HK022 strategy, which allows performing reorganizations of genome elements with the added advantage to control the location and orientation of each recombination event.

Moreover banking on the genome sequence of EIEC HN280, we have defined that in EIEC the integration process is determined by the ISEc11 insertion element and requires a functional RecA protein. These data contribute to clarify relevant steps of the integration process and give further support to the hypothesis that pINV integration might constitute a strategy to ensure plasmid maintenance both in *Shigella* and EIEC.

Publications

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Research Group	Collaborations
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PTR 2018 – 2019 (First Year Report)

ROLE OF HP1/CBX PROTEIN UBIQUITINATION IN CHROMATIN ORGANIZATION

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Genome stability and integrity are essential cell features that require accurate maintenance for the completion of cell division, proliferation as well as for organism survival. Failure in the maintenance of genome stability can result into formation of chromosome aberrations and aneuploidy, two major hallmarks of tumor progression and development as well as severe genetic defects. A proper organization of chromatin structure is an essential prerequisite to preserve genome stability from costant threats to chromosome integrity.

In this proposal, we are addressing the biological relevance of ubiquitination, a particular form of protein modification, on Heterochromatin Protein 1 (HP1), a well conserved chromosomal protein that plays fundamental roles in chromatin architecture, gene expression silencing and homeostasis of chromosome ends (telomeres). While it is largely known that HP1 undergoes a variety of post-translational modifications (PTM) that influence its activity, the role of the ubiquitination is still elusive. We have undertaken a synergic approach to unravel the contribution of HP1 ubiquitination at both cellular (human and Drosophila cell lines) and organismal (Drosophila) levels. We are carrying out a global protein analysis with the aim of identifying specific aminoacids (lysines) in the HP1 protein that interfere with its ubiquitination**.** In addition, we combine genetic, molecular and cytological approaches to investigate (a) how HP1 ubiquitination is relevant for a proper telomere protection in Drosophila; (b) the role of the ubiquitination of Cbx proteins (the human HP1 orthologs) in the global gene expression, and check whether this modification is required for the stability of the Cbx proteins, their sub-cellular localization, their extractability (chromatin-association) and their interaction with telomeric shelterin, a protein complex that normally protects human chromosome ends.

Given that this proposal aims at unraveling how ubiquitination may regulate HP1 functions in telomere maintenance, gene expression and replicative senescence, and since these pathways are fundamental in ensuring genome integrity, we are confident that our results will provide novel mechanisms to prevent genome instability onset that underlie several pathological conditions including cancer.

Publications

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PTR 2018 – 2019 (First Year Report)
DISSECTING ROLES FOR DIVERSE INNATE LYMPHOID CELL SUBSETS IN COLITIS-INDUCED COLORECTAL CANCER

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Our knowledge of the ILC roles in the context of colorectal cancer (CRC) is largely unexplored and several questions remain unanswered. What is the impact of ILC deletion in the pathogenesis of CRC? Can ILCs participate in CRC progression by regulating chronic inflammation? Can CRC alter ILC phenotypes and functions in order to grow and evade surveillance? The proposed research program has the ambition to tackle these fundamental aspects by addressing the following two aims: 1) identification of specific phenotypes and roles of ILCs expressing natural cytotoxicity receptors (NCRs) in the pathogenesis of CRC; 2) discrimination of the factors downstream STAT4 signaling underlying ILC functions and tumor progression. Two parallel and independent WPs have been proposed:

WP1. Investigation of the impact of NCR+ ILCs in CRC development. Different mouse models of CRC have been described recapitulating several traits of the human disease. In the frame of PTR program, we analyzed the features of tumor-infiltrating ILCs using a conventional mouse model of colitis-induced CRC, initiated by an intraperitoneal injection of a single dose of azoxymethane (AOM) and followed by administration of dextran sulfate sodium (DSS) in the drinking water for one week (4 cycles). We began by dissecting the phenotype of NCR+ ILCs isolated from tumors and colonic mucosa (obtained after tumor excision) of AOM/DSS treated mice, as well as, from colon of mice left untreated, used as control. In parallel, we evaluated the phenotype of NCR+ ILCs in the intestinal lamina propria and tumor-infiltrating lymphocytes of CRC patients. Moreover, to define the role of NCR+ ILCs (NK and ILC1) in regulation of inflammation in colitis settings, we targeted these cells in vivo by antibody administration (PK136). Next, to dissect the role of NCR+ ILCs in the pathogenesis of CRC, we plan to selectively delete these cells by genetic approach, using *Ncr1-cre/Il2rg(fl/fl)* mice.

WP2. Evaluation of the role of STAT4 expression in ILCs during CRC. We recently dissected the molecular mechanisms underlying STAT4 expression in intestinal ILC subsets (Team 2; *Eur J Immunol*. 2018. PTR funding acknowledged). Our findings revealed a selective and high expression of STAT4 in NCR+ ILCs, including NK, ILC1 and NCR+ ILC3. We also evaluated the effect of IL-12 and IL-23 in induction of STAT4 activation in ILCs and defined the downstream targets by transcriptomic analysis. We

are currently generating *Stat4(fl/fl)* mice that will be mated with *Ncr1-cre* mice (generated by Team 1) to selectively delete the expression of this TF in NCR+ cells. These mice will be tested for degree of inflammation and CRC development using the models described in WP1. Individuals carrying mutations in IL-12RB1 (shared by IL-12 and IL-23) suffer from Mendelian Susceptibility to Mycobacterial Disease (MSMD, OMIM209950) and fail to normally activate STAT4. Team 1 reported showed that IL12RB1 is important for ILC2 function in humans (*J Exp Med*. 2016) and that human ILC precursors (ILCP) circulate in the blood (*Cell* 2017). Recently, Team 1 identified a novel role for IL-23 in the differentiation of human IL12RB1-deficient ILCP (Croft et al., in preparation). Through generation of a novel mouse model and the analysis of IL12RB1-deficient cells, we plan to define STAT4-dependent and independent effector programs in mouse and human ILC subsets associated with inflammation and cancer.

Evaluation of ILC phenotypes in mouse models of colitis and colitis-induced CRC (Relative to Aim A). We initially dissected the phenotype of ILCs isolated from the colonic lamina propria (LP) of mice left untreated or treated with DSS (day 7). We observed that type 1 ILCs (identified as RORgt+NKp46+NK1.1high cells) were highly enriched as compared to other ILC subsets (data not shown). Among type 1 ILCs, CD49a+ ILC1 were more abundant in untreated mice, while a drastic increase of CD49b+ NK cells was observed in colitis settings (Figure 1A-B). We are currently a) optimizing a 14-parameters flow cytometry panel to further dissect the ILC phenotypes and b) analyzing the profile of cytokine expression, proliferation rate and mechanisms involved in tissue homing of NK and ILC1 (Team 1 and Team 2). Administration of the monoclonal antibody PK136 (anti-NK1.1) at -3, 0, +3 days from the beginning of the DSS cycle led to an efficient depletion (>95%) of both NK cells and ILC1 in the colon (Figure 1C). These experiments also showed that anti-NK1.1 treatment was protective in the DSS model (Figure 1D), providing evidence for a possible role of NCR+ ILCs in promoting inflammation driving CRC development. Next, we analyzed the phenotype of type 1 ILCs isolated from tumors and colonic mucosa (after tumor excision) of AOM/DSS treated mice. As shown in Figure 2A, we observed a reduced number of tumor-infiltrating ILC1, while NK cells remained the main NCR+ ILC subset. Based on these observations, we plan to define the overall role of NCR+ ILCs in regulation of inflammation and CRC pathogenesis by selective deletion of these cells, using Ncr1-cre/Il2rg(fl/fl) mice (Team 1). Moreover, to discriminate the relative contribution of NK cells, we plan to optimize an in vivo protocol consisting on administration of limiting doses of anti-NK1.1 able to selectively target NK cells, leaving colonic ILC1 and NKT cells unaltered (Team 2).

Evaluation of tumor-infiltrating ILCs in CRC patients (Relative to Aim B). We have been profiling the phenotype of ILCs in tumor-infiltrating lymphocytes isolated from biopsies of CRC patients by using multiparameter flow cytometry (BD LSR Fortessa). As shown in Figure 2B, we observed a striking decrease in the frequencies of

NKp44+ ILCs isolated from CRC biopsies (Stage II-IV), as compared to non-tumor tissue (upstream distal resection margin), and an increase of CD16+ NK cells in the tumor. Notably, the abundance of NKp44+ ILCs, mainly consisting of ILC1 and NCR+ ILC3, is not altered in the adenomas currently analyzed. These data paralleled the low frequency of tumor-infiltrating ILC1 and NCR+ ILC3 observed in mice and provide evidence for a possible role of CRC in driving the phenotypic changes occurring in NCR+ ILCs. We are currently expanding the flow panel used for evaluation of ILCs (described in Figure Legend 2B) to include a) markers and transcription factors further discriminating NK cells and ILC1 (CD16, CD103, KIRs, T-BET, EOMES, granzymes and perforin), and type 3 ILCs (CD117, CD161, RORγt) and b) signature cytokines including IFN-γ, TNF-α, IL-22, IL-17, after short ex vivo stimulation. Our aim is to find the relationships among frequency and phenotype of tumor-infiltrating NCR+ ILCs, CRC stage, and prognosis in humans.

Dissecting the role of STAT4 in NCR+ ILCs in mouse models of colitis and colitis-induced CRC (relative to AIM A). Team 2 has recently dissected the molecular mechanisms underlying STAT4 expression in intestinal ILC subsets. We showed a selective and high expression of STAT4 in NCR+ ILCs, comprising NK, ILC1 and NCR+ ILC3 in homeostatic conditions and during acute colitis. We also evaluated the effect of IL-12 and IL-23 in induction of STAT4 activation in ILCs and defined the downstream targets by transcriptomic analysis. Moreover, we evaluated the contribution of STAT4 in induction of IFN-□ expression in NCR+ ILCs. These data were published in the *European Journal of Immunology* and PTR program was acknowledged (*see section 6E*). Team 2 is currently generating *Stat4(fl/fl)* mice) that will be mated with Ncr1-cre mice (Team 1, available) to selectively delete the expression of this TF in NCR+ ILCs. These mice will be evaluated for degree of colon inflammation using the DSS-colitis model and kinetics of CRC development using the AOM/DSS model. Team 1 showed that IL12RB1 affects ILC2 function and identified a role for IL-12 in ILC2 plasticity (*J Exp Med*. 2016). In addition, Team 1 characterized human ILC precursors (ILCP) that circulate in the blood (*Cell* 2017) and can differentiate to mature ILC subsets in vitro. Using this approach, Team 1 recently identified a role for IL-23 in human ILCP differentiation (Croft et al., in preparation). The molecular mechanisms of this novel IL-23 effect is currently being characterized.

Publications

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Research Group

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Figure 1

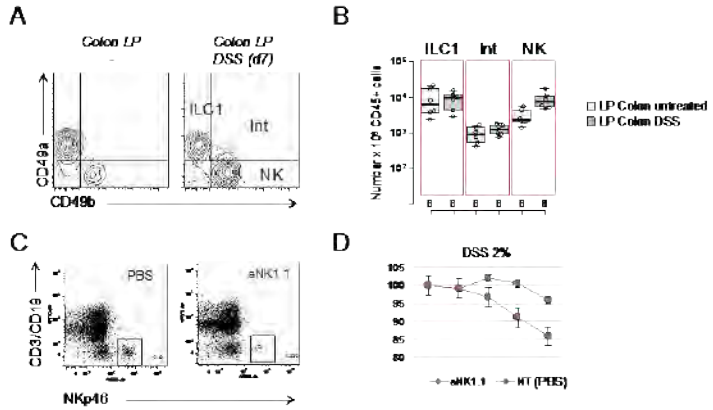

Figure 2

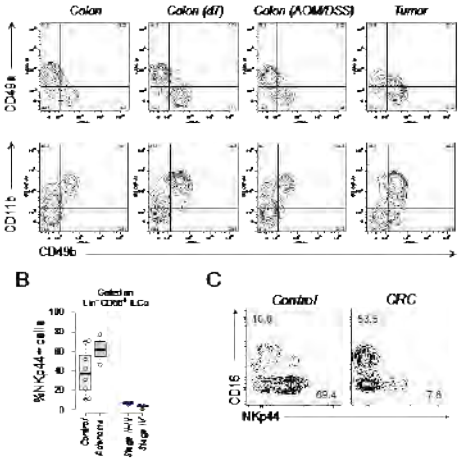

Figure 1. Mice were treated for 7 days with 2% DSS in the drinking water, and weight loss was measured relative to initial weight. Lamina propria lymphocyte were isolated from colon of DSS treated mice and from colon of untreated mice. Cells were stained for CD19, CD3ε, CD4, CD127, NK1.1, NKp46, KLRG1, RORgt and T-BET. A. Flow cytometry dot plots depict the different type 1 ILCs gated as CD3e-CD19-RORγt-NKp46+NK1.1+ cells. B. Box plot shows the frequency of different type 1 ILC subsets in CD45+ cells, relative to A. Each dot represents an individual mouse. C and D. Effects of in vivo anti-NK1.1 administration (i.p.100 mg) on type 1 ILCs frequency (C) and weight loss (D).

Figure 2. Mice were treated with 2% DSS or AOM/DSS. After 12 weeks, tumors/polyps were dissected and tumor-infiltrating ILCs were isolated following enzymatic digestion. As control, ILCs were isolated from healthy colon of AOM/DSS treated mice after dissection of tumors/polyps, and from colon of untreated mice. A. Flow cytometry dot plots depict the different type 1 ILCs gated as CD3e-CD19-RORγt-NKp46+NK1.1+ cells in all conditions. B and C. Specimens were obtained from CRC patients not previously treated with steroid therapies and with no history of immunodeficiency. Lymphocytes were isolated from tumors and intestinal tissue by enzymatic digestion and stained for CD45, CD3e, CD19, CD14, CD56, CD127, CD103, and CD117 to discriminate distinct ILC subsets. (B) Box plot shows the frequencies of human NKp44+ ILCs (gated on CD45+CD3ε-CD19-CD14-CD56+ cells) within the tumors (Stage I-IV), control tissue (upstream distal resection margin) and adenomas. Each dot represents the percentage of NKp44 in an individual patient. (B) Representative dot plots show the expression of CD16 and NKp44, among CD56+ ILCs, within the tumor and control tissue.

ACIP 2018

EXPLORING THE HIDDEN GENOMIC DIVERSITY OF *ANOPHELES GAMBIAE* AND *AN. COLUZZII* SPECIES PAIR TO ACCOUNT FOR SPATIAL DIFFERENCES IN MALARIA TRANSMISSION (EXGENMAL)

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Despite decline of malaria cases and deaths over the last 15 years, the burden is still very high in sub-Saharan Africa due to, among other factors, a very efficient vectorial system characterized by genetic discontinuities among closely related mosquito species. *Anopheles gambiae* and *An. coluzzii* are two very closely related species accounting for the majority of malaria cases/deaths in sub-Saharan Africa. They were first recognized based on fixed differences in the chromosome-X linked rDNA region and later raised to formal species following the discovery of centromeric islands of high divergence, (permeable) pre-mating barriers and strong ecological divergence at the larval stage (Lehman & Diabate, 2008). Due to their public health relevance the 2 species have been the subject of extensive ecological, epidemiological and genomic studies (Miles et al 2017) which have revealed a rich mosaic of different ancestries, shaped by geography, ecology and speciation, and highlighted evidence of strong intra-specific sub-structuring (Caputo et, 2014).
The main aim of this project is to confirm/disprove the hypothesis that the genomic intra-specific sub-structuring, hybridization and introgression shown to occur at the far-west of A. *coluzzii* and *A. gambiae* range (Caputo et al 2011, Vicente et al 2017) have epidemiological consequences on malaria transmission, as suggested by significant differences in *P. falciparum* prevalence in human populations between coastal and inland areas. In addition to the above objectives, the project also aims to strengthen the collaboration between IP-ITALY and IP-DAKAR and IP-Cote D'Ivoire (IP-Cd'I).

The results obtained during the first year of the project are summarized below with reference to the two Work Packages (WPs):

WP1: Development of a new easy-to-use and relatively inexpensive molecular tool to distinguish *A.coluzzii, A. gambiae* and hybrid genotypes. Clear signs of more or less extensive introgressive between *An. gambiae* and *An. coluzzii* have been detected not only at the western edge of *An. gambiae/An.coluzzii* range (Vicente et al 2017; Caputo et al 2014, Caputo et al 2016) but sporadic sprouts of hybridization have been shown also in areas were the two species were thought to be well differentiated (Lee et al 2013). Despite such evidence species identification is based essentially on two

X-centromeric diagnostic markers (i.e. IGS and SINE200-X6.1) which aren't sufficient to capture signs of autosomal introgression.
PI-Italy developed a new MassaArray genotyping approach in collaboration with the Wellcome Trust Center (Oxford; manuscript in preparation) taking advantage of results obtained by Ag1000G project which has allowed the identification of hundreds of Ancestry Informative Markers (AIMs), fixed or very highly differentiated between the two species along their whole range of distribution. Selection of AIMs to be included in the MassArray approach was based on maximization of distribution on different chromosomal arms, location within coding regions and maximization of physical distance between variants. This resulted in a set of 35 SNPs which were validated using the MassArray-genotyping approach on specimens already included in the AG1000G project and 29 SNPs were successfully genotyped in >75% of tested individuals from the whole range of *An. gambiae* and *An. coluzzii* (N=336). After exclusion of few SNPs due to incomplete concordance between Illumina sequencing results and MassArray genotyping approach, the final MassArray assay included 26 of SNPs (10 on chromosome-X, 6 on chromosome-2L, 3 on chromosome-2R, 3 on chromosome-3L and 4 on chromosome-3R telomere).

This novel MassArray genotyping approach was used also to genotype a total of 564 indoor-resting *An. coluzzii* and *An. gambiae* individuals from Guinea Bissau, The Gambia and Senegal, where hybridization between the two species is known to happen, confirming the potential of this approach to detect introgression. In fact, depending on sampling site the presence of admixed individuals varied from 0 to 100% in these samples from far west of the species' range.

Milestone 1.1 - Identification of candidate SNPs for development of PCR genotyping approach
While the above MassArray approach is very useful to investigate fine-scale levels of recombination and introgression along the genome it requires expensive equipment and specialized technicians and is thus not affordable for large-scale studies. PI-Italy thus developed a more cost-effective PCR approach for two autosomal markers identified among the AIMs included in the MassArray assay, offering the possibility to genotype large numbers of specimens and to identify the possible presence of admixed or hybrid specimens. Based on results obtained from the MassArray assay two autosomal SNPs situated on chromosomal arms 3R (3R: 42848) and 3L (3L:129051) were chosen for this novel PCR-approach while SNPs from chromosomal arms 2L and 2R were excluded from the design since they appeared to be heavily influenced by the introgression of the *kdr* mutation from *An. gambiae* to *An. coluzzii* and the wide presence of chromosomal inversions, respectively.

ML 1.2 -Design and validation of the above PCR genotyping approach on specimens already genotyped by Mass Array system in the high hybridization zone (HHZ) and on *An. coluzzii* and *An. gambiae* samples from the rest of range of distribution. For the two

chosen loci Tetra-AMRS-PCR has been developed and fragment length was set in order to allow also a combination of the two PCR assays in a single reaction making thus the approach even more cost effective and less time consuming. Both PCR-assays were tested on ~106 specimens from Guinea Bissau already analyzed for all the 30 AIMs using the MassArray approach and for which it was therefore possible to compare PCR and MassArray genotyping results. For all these specimens' DNA quality was high enough to allow genotyping of both loci on chromosome 3 in a single reaction. PCR-genotyping results of both loci were highly concordant (>97%) with results obtained by the MassArray approach and were confirmed by Sanger-sequencing for a subset of specimens. Results obtained via PCR for a single locus on chromosomal arms 3L and 3R was able to define for >98% of specimens the respective chromosomal arm as pure or introgressed in concordance with results obtained using the multilocus MassArray approach. This new PCR approach should be used in combination with the traditional X-linked species-specific markers (i.e. IGS and SINE200-X6.1, both situated in the X-centromeric region) to identify islands of high divergence on chromosome-X and -3 and to determine their linkage.

WP2: Exploring the impact of genetic diversity between and within *An. gambiae* and *An. coluzzii* on epidemiologically relevant entomological parameters in contrasting settings.

The aim of WP2 is to explore if and how the complex genetic diversity (introgression and intra-specific sub-structuring) reported between *A. gambiae* west (coastal) and east (inland) populations in high hybrid zone (HHZ, Senegal-The Gambia-Guinea Bissau, Caputo et al, 2011; Vincente et al 2017) and between *A. coluzzii* from coastal west-Africa and savannah areas (Tene Fossog et al 2015) has an impact on epidemiologically relevant entomological parameters. To compare whether in the presence or in the absence of high hybridization, coastal and inland populations present phenotypic divergence relevant to malaria transmission, collections have been carried out in two villages in Senegal and in Cote D'Ivoire.
Ethical approval. Immediately after funding and preliminary to the start entomological collections, in order to send the request to the Ethical approval, the three principal investigators have discussed and rearranged the protocol of EXGENMAL project with the group of the ACIP team in IP-Paris (ACIP project team). The project and the entomological protocol was translated in French language to be sent to the ethical committee. On the 3 April 2018 and the 27 June 2018, ethical approval was granted by the "Ministre de la Sante et de Hygiene Publique" of the Republique de Cote 'Ivoire and by and "Ministere de la Sante et de action sociale" of the" Republique du Senegal".

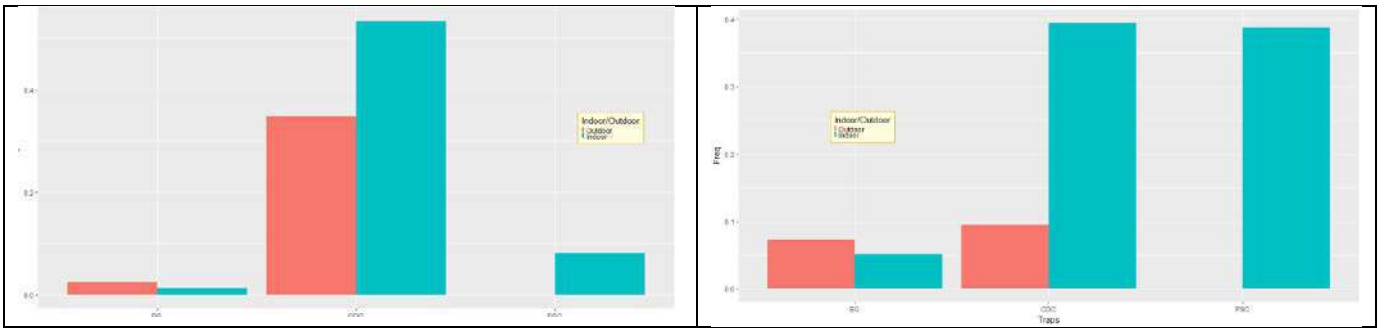
Kick-off meeting in IP-Cd'I (7-16 May 2018). At the beginning of the project, a kick-off meeting aiming to harmonize the technical approach of entomological activities, has been held by the participation of the 3 PIs at IP-Cd'I. During the meeting the details of

the WP-plan were well defined with particular reference to: i) the villages of collections in relation of the activities carried out by National Malaria Control programme; ii) the trap method for host-seeking and resting malaria vector females, iii) protocols for field activities (mosquito storage, morphological identifications, dissections of ovaries from half–gravid females, code and database for data repository): iv) protocols for molecular genotyping (species identifications, PCR protocols developed in the frame of WP1, Plasmodium and blood meal origin identifications, insecticide resistance); v) preparation of database for entomological collections and statistical analysis. Also, a field trip in the village selected for entomological collections near the city of Ayame (South –East of Cd'I,) was organized. During the meeting, the project's coordinator also trained IP-Cd'I personnel for field work activities to be carried out in the following months and guarantee familiarization with and standardization of methods and protocols for mosquito collection and processing. After the meeting, a report has been prepared and shared among all participants.

Mosquito Collections. Due to delay of wire transfer between the Financial Service of different Institut Pasteur (31-May 2018 for Côte d'Ivoire), the field activity has been started few months late comparing to the work plan. According to the WP2 *Anopheles gambiae* s.l. host-seeking and blood-fed resting females have been collected from one coastal and one inland village in Senegal and in Cote d'Ivoire during year-1 rainy/dry season (ML 2.1). Prior to indoor collections free and informed consent have been obtained from inhabitants to perform collections in their rooms. Collections of indoor resting mosquitoes were carried out in human dwellings by using pyrethrum spray catch early in the morning (each night a total of 5 rooms). Collections of host seeking mosquitoes have been performed indoors and outdoors by CDC (10 in and 10 out) and/or BG (10 in and 10 out), respectively. The traps collected each night from 8 PM to 7 AM. Mosquitoes have been sorted to species and gender using morphological taxonomic keys. Anophelines and Culicinae were identified using the morphological identification keys of Gillies & de Meillon and Gillies & Coetzee. *Anopheles gambiae* s.l. female ovaries have been dissected for nulliparous/parous rate assessment and carcasses stored in ethanol 70%. Ovaries has been extracted from freshly half-gravid females and stored in Carnoy's fixative.

Senegal Entomological collections were carried out from September to November 2018 in two villages selected based on earlier surveys (Nwakanma et al, 2013; Niang et al, 2014; Ndiath et al, 2014) in order to have frequencies as balanced as possible of *An. gambiae*, *An. coluzzii* and hybrid genotypes. The first is a coastal village (Madina Djikoye; GPS: 13°38'N, l6 °18'W) located in the northern Sudanese zone (within the 700-1,00 0 mm isohyet) in Kaolack region neighbouring the Gambian border. The second village (Djinkore Manfing, GPS: 13°69' N 13°65'W) lies in the inland Tambacounda region (south-east Senegal) within a southern Sudanese eco-climatic zone. Collections were carried out for 4 nights/month. A total of N=144 and N= 200 Anophelinae have

been collected in the coastal and inland village, respectively. *Anopheles gambiae* sl is the most abundant Anophelinae collected in both villages (relative proportion of 95% at Djinkore and 81% in Madina). *An. funestus*, *An rufipes*, *An.domicola*, *An. rufipes* and *An .ziemanni* have been collected. The relative proportions of *An.gambiae* sl sampled indoor (blue) and outdoor (red) in Madina Djikoye (N=161, left panel) and Djinkore Manfing (N= 137, right panel) with the three different methods (ie BG, CDC, and PSC) are reported in the Figure below.



In both the villages and for each methods of collection, *An.gambiae* decreased from September to December, according to the end of rainy season and beginning of dry season. *An.gambiae* sl samples were differentiated for gonotrophic stages. Most of specimens collected with traps were unfed, while mainly gravid mosquitoes were collected by pyrethrum spray catches (PSC) inside the dwelling. Unfed samples collected with traps (either CDC or BG) were dissected to evaluate the parity rate, which allows an age estimate. In the village of Djinkore the rate of nulliparous was 38% (N=42), 66% (N=15) in September and beginning of November, respectively. In the village of Madina, the rate of nulliparous was 40% (N=57), 36% (14) in September and October, respectively. Sample size of November was so low for further analyses (<10). In order to analyze polytene chromosome, ovaries from *An. gambiae* half gravid females (N=24 and N=18 from Djinkore and Madina, respectively) were dissected and stored in Carnoy preservative for successive cytological analyses.

Côte D'Ivoire. Entomological collections were carried out in December 2018 in two villages selected based on publish literature in order to have both species at balanced frequencies (Zoh et al, 2018). The first was Ayamé coastal village (Piste 4) (GPS: 5°28' N 3°12'W), near the region of Aboisso, nearby the border of Ghana and near a man-made lake on the river. The region is hilly and covered with humid forest and cacao or coffee plantations. Ayamé has significant rainfall most months, with a short dry season. The second was Petessou village (GPS: 8°6' N 5°28'W) the inland Boukè region within a tropical humid zone. The village is located in the central part of the country, nearby a permanent watercourse maintains by very large area of shallows used for rice farming

and market gardening, represent suitable breeding sites for mosquitoes. Four night of collections were carried out in each of the two villages. A total of N=136 and N= 178 *An.gambiae* sl have been collected by 20 CDC traps in Ayame and Petessou, respectively. The relative frequency of *An.gambiae* females collected indoor with CDC traps were 71,3%, 84% in Ayame and Petessou, respectively. Moreover, resting *An. gambiae* females were collected in human dwelling (N=39 Ayamé, N=54 Petessou in 20 rooms each) by the use of PSC, manual aspirators and exit windows traps.

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GPF
A NOVEL APPROACH TO NEUROINFLAMMATION THERAPY BASED ON SEC61 BLOCKADE

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Increasing evidence demonstrate that neuroinflammation, mediated by the activation of glial cells and infiltration of peripheral immune cells in the brain, is critical to the initiation and maintenance of neurodegenerative diseases, and that uncontrolled neuroinflammation can lead to permanent brain damage. Mycolactone is a polyketide-derived macrolide produced by Mycobacterium ulcerans that, in human skin, causes both ulcerative, analgesic, and anti-inflammatory actions (Guenin Macé et al., 2015). It was initially proposed that ulcer formation occurs through a mechanism involving hyperactivation of Wiskott-Aldrich syndrome proteins, and that analgesic effect results from neuronal hyperpolarization induced by the activation of angiotensin II type 2 receptors. More recently, it has been shown that mycolactone displays its cytotoxic and immunosuppressive effects by blocking Sec61-dependent protein translocation (Baron et al., 2016). It was shown that mycolactone suppresses the inflammatory responses of sensory neurons, Schwann cells and microglia, without affecting the cell viability (Isaac et al., 2017). We were interested in the ability of mycolactone to reduce inflammation, preventing the activation and migration of circulating inflammatory cells, for a possible application in amyotrophic lateral sclerosis (ALS), a neurodegenerative disease with neuroinflammatory elements. In particular, having recently shown that systemically-delivered mycolactone gains access to the brain (unpublished observations), we hypothesized that its diffusion capacity, combined with potent anti-inflammatory and analgesic properties, could make mycolactone an attractive drug candidate for suppressing neuroinflammation. The purpose of this application is therefore to test the innovative concept that mycolactone can prevent acute and chronic neuroinflammation, for future testing in mouse models of ALS. In the first year of this project we investigated the effect of chronic treatment with mycolactone on wild type C57BL/6 mice, to characterize the effect of repeated systemic injections. At this aim, we performed repetitive injection (i.p.) of mycolactone at different doses, from 0.5mg/kg, every 4 days (as in Guenin-Macé et al., 2017), to 0.5mg/kg and 0.25mg/kg, 1 time per week. At different time points, these mice have been sacrificed and tissue isolated for HPLC/MS analysis, that will be performed in the next future.

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TRAINING AND EDUCATION

- **RIIP INTERNATIONAL COURSE**
- **PASTEUR SEMINAR SERIES**

RIIP INTERNATIONAL COURSE ROME, JULY 09 – 14 2018

2018 PASTEUR SEMINARS - Scientific Organizer: John Hiscott

18 Jan **Maria Luisa Mangoni** (Dept. of Biochemical Science – Sapienza)
How to squash the "superbug" Pseudomonas aeruginosa? A lesson from the amphibian skin-derived Esculentin(1-21) and its diastereomer.

01 Feb **Lucia Gabriele** (Istituto Superiore di Sanità)
The epigenetic signature of Type I IFN at the interface of cancer and immunity.

15 March **Alessandra della Torre** (Dept. of Public Health and Infectious Diseases – Sapienza)
The ecological genomics of the major Afrotropical malaria vectors in the context of malaria elimination.

29 March **Hergen Spitz** (Academic Medical Center at the University of Amsterdam, Netherlands)
Development and plasticity of human ILC.

12 April **Ulrich Kalinke** (Centre for Experimental and Clinical Infection Research Institute for Experimental Infection Research Hannover Germany)
Viral Defense at the Brain Barrier.

26 April **Martin Roelsgaard Jakobsen,** (Dept. of Biomedicine Aarhus University, Denmark)
The effects of IFI16 and cGAS-STING-mediated immune responses to control HIV and cancer - a complex story.

24 May **Evelyne Tassone** (Dept. of Cell Biology New York University Medical Center)
Effects of KLF4 on bone remodeling induced by prostate cancer cells.

05 July **Heather Marlow** (institute Pasteur Paris France)
Specification and regulation of Cell Identity Programs in Nematostella vectensis.

13 Dec **Stefano Gianni** (Dept. of Biochemical Science – Sapienza)
Understanding the protein folding problem.

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