**PROSTATE CANCER**

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Project title: **PROSTATE CANCER: DISENTANGLING THE RELATIONSHIPS WITHIN THE TUMOUR MICROENVIRONMENT TO BETTER MODEL AND TARGET TUMOUR PROGRESSION.**

Background. Affecting 1 man out of 7 in industrialized countries, prostate cancer (PCa) is one of the most common tumours in men over 60. At diagnosis, 90% of prostate cancers are confined to the organ. Since it is almost impossible to predict the pathological steps that lead to tumour aggressiveness, patients are often treated with partial or radical prostatectomy and/or anti-androgen therapy. However, of the over 600.000 newly diagnosed PCa in the European Union and US every year, one third will progress to the metastatic stage of the disease for which no effective treatments are actually available. It is clearly emerging that the type of genetic and epigenetic alterations driving malignant transformation in the prostate epithelial cells (intrinsic factors) can predict much better than histology tumour behaviour and sometimes the response to specific therapies.

Lethality of PCa is uniquely associated with the metastatic progression of the disease. We generated a genetic in vitro platform modelling the natural history of prostate tumourigenesis.

For this purpose, the immortalized human epithelial prostate cell line RWPE-1 has been engineered to generate a 2D model of PCa with a panel of doxycycline-based inducible vectors to mimic:

ERG over-expression, a very early genomic event in prostate tumourigenesis affecting almost 50% of all PCa patients. ERG is not oncogenic per se, yet it sustains tumour progression when combined with a transformation event such as PTEN dysfunction.

ERG over-expression in combination with partial PTEN downregulation.

ERG over-expression in combination with massive PTEN downregulation.

Hypothesis. We hypothesise that DNA methylation changes are required for ERG over-expression and PTEN down-regulation during PCa progression.

Aims. The overall objective of the project is the identification of the transcription and DNA methylation profiles required for tumour reprogramming by ERG and PTEN modulation in 2D models of in vitro PCa model.

Experimental design. To isolate differentially expressed transcripts required for tumour reprogramming in the 2D model that are dependent or independent from DNA methylation changes, we will or not knockdown DNMT-1, -3A, -3B, the three TETs prior to ERG and PTEN modulation, and the resulting phenotypes will be analysed for tumour and invasive parameters. For each experimental condition and relative controls, Illumina Infinium CytoSNP-850K BeadChip and RNA-seq for coding and non coding sequences will be performed.

The combined differential analysis of the methylation profiles and of the RNA-seq data between each sample, will allow identifying coding and non coding transcripts dependent or not on DNA methylation changes that are induced or repressed by ERG and PTEN modulation and in response to the secretome signalling and that might be required for their action during tumour progression (driver transcripts).

To validate the driving role of those identified transcripts, knock down of the up-regulated or over-expression of the down-regulated transcripts (both coding and non-coding) will be performed prior to ERG and PTEN modulation in the 2D and 3D models, and the resulting phenotypes analysed

Financing

The project is financed by EPIGEN – Progetto Bandiera with 400K€ in three years.

External collaborators

Prof. Andrea Lunardi, University of Trento, Trento

Prof. Valeria Poli, University of Turin, Turin

Prof. Licio Collavin, University of Trieste, Trieste

Prof. Alessandro Weisz, University of Salerno, Salerno

The PhD candidate will perform his/her PhD thesis in the lab of General Pathology at the University of Insubria and in collaboration with Prof. Alessandro Weisz at the University of Salerno for the -Omics experiments.

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**CARDIOVASCULAR DISEASES**

**PI: Prof. Roberto DE PONTI** – roberto.deponti@uninsubria.it

Project title: **MULTIPARAMETRIC EVALUATION OF PATIENTS HOSPITALIZED FOR CONGESTIVE HEART FAILURE TO PLAN THE LONG-TERM FOLLOW-UP**

Introduzione: la dimissione ospedaliera dopo un ricovero per scompenso cardiaco acuto segna un momento chiave nella storia naturale del paziente affetto da questa patologia, poiché rappresenta l'inizio di un complesso percorso di assistenza e cura con integrazione fra ospedale e territorio[1]. La gravità della patologia e delle comorbilità, il contesto familiare attorno al malato, la compliance alla terapia, l'intensità di cure e la frequenza delle rivalutazioni specialistiche rappresentano le colonne portanti del sistema di assistenza al malato. Il corretto funzionamento di questo sistema può garantire al paziente una più lunga sopravvivenza e una migliore qualità di vita, prevenendo le recidive di malattia e le ospedalizzazioni. L'analisi della letteratura mostra come ad oggi non esista un modello gestionale capace di ottenere tali obiettivi[2]. Tuttavia, vi è unanime consenso nel ritenere che la gestione multidisciplinare e quanto più personalizzata del follow-up sia la miglior strategia da applicare. In assenza di evidenze scientifiche a riguardo, il protocollo organizzativo di ogni centro che si occupa di scompenso cardiaco deve tenere conto delle proprie risorse professionali e logistiche, dei servizi specifici dedicati (telemedicina, servizi di day-hospital, terapie palliative) e delle capacità di contenere la domanda assistenziale. E' in questo contesto di domanda crescente e offerta limitata che si pone la necessità di una pianificazione razionale dell'utilizzo delle risorse, per garantire una assistenza proporzionata alla gravità delle condizioni cliniche del singolo paziente.

Razionale: la pianificazione del follow-up ambulatoriale del paziente ricoverato per scompenso cardiaco è attualmente guidata da esperienza e "senso clinico". Dalle analisi di registri internazionali e multicetrici sono stati creati numerosi score di rischio in grado di stimare la mortalità del paziente affetto da scompenso cardiaco a seconda di caratteristiche cliniche facilmente reperibili: anamnesi, segni obiettivi, parametri strumentali e dati laboratoristici sono in grado di stratificare la popolazione affetta da scompenso cardiaco in classi di rischio crescente che, almeno in linea teorica, permetterebbero di identificare soggetti a maggior rischio di eventi a breve termine. Un'analisi quantitativa basata su questi elementi sarebbe pertanto preferibile ad una valutazione puramente qualitativa effettuata in fase di dimissione, permettendo una pianificazione ragionata del follow-up ambulatoriale di questi pazienti e un'ottimizzazione del percorso di cure in una popolazione così varia di pazienti.

Scopi:

- ✧ creazione di protocolli di follow-up ponderati sulle caratteristiche clinico-strumentali del paziente
- ✧ rivalutazione periodica e critica del valore di tali protocolli nella gestione clinica dei pazienti con scompenso cardiaco

Materiali e metodi: mediante l'analisi di parametri clinici, strumentali ed ematochimici acquisiti durante la fase di acuzie e in fase di dimissione, con l'ausilio di appropriati score di rischio a breve e lungo termine, verranno analizzati i dati di pazienti ricoverati per scompenso cardiaco acuto. In Tabella 1 vengono riportati alcuni dei parametri considerati fondamentali per una corretta stratificazione del rischio del paziente ospedalizzato per scompenso cardiaco, secondo un recente documento di consenso[3]. Gli score di rischio che verranno combinati con i dati clinico-strumentali raccolti comprendono  tre score di mortalità per lo scompenso cardiaco validati a

livello internazionale: 3CHF score[4], MAGGIC score[5], Seattle Heart Failure Model[5]. Da queste analisi verrà creato un registro prospettico dei pazienti ospedalizzati per scompenso cardiaco acuto volto ad identificare sottogruppi di pazienti ad alto rischio, a rischio intermedio e a basso rischio. L'identificazione di sottogruppi differenti di pazienti garantisce non solo una valutazione più oggettiva del rischio dei singoli, ma permette la gestione del follow-up secondo protocolli di intensità differente proporzionati alla gravità delle condizioni cliniche, con un utilizzo più oculato di preziose risorse quali: rivalutazioni in regime di day-hospital/MAC, infusioni ripetute di farmaci, progetti di telesorveglianza, valutazioni e analisi strumentali a breve distanza dalla dimissione

Tabella 1: Fattori da considerare nella stratificazione dei pazienti ospedalizzati per scompenso cardiaco (3).

- Età, cardiopatia di base e storia clinica (ospedalizzazioni/accessi in pronto soccorso ripetuti).
- Grado di disfunzione sistolica ventricolare sinistra, di insufficienza mitralica ed eventuale compromissione biventricolare.
- Decorso ospedaliero (andamento della pressione arteriosa, risposta alla terapia diuretica, necessità di inotropi, tollerabilità di ACE-inibitori/sartani e betabloccanti, aritmie, danno d'organo (troponina, funzione epatica, iponatriemia, ecc.), frequenza cardiaca alla dimissione.
- Variazioni ingresso/dimissione e valore assoluto in dimissione dei peptidi natriuretici.
- Numero e gravità delle comorbilità, in particolare grado di disfunzione renale e suo andamento durante il ricovero, diabete, fragilità/complessità.
- Livello di compliance/aderenza del paziente e dell'ambiente familiare.

Pertanto, sulla base delle diverse classi di rischio, verranno elaborati degli specifici modelli di follow-up differenziati per ogni classe di rischio finalizzati soprattutto all'ottimizzazione delle risorse evitando altresì ri-ospedalizzazioni ed eventi maggiori. L'analisi degli eventi avversi in ogni classe di rischio nel follow-up a lungo termine (24 mesi) permetterà di valutare la validità del modello di follow-up per quella specifica classe di rischio.

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**INFECTION AND IMMUNITY**

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Project title: **AGONISTS/ANTAGONISTS OF TOLL-LIKE RECEPTORS (TLRS) IN INNATE IMMUNITY AND IN CELLULAR DIFFERENTIATION WITH REGARD TO HEMATOPOIETIC STEM CELLS.**

TLRs receptors play an important role in the activation of Innate Immunity, indeed, regulation of TLR activity by Agonists and Antagonists is of great importance also to study their involvement in other mechanisms independently of Immunity, such as cell differentiation.

Natural TLR ligands are molecules linked to bacterial and / or viral infections (PAMPS), but also released from dead cells, mainly from necrotic cells and / or extracellular matrix molecules (DAMPS), thus TLRs act as "antenna" receptors of the external environment.

Recently, a role for TLRs, particularly TLR4, has been demonstrated in macrophages that undergo different forms of polarized activation (M1 or M2), using specific cytokines and TLR4 ligands. Preliminary experiments conducted in our laboratories showed that using a TLR4 antagonist, polarization is altered.

In this PhD project, we will study the effects of TLR4 agonists (LPS, HMGB1) and antagonists (CyP) on blood stem cells by trying to modulate their differentiation in Monocytes and Fibrocytes. Using an in vitro model, the mechanisms involved in the differentiation of circulating monocytes will be studied. Particular attention will be paid to the CD14 + CD16 + subpopulation and the effect of the different molecules involved in sterile inflammation

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**INFECTION AND IMMUNITY**

**PIs: Prof. Roberto ACCOLLA and Dr. Greta FORLANI –** roberto.accolla@uninsubria.it, greta.forlani@uninsubria.it

Project title: **NOVEL APPROACHES OF ANTI-TUMOR VACCINATION BASED ON OPTIMAL STIMULATION AND/OR RESCUE OF IMMUNE EFFECTOR CELLS**

Summary program

Although recent therapeutic approaches have revitalized the enthusiasm of the immunological way to combat cancer, still the comprehension of immunity against tumors is largely incomplete. Due to their specific function, CD8+ T cells with cytolytic activity (CTL) have attracted the attention of most investigators because CTL are considered the main effectors against tumor cells. Nevertheless, CTL activity and persistence is largely dependent on the action of CD4+ T helper cells (TH). Thus establishment of tumor-specific TH cell response is key to the optimal response against cancer. CD4+ TH lymphocytes are conventionally primed and activated against antigens, including TAAs, by professional antigen presenting cells (APCs). Priming is mainly induced by dendritic cells (DCs) and less efficiently by macrophages, that present antigens via their cell surface MHC class II molecules.

Our research approach is based on rendering tumor cells MHC class II-positive and thus potential APCs for their own tumor antigens, by introducing the gene expressing the MHC class II transactivator (also designed CIITA), discovered in our laboratory. Previous results from our laboratory have demonstrated that effective rejection or significant growth retardation of the CIITA-driven MHC-II-positive tumor cells of distinct histological origins was accomplished (1). Moreover, successfully “vaccinated” mice were shown to be protected from challenge with the MHC-II negative parental tumor.

Can these experimental observations be integrated in a strategy applicable to human cancer? The straightforward conclusion from the above studies is the evidence of the strong immunogenicity of the MHC-II-tumor peptide complexes present on CIITA-positive tumor cells (2). Based on this evidence we would like to finally establish the potential of CIITA-tumors to act as original priming cells for naïve CD4+ TH cells (3) and, more importantly, from the translational aspect of our research, to construct an innovative tumor vaccine against several human carcinomas and the central nervous system (CNS) deadly glioblastoma tumor by purifying MHC-II-bound tumor peptides isolated from CIITA-modified tumor cells (TUMAPs) following a methodological purification strategy already described. The reason to focus particularly on glioblastoma is motivated by the increasing incidence, severity and lack of resolutive therapeutic tools against this tumor. Indeed, glioblastoma is the most common primary CNS malignancy accounting for 70% of all new CNS cancers.

Our approach will proceed stepwise, first by transfecting human tumor cells in vitro with CIITA, assessing the expression of MHC class II cell surface molecules, subsequently to isolate stable CIITA transfectants and finally proceed to the purification of MHC class II cell surface molecules and their bound peptides. Among those, through mass spectrometry, we will identify the sequence of those peptides expressed only on tumor cells and not on normal cells and proceed, by collaborating with other centers and particularly with Dr. Bassani-Sternberg at the Ludwig Institute for Cancer Research in Lausanne, to the synthesis of the most tumor-specific and immunogenic peptides to be part of a suitable anti-tumor vaccine cocktail.

It is our belief that such a strategy will allow the display and identification of a much broader as well as more representative array of tumor peptides compared to those that professional APCs can display, due to their intrinsic limitation to process and present peptides derived only from exogenously engulfed, phagocytosed material.

The study of anti-tumor immune response in animal models has paved the way not only to increase our knowledge on basic mechanism of antigen presentation and triggering of CD4+ TH cells but also to apply the acquired knowledge for testing innovative strategies of anti-tumor vaccination in clinical setting.

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**NEUROSCIENCES**

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Project title: **SYNAPTIC DEFECTS IN CDKL5-DISORDER: IDENTIFICATION AND CHARACTERIZATION OF NEW TARGETS FOR THERAPEUTIC INTERVENTION**

**Background:** Mutations in the X-linked CDKL5 gene (cyclin-dependent kinase-like 5) cause a neurological disorder characterized by the early onset of drug-resistant epilepsy, intellectual disability, hypotonia, gross motor dysfunctions etc. The loss of CDKL5 impacts on various neuronal aspects such as morphology and synaptic functions, which are likely to underlie the observed epilepsy and cognitive defects in patients. The main interest of our laboratory is to identify the molecular network controlled by CDKL5 in neurons thus increasing the comprehension of the molecular basis of the observed phenotypes as well as the identification of novel druggable targets.

**Project:** The focus of the present project is to characterize the molecular basis of the imbalanced excitation/inhibition characterizing Cdkl5-null mice. The student will make large use of primary neurons from Cdkl5-null mice to analyze the expression and functioning of synaptic proteins, including membrane bound receptors, involved in such phenotype. The project will include phospho-proteomic approaches, molecular biology, imaging techniques, and electrophysiologic studies.

Interested candidates should have a background in molecular and cellular biology and be willing to work with rodents.

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**NEUROSCIENCES**

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Project title: **BRAIN-GUT ALTERATIONS AFTER ANTIBIOTIC TREATMENT IN ADOLESCENT MICE: ROLE OF NELAV MRNA-BINDING PROTEINS.**

Alterations of the enteric microbiota play an important role in the pathophysiology of some diseases, both in the gut, such as irritable bowel syndrome (IBS), a functional disorder characterized also by psychiatric symptoms, and in the brain, such as autism and cognitive disorders. The intestinal bacterial flora, by modifying enteric neuronal circuitries, may communicate with the brain, via the microbiota-gut-brain axis, thus influencing both development and maturation of both enteric nervous system (ENS) and central nervous system (CNS). During neonatal and pediatric age, critical factors associated with birth, feeding, pharmacological treatments with either antibiotics or probiotics, may influence the microbiota composition and function, as well as the development of both ENS and CNS circuitries. In this perspective, it is particularly important to evaluate if changes in the symbiotic intestinal flora may impact on the expression of some factors involved in neuronal development and plasticity in both nervous systems. Among the numerous players, ELAV (Embryonic Lethal Abnormal Vision)-like proteins play a fundamental role in neuronal survival, plasticity during learning and memory, and following neuronal injury, by modulating all steps of transcript fate. ELAV-like protein family includes the ubiquitously expressed HuA and HuR and the neuron specific members HuB, HuC and HuD. HuC/D is present in all myenteric neurons and may participate to neuronal rescue in pathological conditions.

In the CNS, HuC/D proteins can bind and stabilize specific neurotrophic factor mRNAs, such as BDNF mRNA, through particular recognition motifs that interact with adenine, uridine-rich elements in the 3' untranslated region, thereby promoting protein translation. Changes in BDNF transcript and protein levels in the murine hippocampus underlie stress response and depression. BDNF may also participate to alteration of gut function in germ free and specific pathogen free animals and in rodent models of IBS.

We have recently demonstrated that in the small intestine of juvenile dysbiotic mice, protein and mRNA levels of HuC/D, BDNF and BDNF specific receptor, TrkB, increase, possibly representing a form of molecular rescue to compensate for the vulnerability of some population of myenteric neurons after massive antibiotic treatment. In the hippocampus of dysbiotic mice, HuC/D mRNA and protein levels increased, while BDNF mRNA and protein levels decreased. These preliminary data suggest that in the juvenile period, dysbiosis induced by a massive antibiotic treatment, may modify the expression of important molecular pathways involved in the development of neuronal plasticity, both in the ENS and CNS.

The aim of this project will be to investigate more in detail the effect of changes in the expression of HuC/D, BDNF and its receptor TrkB after antibiotic-induced dysbiosis both in the ENS and in CNS of juvenile mice and their potential involvement in the development of gastrointestinal and CNS disease.

The project will comprise the following approaches:

-Functional studies to evaluate whether the molecular changes of HuC/D, BDNF and TrKB pathways in dysbiotic mice may influence the neuromuscular and sensitive gastrointestinal function.

-Studies concerning the use of probiotics in antibiotic-treated mice, in order to evaluate whether the reintegration of the normal intestinal flora may influence the alteration in the HuC/D, BDNF and TrkB pathways.

-Biomolecular studies concerning the expression levels of HuC/D, BDNF and TrkB in the small intestine, hippocampus and prefrontal cortex of neurobehavioral mice models of mental diseases during childhood and adolescence, such as ADHD. In particular, siRNA studies targeting HuC/D RNA will be carried out in order to evaluate whether HuC/D may influence the transcription/translation of the BDNF/TrKB pathway in the different experimental models.

**NEUROSCIENCES**

**PI: Prof. Tiziana RUBINO –** tiziana.rubino@uninsubria.it

Project title: **ROLE OF THE ENDOCANNABINOID SYSTEM IN ADOLESCENT BRAIN DEVELOPMENT.**

Adolescence is a time of important neurobiological and behavioral changes, but is also the period in which several mental illnesses emerge, included psychosis, mood disorders, and substance abuse.

Among all drugs, Cannabis is the most widely abused by teens and several clinical data suggest the presence of a relationship between adolescent Cannabis abuse and the risk for developing psychiatric diseases later in life. Consistently, we have demonstrated that adolescent female rats treated with the psychoactive compound of Cannabis delta-9-tetrahydrocannabinol (THC), develop a depressive/psychotic-like phenotype in adulthood. Interestingly, we observed that only adolescent, but not adult, THC exposure leads to this phenotype, suggesting that adolescence represents a vulnerable period for the psychiatric consequences of THC exposure. However, the molecular underpinnings of this vulnerability remain to be established.

During adolescence, brain undergoes intensive processes of neuronal refinement, especially in cortical regions. Adolescent brain maturation involves a thinning of the gray matter (GM – it contains the cell bodies, dendrites and axon terminals of neurons) as result of synaptic pruning processes, through which "redundant" synapses overproduced in the early years of life are being eliminated; and an increase in white matter (WM – it is made of myelinated axons). Myelin improves neural transmission, contributing to the enhanced brain-regional connectivity and cognitive function that occur during adolescence. Thus, alterations in synaptic refinement as well as in myelination during this sensitive period could confer a vulnerability to psychiatric diseases.

In the brain, the Endocannabinoid System (ECS) is an important neuromodulatory system involved in synaptic plasticity regulation. So far, many works have addressed adolescent brain maturation, but the involvement of the ECS in adolescent brain refinement remains to be elucidated. Recently, we have demonstrated that adolescent THC exposure deeply changes neuronal refinement, altering the expression of proteins involved in synaptic plasticity and brain functionality. Moreover, our preliminary data show that adolescent THC alters the expression of MOG and MBP, two important markers of myelination. Thus, it is alleged that Cannabis consumption during adolescent brain maturation may alter the ECS functionality, interfering with normal brain development, and eventually resulting in a major vulnerability to mental illnesses.

On these bases, our proposal is to thoroughly investigate the role played by the EC signaling in processes occurring in the adolescent prefrontal cortex of female rats. To clearly depict each step of adolescent brain maturation, analyses will be performed every 5 day, from 28 to 75 PND focusing on the events of synaptic pruning and myelination. Next, through the administration of specific modulators of the ECS, we will study the impact of this modulation on markers of plasticity and myelination. Specifically, we will administer AM251, a selective antagonist of CB1 receptor, the major cannabinoid receptor in the CNS; URB597, an inhibitor of the enzyme fatty acid amide hydrolase (FAAH, the enzyme that catalyzes the intracellular hydrolysis of the endocannabinoid anandamide "AEA"), JZL184, a selective inhibitor of monoacylglycerol lipase (MAGL, the enzyme that preferentially catabolizes the endocannabinoid 2-arachidonoyl glycerol "2-AG") and THC.

With this approach, we will be able to elucidate the role played by the specific components of the ECS (CB1R, AEA and 2-AG) during adolescent brain maturation. Moreover, we will also understand the impact of EC tone disruption in triggering brain vulnerability to psychiatric conditions.

**NEUROSCIENCES**

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Project title: **MITHOCONDRIAL DISFUNCTION IN THE PATHOGENESIS OF THE PARKINSON DISEASE.**

**Parkinson's disease** (PD) is a multifactorial disorder whose etiology is not completely understood. Strong evidences suggest that **mitochondrial impairment** and altered mitochondrial disposal play a key role in the development of this pathology. This association has been demonstrated in both genetic and sporadic forms of the disease.

The project **aims** at clarifying the mitochondrial landscape and molecular mechanisms underlying Parkinson's disease, with a specific focus on investigating the consequences of impaired clearance of dysfunctional mitochondria.

As cellular models, we will use both toxin-induced models of PD in immortalized cell lines, thus highlighting the importance of environmental factors in the onset of this pathology and PARK2 patients' fibroblasts, thus having a picture of alterations due to parkin loss in familial cases of PD. In particular, we will focus our attention on **mitochondrial dynamics**, mitochondrial biogenesis, and **mitophagy**. By the use of standard **biochemical assays** (e.g., Western blotting, Immunofluorescence), we will investigate main molecular factors involved in mitochondrial dynamics. Moreover, we will exploit **proteomics** being a global and unbiased approach suitable to unravel alterations of the molecular pathways in multifactorial diseases. To better decipher the molecular landscape affected by mutations in the PARK2 gene, we will switch to the **interactomics** approach. Thanks to biochemical fractionation coupled with mass spectrometry of the same samples analyzed by shotgun proteomics, it will be possible to obtain a high confidence global mitochondrial protein-protein interactions network in PARK2-PD patients. By integrating biochemical assays, proteomics and interactomics results, a bioinformatics model of molecular mechanisms underlying mitochondrial impairment in PD will be generated. Moreover, mathematical **dynamic models** of the altered mitochondrion will be generated in collaboration with informaticians. A better understanding of the molecular factors will help to unravel possible biochemical pathways altered also in the sporadic form of the disease and, possibly, it will shed light on new potential therapeutic targets in PD.

The PhD student involved in the project will choose, based on her/his skills and preferences, on which specific aspects concentrate the PhD thesis. She/he will be asked to design and perform experiments and critically interpret the results.

**ONCOLOGY**

**PIs: Prof. Roberto TARAMELLI, Prof. Francesco AQUATI –** roberto.taramelli@uninsubria.it, francesco.acquati@uninsubria.it

Project title: **FUNCTIONAL CHARACTERIZATION OF THE ROLE PLAYED BY THE RNASET2 GENE IN THE CONTEXT OF HUMAN CANCER PATHOGENESIS**

A growing body of experimental evidence has been gathered in the last decades to indicate that the origin and development of cancer represent a much more complex process than was previously thought.

Indeed, the origin of cancer itself currently represents a heatedly debated issue where several theoretical concepts [such as the somatic mutation theory (SMT), the tissue organization field theory (TOFT) and the cancer stem cells (CSCs) concept] are constantly confronted. In light of such a lack of consensus, cancer research has been recently spread over different lines of investigations in the hope to better clarify the molecular and cellular mechanisms underlying the origin and development of this deadly disease.

Since many years, our research group has focussed its attention on a human tumor suppressor gene whose features make it a particulary interesting target of investigation in the field of cancer research.

The *RNASET2* gene maps to human chromosome 6, a region which has consistently been reported to be deleted or rearranged in a wide range of human solid and haematolgical cancers. It encodes a 256 aminoacid extracellular protein belonging to the highly evolutionary conserved T2 ribonucleases family.

In recent years, several research groups including our have independently reported a clear oncosuppressor role for the *RNASET2* gene. Strikingly, further investigations focused on the mechanisms of action of this gene have disclosed a plethora of biological processes involved in RNASET2-mediated tumor suppression (such as angiogenesis, cell cytoskeleton remodelling or differentiation, innate immune response regulation and apoptosis), in keeping with a highly pleiotropic role for this gene. In our lab, *RNASET2* has been consistently shown to carry out a marked tumor suppressive role in *in vivo* xenograft-based experimental models of human ovarian cancer, by means of recruiting to the tumor mass host-derived M1-polarized macrophages endowed with a marked oncosuppressive activity. Since the RNASET2 protein is known to be actively secreted by cancer cells, these data suggested a non cell-autonomous mechanism of tumor suppression whereby early-stage tumor cells secrete RNASET2 to send a “danger” signal to the surrounding microenvironment in order to trigger an innate immunity-mediated oncosuppressive response. Indeed, further data from our group using ovarian cancer cell models confirmed the role of RNASET2 as a general stress-response gene, allowing us to rank this gene in the growing family of “alarmins” danger signaling factors.

At the same time, however, other features discovered for RNASET2 were compatible with a cell autonomous oncosuppressive role as well. For instance, recent experimental data have demonstrated the ability of RNASET2 to induce a marked cytoskeleton rearrangement in ovarian cancer cells or to control the stemness capacity of breast cancer cells grown in 3D culture conditions.

Taken together, these data clearly define RNASET2 as a highly pleiotropic tumor soppressor protein endowed with a great potential for the development of an effective anticancer therapeutic strategy.

The PhD research program presented here thus aims at further investigating the molecular and cellular mechanisms by which the *RNASET2* gene carries out its multi-faceted role in the context of tumor suppression. To this end, based on the above described experimental data, the research program will address several issues concerning the biological processed involved in RNASET2-mediated tumor suppression, by means of the following experimental tasks:

1) Role of RNASET2 in macrophage activation/polarization

Our previous *in vivo* xenograft-based data clearly showed that *RNASET2*-mediated tumor suppression involves M1-polarized macrophages. To better investigate the functional relationship between *RNASET2* and the monocyte/macrophage cell lineage, the functional relevance of macrophages-derived RNASET2 protein will be investigated *in vitro*. To this aim, a co-culture system based on Transwell inserts will be used to investigate whether RNASET2-primed human macrophages elicit a tumor suppressive response. To this end, we will use the human monocytic leukemia-derived cell line THP-1, a well established model of *in vitro* macrophage differentiation. Parental THP-1 cells (expressing and secreting endogenous RNASET2 at high concentration) and their *RNASET2*-silenced counterpart will be compared with respect to their ability to carry out a tumor suppressive role against PC-3 and DU-145 human prostate cancer cells *in vitro*. To evaluate the putative non-cell autonomous role of macrophages-derived RNASET2, the co-culture system will be assembled without direct cell-to-cell contact. Following co-culture of cancer cells with THP-1-derived macrophages, several cancer-related parameters (apoptotic and proliferation rates, clonogenic capacity and anchorage independent growth) will be investigated at different time points in the human cancer cells by means of *in vitro* assays already established our lab. Moreover, RNASET2-mediated changes in the cytoskeletal organization of the cancer cells will be investigated by TRITC-phalloidin staining following by fluorescence microscopy analysis. To evaluate whether a direct contact between RNASET2-primed macrophages and cancer cells is required to observe a biological response, the above described assays will also be performed following a direct co-culture of GFP-expressing cancer cells with human THP1-derived macrophages. GFP-positive cancer cells will then be separated by THP1 cells by FACS-assisted cell sorting before carrying out the *in vitro* assays. This subtask will provide an independent confirmation of the biological response triggered in human macrophages by extracellular RNASET2.

2) Establishemnt of an RNASET2 mouse syngenic model.

Since our previous *in vivo* data on RNASET2-mediated tumor suppression were obtained in immunocompromised animal models, the *in vivo* oncosuppressive role of RNASET2 in the context of a functional host immune system has not been investigated yet. To this end, a mouse syngenic model will be developed. The murine RNASET2 coding region, which has already been cloned into different expression vectors in our lab, will be used to stably transfect C51 and TSA cells, representing colon and mammary mouse cancer cells, respectively. Transfected clones overexpressing the murine RNASET2 protein (together with empty vector-transfected control clones) will be subcutaneously implanted into immunocompetent BALB/C mice to evaluate the occurrence of an RNASET2-mediated effect on tumor growth cynetics. Tumor samples from both experimental groups will then be compared by both standard microscopy and immunohistochemical assays using a panel of molecular marker (such as Ki-67, CD31, F4/80, CCL3, CD11b) to define the features of both the injected cancer cells and the host microenvironmental compartment.

3) Role of RNASET2 in regulating cell stemness.

Recent data from our lab in 3D breast cancer models have unveiled un unexpended role for RNASET2 in the control of mammosphere formation rate. Since the latter represents a well-established parameter in stem cell research, our data suggest that the control cell differentiation might be included among the various roles played by RNASET2 in cancer cell biology. To better investigate this issue, human breast cancer-derived MCF-7 cells genetically manipulated to express high levels of RNASET2 will be cultured in 3D conditions (in parallel with control, empty-vector transfected cells) in order to generate mammospheres. The gene expression profile of mammosphere pools from both experimental groups will then be evaluated by means of both targeted and whole transcriptome analysis (qPCR and microarray or RNA-seq) in order to evaluate putative differences in the expression level of several differentiation and stemness markers, such as CHD1,CD24, CD44, Twist, Snail, ALDH1A1,EPCAM, □-casein, among others.

4) Investigation of the role of RNASET2 in an invertebrate experimental model.

The recent directives of the EU on the protection of animals used for scientific purposes, which strongly promotes the alternative use of vertebrate models in biological research, prompted us to evaluate the medicinal leech *Hirudo verbana* (*Hm*) as a complementary experimental model to investigate the role of RNASET2 in innate immunity regulation. This invertebrate is cost-effective, easily manipulable and devoid of significant ethical considerations. In addition, its anatomical and physiological characteristics and a less varied repertoire of cell types involved in immune response and wound healing allow to more easily evaluate the cellular and molecular mechanisms linked to these events. Preliminary data from our group have already demonstrated a role for human recombinant RNASET2 in macrophage recruitment in this experimental model. Moreover, injection of bacterial lipopolisaccaride (LPS) in leeches to mimic bacterial infection has been shown to trigger a marked recruitment of both granulocyte and macrophages, which express copious amounts of endogenous RNASET2. These data are in keeping with the notion of RNASET2 as an ancient, evolutionary conserved stress-response protein.

To better investigate the role of RNASET2 in *H.verbana* innate immunity, we have recently cloned the leeche's ortholog *RNASET2* gene into an eucaryotic expression vector, in order to produce leech recombinant RNASET2 protein. Once produced in sufficient amount, the protein will be used to investigate the dosage-sensitive response of *H.verbana* immune system following injection of recombinant RNASET2 in the body wall. An anti-*hm*RNASET2 polyclonal antibody will also be raised to investigate the endogenous expression pattern of this protein and the cell repertoire expressing it. Moreover, *in vivo* RNA interference approaches will be implemented in order to confirm the role of *hm*RNASET2 in the activation of the innate immune response under different pathogenic stimuli.

5) Immunohistochemical assays on human cancers

The *RNASET2* gene is currently defined as an oncosuppressor gene likely involved in the control of several human neoplasic diseases. Indeed, in keeping with the notion of RNASET2 as an "alarmin-like" molecule, preliminary data from a limited sample of human tumors of different origin have shown an initial increase of RNASET2 expression in the early stages of cancer, followed by a steadly downregulation of this gene in later cancer stages. To shed more light into this issue, a wide panel of staged human solid tumor samples (including prostate cancer, ovarian cancer, malignant melanomas, breast cancer and thyroid cancer) will be investigated by immunohistochemical assays to define a putative correlation between RNASET2 expression and the different cancer stage and grade and, at the same time, the putative changes in the cancer microenvironment, in term of both cellular composition (immune cells, fibroblasts, endothelial cells) end ECM components (collagen, laminin, fibronectin, vitronectin etc…).

This experimental approach will prove essential to investigate and possibly confirm the notion of RNASET2 as a wide-spectrum stress response gene endowed with a marked oncosuppressor role.

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**ONCOLOGY**

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Project title: **MUTATION PROFILING AND IMMUNE INFILTRATION STUDY IN ADVANCED BRAF MUTANT COLORECTAL CANCER.**

This study will be carried out in the laboratories of the Department of Pathology of our University, where, in addition to standard diagnostic histopathology and immunohistochemistry, there are fully equipped research laboratories of molecular pathology and electron microscopy.

For a long time our main research interests have been focused on the pathogenetic mechanisms of hereditary and sporadic colorectal cancer (CRC) with the aim of identifying markers with potential diagnostic, prognostic or therapeutic values.

*BRAF* $^{V600E}$ mutation occurs in less than 10% of colorectal cancers and is a strong predictor of poor prognosis particularly in advanced stage. To date, these tumors remain a clinical challenge because they do not respond efficiently to standard chemotherapy, BRAF-targeted therapeutic approaches and even to recently reported combinations with MEK and EGFR inhibitors.

*BRAF*$^{V600E}$ CRCs are correlated with specific clinico-pathological and biological factors such as female gender, older age, right-sided tumor locations, frequent lymph node involvement, advanced primaries and high frequency of Mismatch Repair (MMR) defect associated with microsatellite instability (MSI) [1]. Although *BRAF*$^{V600E}$ CRCs are usually considered a unique clinico-pathological entity, the recent outcome data of trials targeting selected *BRAF*$^{V600E}$ CRC cohorts with monotherapy [2], double-targeted therapy [2,3,5], and triple-targeted therapeutics [6,7] have shown a surprisingly strong heterogeneity in response, suggesting that further biological subdivisions may exist even within this population.

Recently, a concerted effort by the Colorectal Cancer Subtyping Consortium (CRCSC) succeeded in unifying colorectal cancer subtyping. This led to subdivision of CRC into four consensus molecular subtypes (CMS), capturing the main overall gene expression variability in colorectal cancer [8]. The vast majority (> 70%) of *BRAF* $^{V600E}$ CRCs were classified into the same subtype (CMS1) whereas 17% fell into CMS4 and 6% into CMS3. Next, Barras D *et al* found that *BRAF* $^{V600E}$ CRCs can be segregated into two different subtypes based upon gene expression profiles, called BM1 and BM2, suggesting that this heterogeneity may be exploited for drug targeting these patients.

The aim of this project will be an extensive clinico-pathological and molecular study of a multicentric series of 60 advanced *BRAF* $^{V600E}$ CRCs in order to verify whether MSI status allows to stratify these cancers into two different subsets showing specific immunologic characteristics of the tumor microenvironment, different gene mutation profiles and peculiar clinico-pathological features. Moreover we will evaluate the prognostic value of a hot

immunogenic phenotype in the whole series regardless of MSI status in order to verify the potential clinical utility of immune-phenotypical features in these tumors.

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**ONCOLOGY**

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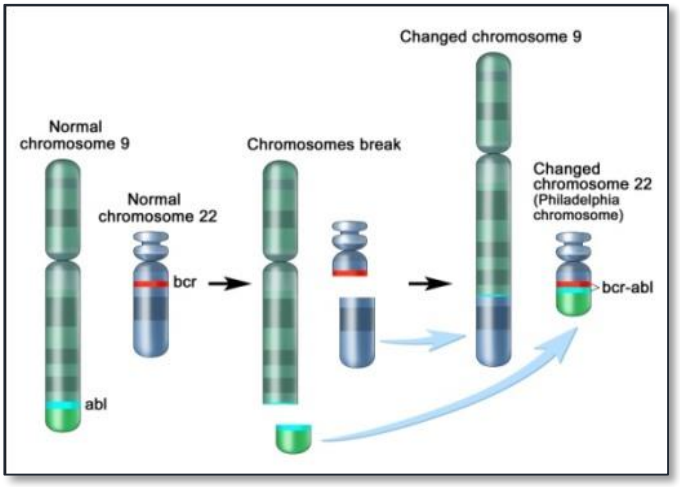
Project title: **NEW MOLECULAR DIAGNOSTIC APPROACHES TO STUDY LEUKEMIC STEM CELLS AND THEIR ROLE IN THE PATHOGENESIS OF CHRONIC MIELOID LEUKEMIA**

La leucemia mieloide cronica (CML) rappresenta il 20% delle leucemie nell'adulto. La CML origina nel 95% dei casi da aberrazione cromosomica, una traslocazione reciproca bilanciata tra i cromosomi 9 e 22 (t(9;22)(q34:q11)) all'interno di una cellula staminale pluripotente, con Formazione del cromosoma Philadelphia




(Ph) (Fig. 1).

*Fig 1. Il cromosoma 9 e il cromosoma 22 si "scambiano" una porzione di DNA generando il piccolo cromosoma Philadelphia.*

La traslocazione ha come risultato la formazione del gene di fusione BCR-ABL1, la cui attività costitutiva promuove a proliferazione e la sopravvivenza cellulare, con resistenza all'apoptosi, l'aumento dell'invasività e la comparsa di metastasi.

Le metodiche classiche attualmente in uso per la diagnosi di CML sono la conta delle cellule del sangue con valutazione della loro morfologia, l'analisi citogenetica, mediante analisi del cariotipo e *Fluorescent In Situ Hybridization* (FISH); queste metodiche consentono la diretta individuazione del cromosoma Philadelphia nelle cellule leucemiche ma sono poco sensibilità.

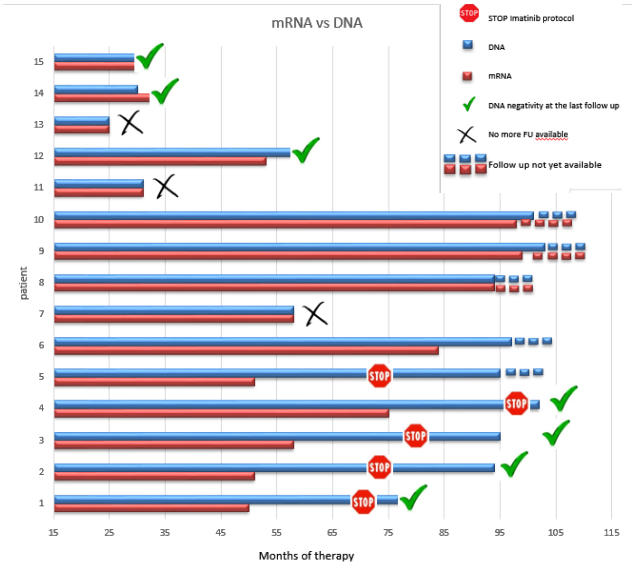
La terapia di prima linea è costituita dall'Imatinib mesilato (Gleevec/Glivec, Novartis Pharma), uno specifico inibitore delle proteine tirosin-chinasiche, che porta ad una inibizione della proliferazione. Lo svantaggio di questo tipo di terapia è la necessità di dover assumere il farmaco a vita e la necessità di monitoraggio continuo per seguire la loro risposta al farmaco e controllare che non ci sia una ricaduta della malattia, mediante determinazione della malattia minima residua (MRD), ossia la percentuale di cellule leucemiche non eradicate dalla terapia.

**gDNA-PCR, la nuova tecnica ad alta sensibilità per l'individuazione di cellule leucemiche nel paziente affetto da CML**

La qRT-PCR è la tecnica più sensibile oggi disponibile per monitorare i livelli di mRNA di BCR-ABL1 dopo la diagnosi e l'inizio del trattamento. I risultati sono espressi come il rapporto tra il numero di trascritti di BCR-ABL1 e i trascritti di un gene controllo. Limiti di questa tecnica sono legati all'efficienza di estrazione, di retrotrascrizione ed alla qualità dell'RNA estratto. Inoltre, è stata riscontrata l'assenza di una correlazione tra i livelli di mRNA ed il numero di cellule leucemiche, in quanto si potrebbero avere cellule che trascrivono in quantità elevata, o cellule quiescenti. I risultati negativi sono, quindi, difficili da interpretare, in quanto l'assenza di trascritto chimerico potrebbe essere imputabile o ad un'effettiva eliminazione delle cellule leucemiche, oppure alla presenza di cellule leucemiche trascrizionalmente silenti. È pertanto fondamentale, di fronte a risultati negativi, riuscire a capire se il paziente sia effettivamente guarito e quindi si possa interrompere la terapia con Imatinib. Attualmente però, la raccomandazione è quella di non interrompere la terapia, nonostante i considerevoli costi ed effetti collaterali negativi della chemio terapia.

Il nostro gruppo di ricerca ha messo a punto e validato una metodica innovativa, basata su una PCR quantitativa in tempo reale (quantitative real-time PCR, qRT-PCR) che amplifica la sequenza genomica della regione di rottura in BCR-ABL1. Ogni paziente affetto da LMC presenta un punto di rottura unico, con una sequenza di fusione specifica. Trovare il preciso punto di rottura porta all'individuazione di un marcatore tumorale paziente specifico che consente il monitoraggio della malattia minima residua durante la terapia. Sono stati sviluppati 16 saggi paziente-specifico di PCR quantitativa su DNA genomico basato sul break-point di BCR-ABL1 con cui sono stati monitorati 16 pazienti affetti da CML in fase cronica e sotto trattamento con TKIs per 5-8 anni. È stata inoltre sviluppata una formula per calcolare il numero di cellule positive al cromosoma Philadelphia. Paragonando i nostri risultati con quelli ottenuti mediante tecniche standard, abbiamo dimostrato che la nostra metodica mostra la presenza di cellule positive al cromosoma Philadelphia in 7 dei campioni che non presentavano livelli misurabili di mRNA chimerico (Fig. 2).

*Fig. 2: Confronto tra metodica qRT-PCR eseguita su RNA e DNA in 15 pazienti affetti da CML.*



Ad oggi sono stati selezionati, presso l'Ospedale Niguarda di Milano, 87 pazienti candidati all'interruzione della terapia con Imatinib e Nilotinib. L'attuazione della nostra metodica prevede una prima caratterizzazione molecolare del break-point genomico di ogni singolo paziente e successivamente il monitoraggio di questo marcatore paziente-specifico nei follow-up.

La tecnica da noi utilizzata si basa su di un arricchimento della regione genomica d'interesse e un successivo "*deep sequencing*" per sequenziare il break-point a livello di singolo nucleotide. Una volta caratterizzato il punto di rottura paziente specifico la malattia minima residua potrà essere monitorata su campioni di sangue periferico nei follow-up successivi all'inizio della terapia.

Lo scopo della presente ricerca è quello di sviluppare un protocollo di stop-Imatinib italiano mediante monitoraggio del DNA genomico con metodica qRT-PCR. Ogni paziente avrà quindi un marcatore specifico caratteristico delle cellule leucemiche, che potrà essere utilizzato per assicurarsi dell'assenza di cellule Philadelphia positive prima dell'interruzione della terapia con Imatinib e Nilotinib.

**PHYSIOLOGY**

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Project title: **IMPACT OF LYMPHATIC DRAINAGE AND EXTRACELLULAR TISSUE REMODELING ON CARDIAC TISSUE FUNCTION**

The cardiac tissue is supplied by an extended network of lymphatic vessels dispersed in the sub-epicardial, myocardial and sub-endocardial areas and in atrioventricular and semilunar valves. As in the majority of body tissues, an efficient and normal lymphatic drainage guarantees the maintenance of a steady state interstitial fluid volume and solute concentration and returns macromolecular debris, leucocytes and cells to the blood stream. On the other hand, lymphatic saturation and/or failure may cause fluid accumulation and acute or chronic tissue edema, altered interstitial fluid solute concentration, deposition of tissue debris and acute and/or chronic inflammation. The result of lymph flow obstruction is an abnormal fibrous tissue deposition and remodeling, a condition that, in the heart, may lead to the development of several acute or chronic heart diseases, such as severe myocardial injury with subendocardial edema, intracellular edema, myofibrillar and mitochondrial degeneration. Two macromolecules may be particularly involved in tissue remodeling: hyaluronic acid (HA), the main anion hydrated macromolecule in the tissue and very sensitive to tissue water content and therefore lymphatic drainage, and collagen 1, which is the extracellular unsoluble macromolecule most overexpressed in inflammation. Of great importance from the clinical standpoint, it is the observation that lymphatic inefficiency and local lymphedema seem to be strictly associated with conduction disturbances and cardiac arrhythmias. Such an observation is particularly interesting on considering that lymph propulsion within large collecting lymphatics depends upon contraction waves triggered in lymphatic smooth muscle cells by spontaneous action potentials sustained by an inward current, carried by the so called hyperpolarization-activated cyclic nucleotide-gated (HCN) channels which belongs to the same channel family carrying the heart spontaneous sinoatrial pacemaker current.

Hence, the present research project aims at investigating the still largely unknown relationship between normal or altered heart frequency and cardiac lymphatic function. In particular we will focus on :

1. *the interplay between external tissue forces and the intrinsic lymphatic contractility in sustaining and modulating cardiac lymph flow in normal and diseased heart*, to establish whether the spontaneous lymphatics contractility matches and/or is modulated by the cardiac frequency. We hypothesize that cardiac lymph flow requires coordination of the cardiac and lymphatic pace-maker firings, likely through HCN channels;
2. *the functional link between cardiac lymphatic impairment and disturbances of cardiac pacing.* In fact, it is at present unknown whether impairment of the cardiac lymphatics induces matrix remodeling and, as a consequence, altered electrical disturbances of cardiac conductive fibers or, viceversa, if a primitive disturbance of the cardiac pacing eventually results in lymphatic inefficiency and, triggering a vicious cycle, altered matrix deposition and irreversible arrhythmia.

To pursue the above aims we will carry on:

A. functional studies:
   - to determine to what extent modification of cardiac frequency in the normal heart may either improve or hinder cardiac lymph flow in sub-epicardial lymphatics
   - to evaluate the pattern and amount of cardiac lymph flow
B. morpho-functional studies:
   - to verify and characterize the presence of smooth muscle cells in the wall of the cardiac lymphatic vessels
   - to map the specific location of vessels with smooth muscle cells in the different cardiac areas (atria or ventricular walls, conduction tissue, valves)

- to relate cardiac lymphatic function to extracellular tissue structure, with particular focus on HA and collagen type 1
- to examine whether smooth muscle cells in cardiac lymphatics express HCN channels and characterize the specific HCN families, to be compared with the HCN channels expressed by the heart conduction system

The results of the present project might provide useful improvements of basic knowledge of the cardiac lymphatic function in healthy and diseased heart and a toll for the potential development of new therapeutic approaches.

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**PHYSIOLOGY**

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Project title: **ENTERING THE CELLS: HOW NANOPARTICLES AND SMALL PEPTIDES CROSS THE BIOLOGICAL BARRIERS, STUDY OF VARIOUS MEMBRANE CROSSING MECHANISMS BY BIOPHYSICAL APPROACHES (ELECTROPHYSIOLOGY AND FLUORIMETRIC METHODS).**

**Background**. Membranes define the limit between the extra and the intracellular environment, they are one of the key structures in cell biology, not only being involved in compartmentalizing and protecting cells, but also severely controlling and regulating the passages of ion and molecules, metabolite and drugs, having an essential role as organizing centers for metabolism or signaling. Ions and small molecules enter cells by passively diffusing across lipid bilayers or through membrane channels. More complex solutes utilize membrane transporters. Additionally, living cells have a specific membrane potential that is determined by membrane conductances and controlled by many processes including electrical or agonist stimulation, ion channels and transporters activity and changes in ionic concentrations at both side of the membrane. Small peptides and peptidomimetic drugs enter the cell by specific electrogenic transporters that coupled the proton gradient to the substrates translocation, therefore substrate movement across the membrane depolarizes the cell and changes the pH. Monitoring these parameters in the presence of different substrates represents a tool for studying function and identify substrates of these proteins [1-6] . Nanoparticles (NPs) readily enter cells by different endocytotic mechanisms making them at the same time potentially dangerous and useful to human health. Recently, our lab has shown the possibility that some NPs might also cross the plasma membrane by a non-endocytotic way gaining a direct access to the cytoplasm. This pathway is usually poorly considered as it challenges the idea of non-permeability of membranes to large hydrophilic molecules. In our recent paper we have started collected information about the characteristics that NPs must have to be able to directly cross the membrane, and we have we have understood that size and aggregation time are fundamental parameters to define their ability to cross the membrane [7,8].

**Aims.** The goal of research project is to define the mechanism and characteristics of NPs that allow direct permeation into the cell and verify if transporters belonging to some SLC families (SoLute Carrier), are also able to interact and transport modify NPs. Cell cultures or Xenopus oocytes will be exposed to NPs of distinct size, differently functionalized, with different aggregation states, and membrane and intracellular modifications will be recorded.Xenopus oocytes heterologously expressing member (wild type or mutants) of SLC15 or SLC6 family will be tested by Two Electrode Voltage Clamp (TEVC) and data about transport currents, pre -steady state current, leakage current will be acquired in the presen ce or in the absence of specific or hypothesized substrate.

**Methods**. In the project, the biophysical data will be mainly collected by electrophysiological techniques: two electrode voltage-clamp on Xenopus laevis oocytes and patch clamp on cell line, but also fluorimetric assay. Proteins expression will require the use of bio-molecular techniques (cloning, sub-cloning, PCR techniques, site direct mutagenesis, RNA transcription and extraction), heterologous expression protocols (RNA and DNA injection or transfection).The presence of protein will be verified by immunochemistry (Western-blot, Single oocyte chemiluminescence, immunocytochemistry).

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