1. Cardiovascular diseases: Acute and long-term impact of COVID-19 on cardiovascular and pulmonary systems. PI: Roberto DE PONTI, roberto.deponti@uninsubria.it

Cardiovascular diseases: Acute and long-term impact of COVID-19 on cardiovascular and pulmonary systems.

Coronavirus Disease 2019 (COVID-19) is the clinical manifestation of infection with Severe Acute Respiratory Syndrome Coronavirus-2 (SARS-CoV-2) [1, 2] and, in some cases, an acute cardiovascular syndrome [6-14]. The clinical course of the infection is characterized by a flu-like syndrome of mild severity in most cases. Nevertheless, in about 15% of cases, it is complicated by interstitial pneumonia [3, 4] which may progress to acute respiratory distress syndrome (ARDS) and shock [1, 2, 5]. In addition, COVID-19 may also exert an adverse impact on the heart and cardiovascular system. Some reports and systematic reviews described acute cardiopulmonary involvement of COVID-19, including pericardial disease, arrhythmias, complete hart block, myocarditis, acute coronary syndromes, pulmonary embolisms, and acute heart failure [6-14].

A recent case series of patients hospitalized for COVID-19 pneumonia showed that the most common cardiac manifestation was acute pericarditis [10]. Of note, cardiac abnormalities showed a late onset after hospitalization and initiation of COVID-19 symptoms and a large proportion of patients experienced electrocardiographic abnormalities immediately before the scheduled discharge from hospital and after 2 consecutive negative nasopharyngeal swabs [10].

Cardiopulmonary abnormalities developed during the acute phase of SARs-CoV-2 infection may have a relevant clinical impact on the course of the disease. However, it is not entirely clear whether these adverse reactions may be linked to an increased long-term cardiovascular risk. In this context, it is worth mentioning that SARS-CoV-2 uses the angiotensin-converting enzyme 2 (ACE2) receptor to initiate the virus entry into human cells [11, 12]. The real challenge in the field of therapeutic strategies is to modulate SARS-CoV-2 binding to ACE2 without blocking the crucial protective properties of this enzyme. Indeed, it has been suggested that the loss of ACE2 receptor activity as consequence of the viral elimination and down-regulation processes leads to less angiotensin II inactivation and less generation of antiotensin$_{1-7}$ [11, 13, 14]. The imbalance between angiotensin II over-activity and angiotensin$_{1-7}$ deficiency reduce the activation of the Mas receptor and endothelial nitric oxide synthase, triggers inflammation, thrombosis, and other adverse reactions, eventually worsening COVID-19 [11, 13, 14].

Even after the acute phase, patients may experience cardiovascular sequale (so-called "Long COVID syndrome" or "Post-COVID syndrome"), such as inappropriate sinus tachycardia, arrhythmias, chest pain, dyspnea, deep vein thrombosis, syncope and impaired exercise capacity. In fact, in patients recovering from COVID 19, even not hospitalized, MRI shows abnormal findings in the majority of the cases [15, 16]. Finally, there are only few data on cardiopulmonary exercise testing in patients with prior COVID-19 and, in a case series, a reduction in peak oxygen uptake and decreasing oxygen pulse was observed [17]. This indicates that pulmonary dysfunction and gas transfer inefficiency is not the sole reason for exercise limitation and cardiac dysfunction should be also considered.

In this context, several research lines can be developed to further elucidate the cardiopulmonary involvement of COVID-19 in the acute phase of the disease and after recovery. They include: (i) evaluation of the main features of cardiopulmonary involvement during the acute phase of infection (retrospective data collection and meta-analysis) with particular focus on new-onset arrhythmias, alteration of left ventricular function, and prevalence and pathophysiology of COVID-related acute coronary syndromes, (ii) evaluation of the long-term sequaele of cardiopulmonary involvement of COVID-19 (prospective data collection) with a focus also on the mechanisms underlying the "post-COVID syndrome", and (iii) characterization of the potential mechanisms linking the renin-angiotensin-aldosterone system activity and COVID-19 sequelae.

Results have the potential to reinforce the recommendation and to carefully re-asses the therapeutic choices based on the clinical conditions of COVID-19 patients during the acute phase of infection and long-term, after recovery.

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2**. Genetics: Molecular and cellular dissection of the oncosuppressor pathways deployed by the highly pleiotropic human RNASET2 gene.** PI: Francesco ACQUATI, francesco.acquati@uninsubria.it

Project title: "Functional characterization of the pleiotropic oncosuppressive roles played by the human RNASET2 gene". - P.I.: Prof. Francesco Acquati

Cancer research has been revolutionized in the last two decades following the gathering of a growing body of experimental evidence strongly suggesting that cancer development and growth represent a much more complex biological process than was previously thought. In this context, it is nowadays widely acknowledged that the tumor microenvironment play a key role in the process of tumorigenesis. Our research group has long been involved in the functional, cellular and molecular characterization of the human *RNASET2* gene, whose features make it a particular interesting target of investigation in the field of cancer microenvironmental research.

In the last decade, several research groups (including our) have reported a marked oncosuppressive role for the *RNASET2* gene. Noteworthy, independent lines of investigation have disclosed an unexpected plethora of biological processes involved in *RNASET2*-mediated tumor suppression, such as cell response against cancer-associated stresses, angiogenesis, cell proliferation, cytoskeletal remodelling, apoptosis and innate/adaptive immune response regulation. In our lab, both xenograft-based and syngeneic murine models have recently shown the ability of extracellular, cancer cell-derived RNASET2 protein to elicit a strong antitumoral immunological response, prompting us to propose an "alarmin-like" role for this molecule. At the same time, many other features discovered for RNASET2 are clearly compatible with a cell-autonomous oncosuppressive role which seems to act independently of the tumor microenvironment.

Taken together, these data clearly point a*t RNASET2* as a highly pleiotropic oncosuppressor gene carrying out its role at both the cell-autonomous and non-cell autonomous levels, thus making it a very promising candidate to develop a multi-target anticancer therapeutic approach based on a single molecule.

The PhD research program presented here 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 and, more generally, host defense. To this end, the research program will address several issues concerning the biological processed involved in *RNASET2*-mediated tumor suppression.

1) <u>Non-cell autonomous mechanisms: role of RNASET2 in activation of the immune system</u>

Our previous *in vivo* experimental data clearly showed that *RNASET2*-mediated tumor suppression involves the recruitment of M1-polarized macrophages form the stromal compartment. 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 will be used to investigate whether RNASET2-primed human macrophages elicit a tumor suppressive response and, viceversa, whether RNASET2 ovexpressing cancer cells can modulate the macrophage polarization pattern. To this end, the human monocytic leukemia-derived cell line THP-1 (a well established model of *in vitro* macrophage differentiation) will be used coupled to several human cancer cell lines available in our laboratory. To evaluate the putative non-cell autonomous role of macrophages-derived RNASET2, the co-culture system will be assembled both with and without direct cell-to-cell contact. Following co-culture of cancer cells with THP-1-derived, RNASET2 genetically engineered macrophages, several cancer-related parameters will be investigated at different time points in the human cancer cells panel by means of *in vitro* assays already established our lab. These experiments will be validated by using recombinant RNASET2 expressed in our lab in proper heterologous systems. Moreover, a detailed survey of the endogenous expression pattern of human RNASET2 in other cellular components of both innate and adaptive immune system (such as granulocytes, dendritic cells, NK and lymphocytes) will be carried out, and the relevant cell types showing an interesting expression pattern will be further investigated.

2) Cell autonomous mechanisms: Investigation of the intracellular pathways undergoing RNASET2-mediated tumor suppression.

Recent data have shown a previously unexpected role for human RNASET2 in the mitochondrion, where this enzyme was shown to process and release back in the cytoplasm a truncated (TERC-53) form of the RNA component of human telomerase (TERC). Very little is currently known about the role of RNASET2-mediated TERC processing in the biology of human cells. Some authors argue that TERC-53 has a role in cellular senescence, another cancer-related process where RNASET2 might be involved. We aim both to investigate this topic by validating RNASET2-mediated TERC processing in several RNASET2-engineered human cancer cell lines available in our lab and to directly mimic TERC processing by transfecting TERC-53 transcripts into the above-mentioned cell lines, in both physiological and stress culture conditions. The biological response of TERC-53-transfected cells will be investigated in detail, in term not only of a putative cell senescent phenotype, but also for known *RNASET2*-mediated changes in several cancer related parametes.

The ribonucleolytic activity of RNASET2 has been reported to be dispensable for some of its tumor suppressive roles. Of note, besides the two highly conserved CAS I and CAS II catalytic sites, a putative TRAF-2 binding motif located near the C-terminal end of the RNASET2 protein has been identified and proposed to play a role in RNASET2-mediated apoptosis. Of note, the yeast Rny1p T2 RNase also triggers apoptosis in a catalytically-independent manner. TRAF-2 is a key intracellular signaling mediator acting of downstream of TNFa ligand family members to mediate several biological responses, ranging from apoptosis to inflammation. To better define the functional role of the putative TRAF-binding site within RNASET2, a MCF7 breast cancer-derived RNASET2-null cell line will be used as an experimental model. Our preliminary results have shown that RNASET2 overexpression in this cell line triggers a range of *in vitro* responses that are typically associated with this oncosuppressor protein, including an increased apoptotic rate. To better define the role of the RNASET2 putative TRAF-2 binding site, a recombinant vector for constitutive expression of an RNASET2 protein lacking the TRAF-2 binding motif will assembled in our lab. The wild-type or TRAF-2-deleted RNASET2 expression vectors will be co-transfected with a TRAF-2-expression vectors in MCF7 cells in order to define which of the several biological responses are abrogated following TRAF-2 motif deletion. Protein immunoprecipitation and pull-down assays will be also carried out to assess the physical interaction between RNASET2 and TRAF-2. A catalytic mutant RNASET2 expression vector, already available in our lab, which we will used as a control. Whenever a differential RNASET2-mediated biological response attributable to the TRAF-2 deletion will be found, the genome-wide transcriptional profile of both wild-type and TRAF2-deleted transfected MCF7 cells will be investigated in order to better define the molecular pathways involved in the TRAF-2-mediated oncosuppressive roles carried out by RNASET2.

3) Immunohistochemical assays on human cancers

The *RNASET2* gene is currently defined as an oncosuppressor gene likely involved in the control of several human cancer types representing different organs. 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. However, this trend has not been observed for all human cancer types. Therefore,to shed more light into this issue, a pan-human cancer investigation on *RNASET2*-

associated survival will be done by investigating Kaplan-Meier plots, in order to better define the cancer types where overall survival is clearly associate with an increased expression of RNASET2. From such list of RNASET2-sensitive cancers, a selected panel of staged human tumor samples 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, "alarmin-like" gene endowed with a marked oncosuppressor role.

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3. **Infection and Immunity: Virus-host interaction and oncogenesis**. PI: Greta FORLANI, greta.forlani@uninsubria.it

Functional characterization of HBZ cellular interactors involved in ATL progression

Human T-cell leukemia virus type 1 (HTLV-1), the first identified human oncogenic retrovirus, is the etiological agent of a severe form of adult T cell leukemia/lymphoma (ATL) and of HTLV-Associated Myelopathy/Tropical Spastic Paraparesis (HAM/TSP), a progressive neurological disease. Two viral proteins, the viral transactivator Tax-1 and the HTLV-1 bZIP factor (HBZ), play crucial role in HTLV-1 associated oncogenesis. Tax-1 seems to be crucial in the onset of the oncogenic process mostly by disarranging several cellular activation pathways and particularly the NF-kB pathway. However, Tax-1 expression is lost in most of ATL cases suggesting that it might be dispensable for maintaining the neoplastic state, while HBZ is expressed at all stages of infection and neoplastic transformation, suggesting that it may be required in the maintenance of the oncogenic process. We have demonstrated

that the subcellular distribution of HBZ in the various phases of the disease may be relevant to the progression of HTLV-1 infection, indeed we found that HBZ localized exclusively in the cytoplasm in PBMC of both asymptomatic carriers and HAM/TSP patients while ATL patients can express HBZ not only in the nucleus but also in the cytoplasm. Thus, neoplastic transformation is accompanied by a dichotomy of HBZ localization in that exclusive cytoplasmic localization, as observed in AC and in HAM/TSP patients, is progressively modified to include nuclear localization of the protein. Aim of this project is to characterize the cytoplasmic and nuclear specific subcellular compartments of HBZ protein and the HBZ interactors in the different stage of the disease to elucidate the mechanisms responsible for the peculiar subcellular distribution and thus for the oncogenic function of the viral protein. Preliminary results obtained in ATL-2 leukemic cell line expressing HBZ in the nuclear compartment have revealed that HBZ interacts with factors involved in the splicing machinery and in Non-mediated RNA decay signaling, processes that are known to affect neoplastic transformation. The functional characterization of some these cellular factors might represent a crucial tool to add pieces to the complex puzzle of HTLV-1-driven oncogenic transformation and ATL progression.

4. **Microbiology : The measure of total and species-specific Torquetenovirus (TTV) viraemia as a predictive marker of immune system function**. PI Andreina BAJ, andreina.baj@uninsubria.it

The measure of total and species-specific Torquetenovirus (TTV) viraemia as a predictive marker of immune system function

Once thought to be only present in the host during disease, viruses have been recently demonstrated to be numerous in various districts in healthy subjects, and the term "virome" has been coined to describe the collection of viral species present in each human organ, as a kind of viral "flora" made up of bacteriophages, endogenous retroviruses, eukaryotic viruses not associated with disease and viruses able to cause acute, chronic or latent illness. More recently, monitoring the human virome has been suggested as a promising and novel area of research for identifying new biomarkers which would help physicians in the management of diseased patients. Thanks to the next-generation sequencing, the human virome has been studied in several districts, like the respiratory tract, gut, and skin, and in different clinical conditions. Thus, to date, we know that some components of the human virome are identified only in few districts of a limited number of individuals, while others are present in almost all body districts of a very high percentage of people.

Torquetenovirus (TTV) is the prototype of these latter components being the most representative and abundant virus of the human virome. TTV is presently classified in the Anelloviridae family, and it possesses several remarkable properties, including a particularly small single-stranded circular DNA genome, an extremely high degree of genetic heterogeneity (at least 29 TTV species have been identified so far), a remarkable ability to produce chronic infections with no associated clinical manifestations, and a high prevalence in the populations worldwide regardless of age, sex, and socio-economic status. Starting from these properties, evidence is increasing regarding the successful interplay of TTV with its host, and the control of TTV replication exerted by the immune system.

The goals of this project are to expand our knowledge in the TTV/immune system interplay investigating TTV and its genetic species as candidate surrogate markers to infer immune depression level, immune-reconstitution process, graft and/or infections risk, and, finally, the overall clinical outcome in adult patients receiving immunomodulant drugs. A precise understanding of how and how much immunity modulates TTV replication is of utmost importance given the intriguing idea of using PCR monitoring of TTV viremia as a robust way of assessing global immune function.

5. **Neuroscience: Characterization of the molecular mechanism underlying GABA-receptor defects in CDKL5 deficiency disorder"** PI Charlotte KILSTRUP-NIELSEN, c.kilstrup-nielsen@uninsubria.it

Characterization of the molecular mechanism underlying GABAA-receptor defects in CDKL5 deficiency disorder PI: Charlotte KILSTRUP-NIELSEN

Background: mutations in the X-linked cyclin-dependent kinase-like 5 gene (*CDKL5*) cause CDKL5 deficiency disorder (CDD), a neurodevelopmental pathology characterized by early onset of intractable seizures, severe intellectual disability, autistic traits, and hypotonia. The majority of patients are heterozygous females that either do not express CDKL5 or express hypo-functional variants of CDKL5. CDKL5 functions have been investigated in *Cdkl5*-KO mouse models that recapitulate most features of the human pathology. Such studies converge on a role of CDKL5 in regulating synaptic functions through microtubule dynamics and its control of neuronal receptor expression and composition. Recent unpublished data from our lab show that loss of CDKL5 leads to an altered membrane expression of $GABA_A$-rectors, which represent the main inhibitory receptors of the nervous system. The precise molecular mechanism through which CDKL5 causes aberrant $GABA_A$-receptors expression is still not known but we envisage that a deeper understanding would pave the way for therapeutic drug-based strategies.

Objective.

In this project, which has recently received a three-years funding from Fondazione Telethon, the student will make large use of molecular, biochemical, and imaging approaches to study how CDKL5 influences synaptic $GABA_A$-receptor expression possibly through an effect on the post-synaptic scaffolding complex and microtubule dynamics. Primary cultures of *Cdkl5*-KO neurons and cerebral tissue from KO mice will be used together with cell cultures.

Candidates: Interested candidates should be highly motivated to work in a team and have a background in molecular and cellular biology and be willing to work with rodents.

For further details about the project please contact: c.kilstrup-nielsen@uninsubria.it

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6. Neuroscience: Unveiling the mitochondrial Rab GTPases role in Parkinson's Disease. PI: Tiziana ALBERIO, tiziana.alberio@uninsubria.it

Unveiling the mitochondrial Rab GTPases role in Parkinson's Disease RESPONSABILE: Tiziana Alberio

Mitochondrial dysfunction is crucial in Parkinson's disease (PD) pathogenesis. Recently, mutations in Ras analog in brain (Rab) proteins have been linked to PD, supporting the idea that alterations of intracellular protein trafficking could be a central pathogenetic mechanism. In this frame, our working hypothesis is that Rab protein levels and/or interactions are altered in PD, thus resulting in an impairment of mitochondrial protein complexes composition and in the improper disposal of dysfunctional mitochondria. The hypothesis will be tested using different models (e.g., skin fibroblasts, reprogrammed dopaminergic neurons and post-mortem PD midbrains) representing both familial and sporadic PD patients. The project will include both a proteomics/systems biology part and the assessment of vesicular trafficking by functional assays. This will be crucial to provide new insights into the disease mechanisms and to suggest new therapeutics.

7. Neuroscience: Role of the enteric microbiota and neuroimmune interplay in a murine model of Inflammatory Bowel Disease. PI Cristina GIARONI, cristina.giaroni@uninsubria.it

Role of the enteric microbiota and neuroimmune interplay in a murine model of Inflammatory Bowel Disease

The gut saprophytic commensal flora has a fundamental role in the modulation of several local functions including regulation of host immune system and defense against pathogenic microorganisms. Alterations in the symbiotic relationship between the microbiota and the enteric microenvironment, comprising cells of the innate and acquired immune system and enteric neurons, underlay development of complex gut disorders, including chronic inflammatory bowel disease (IBD). In recent years, we have focused on hyaluronan (HA), an unbranched glycosaminoglycan (GAG) component of the extracellular matrix, as a new molecular player involved in neuroadaptive changes of enteric neuronal circuitries during intestinal inflammation and ischemia/reperfusion injury. Accumulation of HA in the epithelial layer, in blood vessels within the submucosal layer, in the smooth muscle layers and within myenteric ganglia was indeed observed both in experimental rodent models of colitis and in bioptic specimens of IBD patients. Interestingly, enteric neurons may respond to microbial factors mainly via specific pathogen-associated molecular pattern recognition receptors (e.g. Toll-like receptors, TLRs), whose signaling is modulated by HA. Rodent models of chemically-induced colitis have highlighted the beneficial effect of HA in reducing dysbiosis and in relieving epithelial damages via TLR4, and recent data, obtained in our laboratory, have immunohistochemically demonstrated that HA preserves myenteric neuron homeostasis in a rat model of IBD. Since TLRs are targets of HA action during inflammation, the GAG may be involved in development of myenteric neural plasticity by tuning adaptive signals at the intersection between the microbiota-innate immunity axis and the ENS. Thus, the present study aims at evaluating the role of HA in the development of neuromuscular dysfunction in a well-established murine model of colitis, by means of functional, immunohistochemical and biomolecular approaches.

8. Oncology: Morphological and molecular profiling of high-grade neuroendocrine neoplasms in different anatomical sites: one disease for all location or not ? PI: Silvia UCCELLA, silvia.uccella@uninsubria.it

"Morphological and molecular profiling of high-grade neuroendocrine neoplasms in different anatomical sites: one disease for all locations or not?"

Background
High-grade neuroendocrine neoplasms (HG NENs) encompass a heterogeneous spectrum of aggressive malignancies that historically recognize their prototype in small cell lung carcinoma. They can virtually arise in any epithelium-lined organ, with lung, colon, and stomach as the most common primary sites. The lack of an effective therapeutic approach, the frequent scarcity of diagnostic material due to advanced inoperable disease, the rarity of cases in extrapulmonary
anatomical sites have contributed, for a long time, to hide their kaleidoscopic nature, in favor of an apparently monolithic "ugly and bad disease." Starting from the early 2000s, the increasing knowledge about molecular mechanisms of cancer provided by the more and more comprehensive genomic analyses have provided new tools to understand and treat neoplastic diseases. Indeed, the "molecular revolution," brought in the last decade by the advent of high-throughput genomic technologies, has enormously enhanced the possibilities of tailored diagnostics and therapeutics, leading to the concept of "precision medicine." The application of these comprehensive approaches to HG NENs have unearthed their complexity and have shed light on their relationships with well differentiated NENs (neuroendocrine tumors, NETs)
as well as with non-neuroendocrine carcinomas arising in the same anatomical sites. One of the consequences of this process was the recognition of a subset of HG NENs which are not characterized by a poorly differentiated neuroendocrine morphology, but still show a high proliferation activity that, in the digestive tract, have been named grade 3 NETs (G3 NETs) and, in thoracic organs, highly proliferating carcinoids. Although rarely, NENs may arise in anatomical sites other than the thoracic cavity and the digestive system. Among these rare sites, the upper respiratory tract, the salivary glands, and the urogenital system are the most common locations. In most of these locations, HG-NENs are still poorly defined entities, although they are commonly diagnosed and treated. Indeed, their boundaries

and relationships with non-neuroendocrine carcinomas of the same sites are still to be established and need to be supported by molecular evidence.

Design

Our group has contributed to the analysis of the morphological, immunophenotypical and molecular features of HG-NENs for a long time, mainly focusing on poorly differentiated NECs of gastroenteropancreatic and pulmonary locations and their relationships with non-neuroendocrine carcinomas of the same sites. We are now starting new projects for the study of HG-NENs in so-called rare sites, as we have been able to collect a consistent series of cases from head and neck, breast, urological and gynecological sites. Our study will include three main interdependent phases, which will allow us:

- To perform a rigorous histopathological review and re-classify all cases according to the most recent guidelines and diagnostic concepts. This will be accomplished under the guidance of the tutor, who is a well-recognized expert in the neuroendocrine pathology field
- To identify significant immunophenotypical markers for diagnostic, prognostic and predictive purposes. To this purpose, a well-equipped immunohistochemistry laboratory is present in the receiving structure.
- To perform a molecular analysis using targeted next generation sequencing (NGS) with customized panels for NECs and NETs, exploring the key genes involved in the cancerogenesis of NETs and site-related carcinomas. This is feasible thanks to the availability of a molecular pathology labotatory with long-standing experience.

We expect that our results will shed light on the presence of site-specific features of HG-NENs that will not only allow a better understanding of these entities, but also, and most importantly, might open the road to the identification of targetable pathways that can be useful for improving patients' outcome in these aggressive diseases.

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9. Oncology: Finding drivers of methylation in endometrial cancers. PI: Daniela FURLAN, daniela.furlan@uninsubria.it

Background

Endometrial cancer (EC) is the most common gynaecological malignancy and fourth most common cancer in the developed countries. Both incidence and mortality rates have been rising in the last decade and are predicted to increase further due to risk factors such as diabetes and obesity.

Currently, clinical and histopathological factors such as stage, histotype, grade, depth of invasion, and lymph vascular space invasion are used to stratify patients into risk groups to guide surgical management, adjuvant therapy and follow-up. However, these clinicopathological variables do not sufficiently predict patient outcomes. Recently the results of next-generation sequencing studies have expanded knowledge of recurrently altered signalling pathways in EC and have laid the foundations for rational design of molecular-based clinical trials. Recently the Cancer Genome Atlas Network (TCGA) has reported a comprehensive genomic and transcriptomic analysis of EC. On the basis of integration of mutation spectra, copy-number aberrations, and microsatellite instability status, ECs were categorised into four genomic classes: 1) *POLE* (ultramutated) tumours characterized by very high mutation rates and hotspot mutations in the exonuclease domain of *POLE*; 2) a microsatellite-unstable (MSI, hypermutated) group of endometrioid tumours characterized by microsatellite instability due to *MLH1* promoter methylation and high mutation rates; 3) copy-number low ECs, comprising microsatellite-stable grade 1 and 2 endometrioid cancers with low mutation rates, characterized by frequent *CTNNB1* mutations; 4) copy-number high (serous-like) tumours, characterized by extensive copy-number alterations, low mutation rates, recurrent *TP53, FBXW7* mutations and poor outcome. Although the TCGA genomic characterization of EC has not yet entered in the clinical practice, there is a strong scientific rationale that the identification of the four TCGA subtypes of EC might permit a reclassification of these tumors, which could directly affect treatment decisions and guide clinical trials of targeted therapies.

The second important aspect regards the epigenetic profiles of ECs. DNA methylation is highly dysregulated in cancers displaying aberrant CpG island hypermethylation and long-range blocks of hypomethylation.

Although very few reports have been reported on the epigenetic landscapes of ECs, there is accumulating evidence that DNA methylation changes may contribute to carcinogenesis in the endometrium. Aberrant methylation of tumor suppressor genes is detectable in EC precursor and has been shown to distinguish between benign tissue and cancer. These findings suggest that some methylation markers may have value in EC screening, early detection and prevention. So far, more than 50 hypermethylated tumour suppressor genes have been identified including the most famous genes: *MLH1, PTEN, p16, APC, MGMT, RASSF1, PR and CDH1*. On the other hand, few publications have described hypomethylated oncogenes in endometrial cancer (such as *BMP, CTCFL, PARP1, CASP8*). Genes with aberrant DNA methylation are involved in various biological pathways such as cell adhesion, cell proliferation, signalling transduction, cell cycle regulation, microtubule stabilization and also apoptosis.

Working hypothesis and translational implications

Specific driver gene mutations are tightly tied to tumor DNA methylation landscapes in a site-specific manner. Well known driver genes associated with CpG island hypermethylation include *BRAF* in colorectal carcinomas and *IDH1* in gliomas.

This aspect has not so far been elucidated in EC and there is a gap in our knowledge about driver gene mutations and pathways causing promoter methylation (CpG island methylator phenotype; CIMP) and how these contribute to the four main categories of EC as defined by the TCGA research network.

In particular, there are no clear drivers of the microsatellite instability and CIMP, which occur in a quarter or more of endometrial carcinomas. Of the commonly mutated genes in EC (including *POLE, POLD, PTEN, PIK3CA, PIK3R1, KRAS, FGFR2, ARID1A, TP53, FBXW7*) *PTEN* has been associated with DNA hypermethylation while *TP53* and *CTNNB1* mutations correlate with DNA hypomethylation. However there is no clear evidence that *PTEN* may be a driver gene of promoter methylation in EC.

We propose that the identification of drivers of methylation in EC may be useful to recognize different clinico-pathological subsets of ECs to guide treatment decisions.

Specific aims

Aim 1: Targeted next generation sequencing (NGS) will be carried out in a consecutive series of 150 sporadic ECs diagnosed at Ospedale di Circolo, ASST Settelaghi in Varese (Italy) in the last three years. This analysis will include all the commonly mutated genes in ECs that will be evaluated for single nucleotide variants (SNV), for small insertion/deletions (Indel) and for Copy Number Variation (CNV). The mutation profiles of these tumors will be correlated with MSI status and with the tumor clinico-pathological features.

Aim 2:
Specific DNA methylation patterns will be evaluated in the same cohort of 150 ECs performing both DNA hypomethylation and DNA hypermethylation analyses in order to correlate precise genetic mutations with CpG island/promoter methylation and with DNA hypomethylation profiles.
We will perform these analyses on bisulfite converted tumor DNAs using NGS analysis and we will include both hypermethylated tumour suppressor genes and hypomethylated oncogenes in ECs. Moreover we will analyse LINE-1 methylation sequences using bisulfite-pyrosequencing to evaluate global levels of DNA methylation in ECs.

Collaborators
Dr. Annabelle Lewis, Department of Life Sciences, Brunel University, London, UK
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10. Oncology: Prostate cancer: disentangling the relationships within the tumour microenvironment to better model and target tumour progression. PI: Ian Marc BONAPACE, ian.bonapace@uninsubria.it

Title of the Project: Prostate cancer: disentangling the relationships within the tumour microenvironment to better model and target tumour progression.

Background. 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, one third of the patients will progress to the metastatic stage of the disease for which no effective treatments are available. It is clearly emerging that the type of genetic and epigenetic alterations driving malignant transformation in the prostate epithelial cells can predict much better than histology tumour behaviour and sometimes the response to specific therapies. Recently, tumour-microenvironment interplay has been proposed to play a relevant role in tumour progression towards Castration Resistant PCa (15-18). Notably, many of the above features are acquired through Cancer Associated Fibroblasts (CAF)-induced DNA methylation-dependent epigenetic modifications.
Aims. The overall aim is the identification of molecular determinants of the cross-talk between PCa cells and CAFs, determining PCa onset and progression. These goals will be pursued making use of stromal cultures from PCa samples (normal fibroblasts-NFs, and CAFs), and of 2D cell lines cultures modelling tumour progression via genetic manipulation. These models will be used to study the relationships between oncogenic mechanisms due to cell-intrinsic (genetic and epigenetic) and extrinsic (microenvironment) processes, involved in prostate tumour onset and progression.
Experimental design. 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:
1. 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.
2. ERG over-expression in combination with massive PTEN downregulation.
By RNA-seq and Illumina Infinium CytoSNP-850K BeadChip, we will identify differentially expressed and methylated transcripts required for tumour reprogramming in the 2D model. By quantitative Mass

spectrometry (MS) performed on the conditioned media of the induced cells, we will also identify secreted factors governing CAF-epithelial crosstalk and prostate tumorigenesis.
To validate the driving role of those identified transcripts and secreted proteins, knock down of the up-regulated or over-expression of the down-regulated transcripts will be performed prior to ERG and PTEN modulation in the 2D model, and the resulting phenotypes analysed. The identified secreted proteins will be tested for tumour promoting phenotypic effects on normal fibroblasts and CAFs isolated from radical prostatectomies in collaboration with the "Molinette" Hospital of the University of Turin.

Financing
The project is financed by PRIN 2017 – Progetti di Interesse Nazionale with 125000€ in three years.

External collaborators
Prof. Valeria Poli, University of Turin, Turin
Prof. Paolo Gontero, University of Turin, Turin
Prof. Andrea Lunardi, University of Trento, Trento
Prof. Licio Collavin, University of Trieste, Trieste
Prof. Marco Gaspari, University of Catanzaro, Catanzaro

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. Valeria Poli at the University of Turin for the -Omics experiments.

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11. Oncology: Phenotype and functional characterization of the immune-landscape of gynaecological cancers: a focus on ovarian and endometrial cancers. PI: Lorenzo MORTARA, lorenzo.mortara@uninsubria.it

Project Title: Phenotype and functional characterization of the immune landscape of gynecological cancers: a focus on endometrial and ovarian cancers

Advanced and recurrent gynecological cancers, including endometrial (ECa) and ovarian (OvCa) cancers associated with poor prognosis and lack of effective treatment. ECa and OvCa accounts among the most common cancer in the female genital tract in developed countries, and with its increasing incidence due to risk factors, such as aging and obesity, tends to become a public health issue. Despite progress in therapy improvements patients still develop a recurrence after first-line treatments, dependent on the tumor and non-tumor complexity/heterogeneity of the neoplasms and its surrounding tumor

microenvironment (TME). The TME has gained greater attention in the design of specific therapies within the new era of immunotherapy. It is well known that environment composition at the maternal-fetal interface parallels with pro-tumoral microenvironments (TME) identified in many cancers, that shares immunesuppression/tolerogenesis and angiogenesis as hallmarks.

Base on this evidence and on the expertise of our group in investigating the similarities between immune cells in the decidua and cancer, the candidate will be involved in the investigation of immune cell subset frequency (monocytes/macrophages, Natural Killer (NK) cells, myeloid-derived suppressor cells, and T cells), activation/polarization state and pattern in clinical samples (peripheral blood and tumor tissues) of patients with ECa and OvCa. Also, the interaction of the characterized and isolated immune cells (with a focus on NK cells) with different non-immunological components of the TME, including endothelial cells, fibroblasts and tumor cells will be investigated using 2D and 3D *in vitro* models.
The candidate will be also involved in *in vivo* studies, to translate the major insights from the clinical samples, using murine models recapitulating the cancer type of interests.

The candidate will have the possibility to acquire strong expertise in the phenotype and functional characterization of the immune landscape in cancer patients and murine models, by multicolor (up to 20 parameters) flow cytometry, cell sorting, and investigation of cell-to-cell interactions by different cellular, molecular and biochemical approaches.

The project will be run within a collaboration by the University of Insubria with IRCCS MultiMedica, Milano and the Istituti Fisioterapici Ospitalieri - Istituto Regina Elena, Roma.

The project will be run in the Laboratory of Immunology and General Pathology, University of Insubria, under the supervision of Prof. Lorenzo Mortara in collaboration with Dr. Matteo Gallazzi, and Dr. Antonino Bruno, Laboratory of Immunology, IRCSS MultiMedica, via Fantoli 16, Milano.

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12. Oncology: New molecular diagnostic approaches to study leukemic stem cells and their role in the pathogenesis of chronic myeloid leukemia. PI: Giovanni PORTA, giovanni.porta@uninsubria.it

Titolo del progetto:
LA LEUCEMIA MIELOIDE CRONICA : modelli patogenetici in vivo, in vitro e in silico per nuovi approcci diagnostici e terapeutici.

La leucemia mieloide cronica (CML, Chronic Myeloid Leukemia) rappresenta il 20% delle leucemie nell'adulto e origina, nel 95% dei casi, da una traslocazione reciproca bilanciata tra i cromosomi 9 e 22 (t(9;22)(q34:q11)) all'interno di una cellula staminale pluripotente, con conseguente formazione del cromosoma Philadelphia (Ph) e del gene di fusione BCR-ABL1, la cui attività tirosin-chinasica costitutiva è responsabile dell'aumento di proliferazione, resistenza all'apoptosi, invasività e comparsa di metastasi.
La diagnosi di CML richiede l'identificazione di blasti nel sangue periferico e del cromosoma Philadelphia tramite analisi citogenetica del cariotipo o Fluorescent In Situ Hybridization (FISH), metodiche dirette ma con sensibilità ridotta.
La terapia di prima linea è costituita dall'Imatinib mesilato (Gleevec/Glivec, Novartis Pharma), uno specifico inibitore delle proteine tirosin-chinasiche, che riduce la proliferazione delle cellule leucemiche. Di contro, è necessario assumere questo farmaco a vita e monitorare periodicamente, mediante determinazione della malattia minima residua (MMR), la quantità di trascritto BCR-ABL1, per valutare la risposta al farmaco e controllare che non ci sia una ricaduta della malattia.

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 durante il trattamento. I risultati delle analisi con questa metodica sono espressi come il rapporto tra il numero di trascritti di BCR-ABL1 e i trascritti di un gene controllo.
Analizzando il mRNA, questa tecnica è limitata dall'efficienza di estrazione, di retrotrascrizione ed alla qualità dell'RNA estratto. Inoltre, è stata riscontrata l'assenza di una correlazione diretta tra i livelli di mRNA ed il numero di cellule leucemiche: le cellule potrebbero trascrivere sia elevate quantità che essere 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, o se sono presenti cellule leucemiche trascrizionalmente silenti.
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 di BCR-ABL1. Ogni paziente affetto da LMC presenta un punto di rottura unico, con una sequenza di fusione specifica: il preciso punto di rottura costituisce un marcatore tumorale paziente specifico che consente il monitoraggio diretto della MMR durante la terapia, poiché ogni cellula leucemica possiede un cromosoma Philadelphia. La tecnica da noi utilizzata si basa su un arricchimento della regione genomica d'interesse e un successivo "deep sequencing", che permette di identificare la posizione del break-point a livello del singolo nucleotide. 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 campioni di 7 pazienti che non presentavano livelli non misurabili di mRNA chimerico (Fig. 2).

Ad oggi sono stati selezionati, presso l'Ospedale Niguarda di Milano, 87 pazienti candidabili all'interruzione della terapia con Imatinib e Nilotinib, di cui è stato caratterizzato molecolarmente il break-point genomico.

Attualmente stiamo utilizzando i saggi paziente-specifico per monitorare la MMR nei follow-up di questi pazienti.

Stiamo conducendo delle analisi in silico sulle sequenze a cavallo dei breakpoint per valutare la funzione delle sequenze ripetute nella patogenesi dell'aberrazione cromosomica.

La linea cellulare KCL22 è utilizzata come modello in vitro della CML per lo studio della modulazione dell'espressione genica in risposta al trattamento con inibitori delle tirosin chinasi, con particolare attenzione ai geni coinvolti nella differenziazione nella nicchia staminale.

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Project title: CHRONIC MIELOID LEUKEMIA: in vivo, in vitro and in silico pathogenetic model for new diagnostic and therapeutic approches

Chronic Myeloid Leukemia (CML) represent the 20% of leukemia in adults. It originates in 95% of cases from chromosomal aberration, a balanced translocation between chromosomes 9 e 22 (t(9;22)(q34:q11)) in a pluripotent staminal cell, resulting in Philadelphia chromosome (Ph) formation.
The translocation results in the formation of the fusion gene BCR-ABL1, whose constitutive activity promotes proliferation and cellular survivial, due to apoptosis resistance, increased invasiveness and formation of metastasis.
Classical methods to diagnose CML are associated to blood cell counts, evaluating morphology, cytogenetic analysis throughout karyotype and Fluorescent In Situ Hybridization (FISH); these techniques allows the direct identification of Ph chromosome in leukemyc cells but they have low sensitivity.
First line therapy is represented by Imatinib mesylate (Gleevec/Glivec, Novartis Pharma), a specific tyrosine-kinase inhibitor, that lead to proliferation inhibition. The drawback of this therapy is due to lifetime assumption of this drug and continuous monitoring required to follow drug response and eventually identify CML relapse, by monitoring the Minimal Residual Disease (MRD), i.e. the percentage of leukemic cells not eradicated by the therapy.

dDNA-PCR, a new high-sensitivity technique to identify leukemic cells in CML affected patients

qRT-PCR is nowadays the most sensitive technique to monitor BCR-ABL1 mRNA after diagnosis and treatment initiation. Results are expressed as the ratio between BCR-ABL1 and a control gene transcripts. Estraction efficiency, RNA quality and retrotranscription efficiency are the main limits associated with this technique. Moreover, there is no linear correlation between mRNA levels and the number of leukemic cells, i.e. it is possible to have higly transcribing or quiescent cells. Negative results (i.e. no detection of mRNA) are hard to be interpreted, because the absence of chimaeric transcript can be due to effective eradication of leukemic cells or to quiescent cells. For this reason, it is essential to have a way to clearly determin if the leukemia has been eradicated and Imatinib therapy can be stopped.
Currently reccomendations is not to interrupt the therapy, despite considerable costs and collateral effects of chemotherapy.

Our group has developed and validated a new method, based on a quantitative real-time PCR (qRT-PCR) that amplifies the genomic sequence corresponding to the BCR-ABL1 breakpoint. Each CML patient has an unique breakpoint and an unique nucleotide sequence and the precise identification of it results in a tumoral, patient-specific marker that allows us to monitor of MRD during therapy.
The sequences spanning the break-point is now under study for an in silico analysis to evaluate the repetitive sequences function in the pathogenesis for chromosomal aberration..
Molecular analysis of KCL22 cell line have been carried out to build a cellular model of chronic myeloid leukemia for in vitro study of gene expression in cell differentiation in the stem niche

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