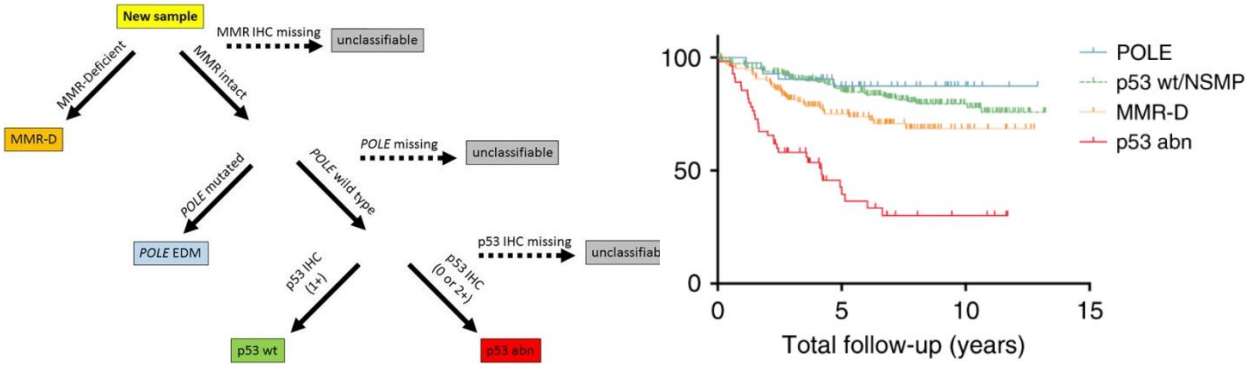
Characterization of early-stage low-grade recurrent endometrial cancer: adoption of the molecular classification to identify patients at higher risk.

Background: Endometrial cancer (EC) is the most common gynecologic malignancy in the developed countries. Approximately 70% of EC cases present at an early-stage of disease, and generally have good prognosis (80% overall 5-year survival). An essential question is how to identify the subset of tumors at a higher risk of recurrence. Currently, clinical and histopathological factors such as stage, histotype, grade, depth of invasion, and lymphovascular space invasion are used to stratify patients into risk groups to guide surgical management and adjuvant therapy. However, these clinicopathological variables do not sufficiently predict patient outcomes. Despite good prognosis for patients with low-risk endometrial cancer, a small subset of women with early-stage low-grade EC experience relapse; indeed, recent reports showed approximately 3% of patients with no identifiable clinical or pathologic risk factors developing recurrence.

DNA sequencing efforts, specifically The Cancer Genome Atlas (TCGA), have resulted in the identification of four prognostically-significant molecular subtypes: copy-number low (CN low), and copy-number high (CN high), microsatellite instability-high (MSI-H, hypermutated), and POLE-mutated (ultramutated).



The genomics-based clinical classifier for endometrial cancer (Talhouk et al., Cancer 2017).

CN low tumors are typically low-grade endometrioid carcinomas, which are defined by low genomic copy number alterations, low tumor mutation burden (TMB); they harbor infrequent mutations in TP53, and frequent mutations in PTEN, PIK3CA, ARID1A, CTNNB1, and KRAS. In contrast, CN high tumors consist of serous carcinomas and some high-grade endometrioid carcinomas. MSI-high tumors, which include low-grade and high-grade endometrioid carcinomas, have mismatch repair (MMR) protein defects superimposed on the mutations seen in CN low tumors. And finally, POLE-mutant tumors are defined by hotspot mutations in the exonuclease domain of DNA polymerase epsilon (POLE). POLE-mutant tumors have dramatically better progression-free survival, regardless of histotype and grade, whereas copy-number high tumors have the poorest outcome. The largest group (copy number low and MSI-high tumors) have an intermediate prognosis.

The objective of the study is to characterize the molecular subtypes of the recurrent early-stage low-grade EC, to define a subset of patients that might benefit from adjuvant treatment and one where clinical follow-up could be omitted.

Objectives: To assess the molecular characterization of apparent early-stage low-grade endometrial cancer that experience recurrence.

Specific aim: To identify the molecular subtypes (according to the genomics-based clinical classifier for endometrial cancer) of stage IA G1 endometrioid endometrial cancer that experience recurrence.

Study design: A propensity match case-control comparative analysis (1:2) between patients who experienced recurrence vs. those with uneventful follow-up (at least three years follow up).

Intervention: Tumor DNA analysis, Proactive Molecular Risk Classifier for Endometrial Cancer (ProMisE), to define the molecular classification of recurrent low-grade EC (case), and uneventful propensity match group (control). Specifically, immunohistochemistry to identify the presence or absence of mismatch repair (MMR) proteins sequencing for polymerase-ε (POLE) exonuclease domain mutations (POLE EDMs), and IHC for tumor protein 53 (p53) (wild type vs. null/missense mutations; p53 wt and p53 abn, respectively).

Study population: Patients who underwent hysterectomy with o without retroperitoneal staging (pelvic +/- para-aortic lymphadenectomy or sentinel lymph node) for apparent early-stage endometrial cancer at Del Ponte Hospital between 01/01/2000 and 31/12/2018.

Inclusion Criteria: Endometrioid histotype; Grading: G1; Myometrial invasion < 50% at final pathology.

Exclusion Criteria: Positive node (either macrometastasis or low volume metastasis) at final pathology

Variables and Outcomes:

Demographic characteristics: age at surgery: continuous and categorized (es: <50; >50); BMI: continuous and categorized (es: <30; >30)

Tumor characteristics: Final histology; Grading; LVSI;

Surgical characteristics: Approach: open; minimally invasive; lymph node status evaluation (lymphadenectomy/sentinel lymph node biopsy)

Adjuvant treatment: External Beam Radiation therapy / Brachytherapy / Chemotherapy

Oncological outcomes: Recurrence; site of recurrence; Death; Disease-free survival (months); Disease-specific survival (months); Overall survivalshs)

ONCOLOGY

PI: Prof. Giovanni PORTA – giovanni.porta@uninsubria.it

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).
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 (MMR), 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).
Ad oggi sono stati selezionati, presso l'Ospedale Niguarda di Milano, 87 pazienti candidati all'interruzione della terapia con Imatinib e Nilotinib di cui è stata fatta la caratterizzazione molecolare del break-point genomico. Successivamente questo marcatore paziente-specifico verrà utilizzato per il monitoraggio della MMR 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.
Project title: NEW MOLECULAR DIAGNOSTIC APPROACHES TO STUDY LEUKEMIC STEM CELLS AND THEIR ROLE IN THE PATHOGENESIS OF CHRONIC MIELOID LEUKEMIA
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 vaalidated 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.
We have characterized the breakpoint sequence of 16 chronic phase CML patient, developing patient-specific gDNA qRT-PCR assays used to analyze TKIs treated follow ups for 5-8 years.
A mathematical formula has also been developed to calculate the Ph+ cells.
Analyzing in parallel our results with those obtained from the standard qRT-PCR on mRNA we demonstrated that our technique identifies the presence of leukemic cells in 7 samples that were negative on mRNA analysis.
Nowadays, 87 candidates to stop TKIs have been characterized on their genomic breakpoint. We will use this patient-specific markers to monitor MRD in follow up.
Our technique is based on a targeted enrichment of the genomic region involved in recombination, followed by "deep sequencing" and identification of the breakpoint at a nucleotide level.
Once obtained the breakpoint localization, MRD will be monitored on follow-up peripheral blood samples.
The aim of this research is to develop a stop-Imatinib protocol through gDNA qRT-PCR monitoring. Each patient will have a patient-specific molecular marker that detect uniquely leukemic cells and that can be used to direcly determine presence/absence of Ph+ cells before stopping the therapy.
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Neurotransmitter transporters in Parkinson's disease

PI: Cristina Roseti

Parkinson's disease (PD) is a chronic progressive neurodegenerative disorder characterized by both motor and non-motor system symptoms. Typical signs of pathological state are loss or degeneration of the dopaminergic neurons in the substantia nigra and development of Lewy Bodies (LBs). Lewy Bodies contain protein's aggregates including α-synuclein: polymorphism involving Synuclein Alpha gene (SNCA), have been associated with increased risk of PD. Over the years, mutations in additional genes, like Parkin (PRKN), PTEN Induced Kinase 1 (PINK1) and Protein Deglycase (DJ-1) have been identified as potential risk factors.
Leucin Rich Repeat Kinase 2 (LRRK2) is the major genetic contributor both in familiar and idiopathic PD.
In particular, studies revealed that LRRK2 p.G2019S mutation appears in approximately the 3-7% of familial PD cases with much higher prevalence in specific populations. Reduction of dopamine levels lead to loss of excitatory/inhibitory balance, resulting in glutamatergic hyper-excitability of output regions of the basal ganglia.
The aim of the project is to evaluate the alteration of excitatory and inhibitory neurotransmission, respectively investigating glutamate and GABA transporters and receptors activity, correlated to the presence of LRRK2 wild-type or LRRK2 G2029S mutant. The function will be investigated mainly by electrophysiology (two electrode voltage clamp) and the expression by immunochemistry methods.
On this point of view, is of particular importance to understand the role of excitatory amino acid transporter (EAAT), acting in the clearance of extracellular glutamate causing hyper-excitability. For this reason, EAATs transporter will be tested in association with LRRK2 in both physiological and pathophysiological conditions.
Electrophysiological studies will be carried out with heterologous co-expression of LRRK2 and EAAT2 in Xenopus Laevis oocytes. Moreover, cell membrane transplantation in Xenopus oocytes derived from brain tissues of PD patients will be also used. Molecular biology techniques will be used to prepare the constructs for the expression of wild-type or mutant cDNA and cRNA for heterologous expression.
The project goal is to understand the basic mechanism in Parkinson disease that correlate LRRK2 and Glutamate transporters, considering that some small-molecule LRRK2 kinase inhibitors has been proved to be neuroprotective in models of PD and that strategies for correcting the effects of mutations in the LRRK2 can be particularly important for treating patients.

2020 PhD Program in Experimental and Translational Medicine

Title of the Project: 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:
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 partial PTEN downregulation.
3. 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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Titolo Progetto: Cardiovascular diseases: Variations and clinical relevance of humoral biomarkers in patients with cardiovascular diseases. PI: Roberto DE PONTI, roberto.deponti@uninsubria.it

ABSTRACT.

Currently cardiovascular diseases (CVDs) continue to be the leading cause of death worldwide. They include a group of disorders of heart and vessels of which cerebrovascular disease, coronary artery disease, heart failure and cardiac arrhythmias are certainly the most frequently encountered1. Over time, various modifiable or non-modifiable risk factors have been identified, as well as some cardiovascular risk scores2,3,4. In this scenario, an increasingly important role has been acquired by humoral biomarkers, defined as "indicators of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention"5. Their measurement has revolutionized the work-up of patients with suspected CVDs, although they cannot provide a conclusive diagnosis on their own, being more useful in risk prediction and in the prognosis assessment. The most interesting aspect is that biomarkers reflect several pathophysiological processes that can occur in CVDs, e.g.:
1. myocardial injury (High-sensitivity Cardiac Troponins);
2. myocardial stress (N-terminal Pro-B-Type Natriuretic Peptide);
3. inflammation (C-reactive protein, interleukin-6, ferritin);
4. atherosclerosis and plaque instability (matrix metalloproteinases, TMAO, oxidized low-density lipoprotein);
5. coagulopathies (d-dimer, fibrinogen)6
Several other biomarkers have been identified over the years, but few of them have made their way to clinical practice due to some inherent limitations in terms of sensitivity and specificity. For this reason, an approach that considers them in an integrated way can make up for this lack and make the difference in the future of the personalized cardiovascular medicine. Moreover, the development of new technologies and the expanding research in the field of biology, biochemistry, and genetics could help identify new pathways, leading to better and more useful way of using biomarkers in clinical practice. Finally, the acute cardiovascular syndrome observed during COVID-19 pandemia may represent a new and interesting model to investigate with the aim of understanding the mechanism of myocardial injury in this new syndrome.
The aim of this research project is to evaluate the above-mentioned biomarkers in several cardiovascular diseases, assessing the correlation between their variations and the relevant clinical presentations in order to clarify their role in predicting risk and/or prognosis in clinical practice. The research methodology used will include meta-analysis of the available evidence and retrospective or prospective single-centre or multicenter data collection.
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Incidence and treatment of multidrug resistant pathogens with the new antimicrobial agents.

PI: Prof. Paolo Grossi

Multidrug resistant (MDR) pathogens are currently one of the major threats worldwide and particularly in italian hospitals. Few new drugs are currently available for the treatment of some Gram-positive and particularly Gram-negatve MDR.

Objective of the project is the study of the incidence of the different MDR pathogens (MRSA, VRE, Carbapenen-resistant enterobacteriaceae and non fermentant Gram-negatives) in the ASST-Sette Laghi Hospital and to evaluate efficacy and safety of the new molecules availble for their treatment.

The project will be performed in collaboration with the Microbiology laboratory in order to identify all relevant pathogens isolated from different clinical samples on order to differentiate colonizers from those responsible of symptomatic infection.

Characterization of the effect of pathological CDKL5 derivatives on microtubule dynamics and neuronal receptor functions

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 mostly been investigated in Cdkl5-KO primary neurons and mice. Such studies converge on a role of CDKL5 in regulating synaptic functions through microtubule dynamics and its control of neuronal receptor expression and composition. Moreover, studies from our and other labs have identified and validated drugs that can rescue various deficits linked to loss of CDKL5. However, it remains completely unknown how the presence of hypo-functional CDKL5 derivatives affect neuronal functions. Such information is mandatory to fully understand the potential of drug-based therapies and those based on the re-expression of functional CDKL5.
Objective. The aims of the project are to:
i) generate cellular / neuronal models expressing selected pathogenic CDKL5 derivates;
ii) characterize the above models for microtubule dynamics and neuronal receptor functioning;
iii) evaluate how selected drugs impact microtubule dynamics and neuronal functions in the presence of pathogenic CDKL5 derivatives.
Research design and methodological approach:
i) Stable cell lines and primary neuronal cultures expressing pathological CDKL5 mutations will be generated through CRISPR/Cas9 mediated genome editing and through viral mediated expression.
ii) Microtubule dynamics and cellular morphology/neuronal maturation, excitatory and inhibitory receptor expression and functioning will be analyzed through live imaging studies, biochemical approaches, and electrophysiological studies.
iii) Selected drugs, which have already been tested in our laboratory (acting on MT and neuronal receptor dynamics), will be tested for their capacity to revert CDKL5-dependent phenotypes.
Interested candidates should have a background in molecular and cellular biology and be willing to work with rodents.
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Neuroscience: The role of the neprilysin mitochondrial protease in the pathogenesis of Parkinson's disease. Mauro Fasano – mauro.fasano@uninsubria.it Tiziana Alberio – tiziana.alberio@uninsubria.it

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. Altered dopamine (DA) homeostasis is recognized as an early event in dopaminergic neurons degeneration. In this frame, we recently demonstrated, using a novel degradomics strategy, that DA imbalance can lead the aberrant activation of mitochondrial proteases. In detail, neprilysin emerged as a hyperactivated protease and we first demonstrated its mitochondrial localization.

The aim of the present proposal is to obtain an in-depth characterization of neprilysin activity within mitochondria. To achieve this aim, the experimental plan will include: i) the investigation of the sub-mitochondrial localization of the neprilysin protease in SH-SY5Y cells, ii) the definition of the complete set of neprilysin protein targets, both mitochondrial and not, in SH-SY5Y cells, iii) the quantification of neprilysin protein targets in conditions of altered DA homeostasis in SH-SY5Y cells, and iv) the verification of the levels of some candidates in PD patients-derived samples. The SH-SY5Y cell line will be used as a cellular model in which altered DA homeostasis can be easily induced and high reproducibility can be obtained. On the other hand, post-mortem brain tissues and peripheral blood mononuclear cells will be employed as patients-derived samples for the verification step of the project.

The PhD student involved in the project will be asked to design and perform experiments and critically interpret the results, to participate to lab meetings and to take part to the scientific dissemination of the results.

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Titolo del progetto: Nuove strategie di vaccinazione antitumorale e immunoterapia applicate al glioblastoma, basate sulla stimolazione ottimale delle cellule T CD4+ specifiche per gli antigeni tumorali

Tutor: Dott.ssa Forlani Greta

Abstract

Studi di vaccinazione tumorale sia preventiva che terapeutica condotti nel nostro laboratorio con modelli sperimentali preclinici, hanno dimostrato che il sistema immunitario, attivato da vaccinazioni con cellule tumorali trasfettate stabilmente con CIITA, il principale regolatore dell'espressione delle molecole di istocompatibilità (MHC) di classe II, è in grado non solo di prevenire lo sviluppo e la crescita del tumore ma, in alcuni casi, di bloccare completamente la progressione di tumori già insediati. La stimolazione persistente delle cellule T helper CD4+ (TH) e la successiva attivazione degli effettori della risposta immunitaria adattativa, primi fra tutti, le cellule T CD8+, costituiscono le basi immunologiche del rigetto. Partendo dalle sopradescritte basi sperimentali abbiamo dimostrato che l'espressione ottimale di molecole MHC-II dipendente da CIITA, può rendere le cellule tumorali dei surrogati di cellule presentanti l'antigene, in grado di presentare, attraverso le neoespresse molecole MHC-II, i loro antigeni tumorali a cellule TH tumore-specifiche, responsabili dell'induzione di una risposta immunitaria anti-tumorale. In questo contesto vogliamo proporre un programma di ricerca che, a partire dal modello animale, sia finalizzato a investigare la fattibilità del nostro approccio sperimentale nell'ambito del glioblastoma (GMB), considerato il tumore più maligno del sistema nervoso centrale (SNC), e per cui, a oggi, non sono presenti strategie terapeutiche risolutive. Inoltre, è sempre più evidente che, per indurre una risposta antitumorale specifica e duratura nel tempo, sia necessario coadiuvare le strategie immunologiche sopra descritte con nuovi approcci terapeutici basati sulla conoscenza profonda dei meccanismi alla base dell'innesco e del mantenimento della risposta antitumorale. Il carattere protettivo di tali vaccini sarà quindi testato in somministrazione singola o in associazione con altri trattamenti anti-tumorali, quali gli inibitori degli "immune checkpoints" e i virus oncolitici, che sembrano promettenti strumenti terapeutici nella cura del glioblastoma e possono essere applicati ad altri tipi di tumore.
Title: Construction of anti-glioblastoma vaccines by increasing recognition and persistence of CD4 T cells for tumor antigens
2
ABSTRACT
Although relevant progress has been made in immunotherapy of cancer by the use of immune checkpoint inhibitors and in part therapeutic vaccines, unfortunately both approaches work poorly for glioblastoma, the most malignant tumor of central nervous system. In particular, vaccination strategies using MHC class I-bound tumor-specific peptides have encountered critical difficulties due to the limited effect of these vaccines in stimulating and maintaining MHC class-I restricted tumor specific CD8+ effector cells (CTL). Our working hypothesis prioritizes instead the triggering of tumor-specific MHC class II (MHC-II)-restricted CD4+ T helper (TH) cells, as these cells, hierarchically, are fundamental to both initiate all adaptive immune responses and maintain the proliferation and cytolytic activity of CTL, the terminal effectors of anti-tumor immunity. Thus, in our view, without a strong and persistent activation of tumor specific TH cells, the success of immunotherapeutic vaccination approaches for cancer will be limited. This will be attempted by transferring the MHC-II transactivator (CIITA) discovered in our laboratory, into glioblastoma cells. Our major aim is to construct an operating framework that will lead us to apply the resulting knowledge to clinical setting for a better approach of therapeutic vaccination and treatment of

glioblastoma. CIITA will be expressed in murine GL261 glioblastoma cells which then will be used to assess in vivo their immunogenicity and vaccination potential with respect to the parental tumor. Approaches of intratumor injections in established GL261 tumors with adenoviral vectors containing CIITA, in association with oncolytic viral vectors, will be carried out to assess the immunotherapeutic potential of MHC-II induction in established tumors and the synergy in stimulating TH cells by virus-induced cell death. Finally, stably CIITA-transfected human gliobastoma cell lines will be produced to investigate the MHC class II-bound tumor peptidome derived directly from tumor cells. The murine model will establish the immunogenicity and the immunotherapeutic potential of CIITA-dependent MHC class II-expressing glioblastoma cells as a consequence of optimal stimulators of the hierarchically important TH cells. The use of CIITA-containing vectors could give additional options to increase the anti-tumor immune response and therapeutically treat established tumors. The study of the MHC-II-bound peptidome of human glioblastoma cell lines will assess and possibly define the potential best candidates for a future anti-human glioblastoma vaccine. The cumulative results of our combined approaches will provide new knowledge on how to increase and sustain the adaptive immune response against glioblastoma and hopefully offer novel therapeutical tools to improve the treatment of gliobastoma in clinical settings.

Role of vascular lymphatic drainage in extracellular cardiac tissue remodelling and function

PIs: Daniela NEGRINI and Andrea MORIONDO

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, drainage the lymphatic drainage guarantees the maintenance of the steady state volume and solute concentration of the interstitial fluid 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 oedema, altered interstitial fluid solute concentration, deposition of tissue debris and acute and/or chronic inflammation. As a results, lymph flow obstruction leads to an abnormal fibrous tissue deposition and remodelling, 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 oedema, intracellular oedema, myofibrillar and mitochondrial degeneration. In addition, lymphatic inefficiency and local oedema seem to be strictly associated with conduction disturbances and cardiac arrhythmias. Such an observation is particularly interesting on considering that lymphatic smooth muscle cells express and are activated through 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 unknown relationship between lymphatic smooth muscles and cardiomyocytes function. In particular we aim to 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 remodelling and, as a consequence, altered electrical disturbances of cardiac conductive fibres or, vice-versa, 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 aim at investigating:

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

TITLE: The role of epigenetics in nanotoxicity

TUTOR: Dr. Roberto Papait

ABSTRACT:

Nanoparticles (NPs) are particles characterized by having a size in a 1 – 100 nm range, and in the past 20 years have obtained increasing attention in the biomedical field due to their vast potential and therapeutic applications: NPs are used as vectors for drug delivery, hyperthermia, photoablation therapy, bioimaging and biosensors. Their increasing use makes it essential to define the risk assessment of these nanomaterials. Epigenetics is a complex network of mechanisms that controls gene expression in a potentially heritable way, but without altering the primary nucleotide sequence. These mechanisms regulate gene expression by modulating chromatin structure and DNA-based biological processes, such as the binding of transcription factors to promoters and transcription elongation. Epigenetic dysregulation causes several diseases such as cancer, neuronal disorders and cardiovascular diseases. Recent reports suggest that nanomaterials could cause alteration of epigenetic mechanisms. As an example, ZnO NPs cause a hypomethylation of LINEs, a class of mobile genetic elements that play a key role in maintaining genomic stability and correct gene expression programs. In addition, it has been shown that, carbon nanotubes (CNTs) and AuNPs impact on the levels of DNA methylation, and thus on the relative expression of important genes of DNA repair, e.g., ATM (ataxia telangiectasia mutated) and trp53 (tumor protein p53). Despite these studies, nanotoxicity at epigenetic level remains largely unknown. Thus, the overall scientific objective of this project is to define this aspect through the study of the effects of iron NPs on epigenetic mechanisms. To this end, the aims of this project are as follows:

- Aim 1: Define the impact of several types of Fe NPs on the genomic distribution of canonical DNA and histone modifications, and on the expression of long non-coding RNAs, through genome wide approaches, which combine techniques used for the study of the epigenome (e.g., ChIP-seq and MeDIP) with those employed for the study of transcriptomics (RNA-seq).
- Aim 2: Identify and characterize the molecular pathways through which Fe NPs promote epigenetic changes through the identification epigenetic enzymes and the modulator on which these nanoparticles act.

This project findings will contribute to clarify the underlying mechanisms of nanotoxicity. Moreover, they will allow to identify new tools, based on the analysis of epigenetic parameters, capable to monitoring nanotoxicity.

Neuroscience:

Role of the endocannabinoid system in adolescent brain development in male rats.

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Epidemiological data provide evidence that intense exposure to Cannabis during adolescence, especially to the strains with high THC content, is an important contributing factor to psychiatric vulnerability. However, the molecular mechanisms underlying this association are still poorly understood. During adolescence, the brain undergoes intensive processes of neuronal refinement, especially in cortical regions, resulting in a thinning of the gray matter and an increase of white matter. Derangements from these developmental trajectories could confer a vulnerability to psychiatric disorders. The endocannabinoid system (ECS), the molecular target of THC, the psychoactive component of Cannabis, is an important neuromodulatory system involved in the modulation of synaptic plasticity mechanisms. However, its role in events relevant for the adolescent brain remodeling still needs to be clarified. Results obtained in our laboratory suggest that adolescent THC exposure in female rats deeply changes neuronal refinement, altering the expression of proteins involved in synaptic plasticity and brain functionality. Moreover, 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, at least in female animals.

On these bases, the aim of this study is to investigate the impact of ECS modulation on markers of synaptic plasticity (i.e. PSD95, AMPA and NMDA subunits) and myelination (MOG and MBP), during the adolescent window in male rats, to compare the results with the ones obtained in female animals. To dissect precisely the dynamic of the involvement of the ECS in neuronal refinement and myelination processes occurring in the prefrontal cortex of adolescent rats, we will modulate the endocannabinoid tone through the administration of specific drugs acting on the ECS components. 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 (CB1 receptors, AEA and 2-AG) during adolescent brain maturation in male rats. Moreover, we will also understand the impact of EC tone disruption in triggering brain vulnerability to psychiatric conditions.

Characterization of early-stage low-grade recurrent endometrial cancer: adoption of the molecular classification to identify patients at higher risk.

Background: Endometrial cancer (EC) is the most common gynecologic malignancy in the developed countries. Approximately 70% of EC cases present at an early-stage of disease, and generally have good prognosis (80% overall 5-year survival). An essential question is how to identify the subset of tumors at a higher risk of recurrence. Currently, clinical and histopathological factors such as stage, histotype, grade, depth of invasion, and lymphovascular space invasion are used to stratify patients into risk groups to guide surgical management and adjuvant therapy. However, these clinicopathological variables do not sufficiently predict patient outcomes. Despite good prognosis for patients with low-risk endometrial cancer, a small subset of women with early-stage low-grade EC experience relapse; indeed, recent reports showed approximately 3% of patients with no identifiable clinical or pathologic risk factors developing recurrence.

DNA sequencing efforts, specifically The Cancer Genome Atlas (TCGA), have resulted in the identification of four prognostically-significant molecular subtypes: copy-number low (CN low), and copy-number high (CN high), microsatellite instability-high (MSI-H, hypermutated), and POLE-mutated (ultramutated).

The genomics-based clinical classifier for endometrial cancer (Talhouk et al., Cancer 2017).

CN low tumors are typically low-grade endometrioid carcinomas, which are defined by low genomic copy number alterations, low tumor mutation burden (TMB); they harbor infrequent mutations in TP53, and frequent mutations in PTEN, PIK3CA, ARID1A, CTNNB1, and KRAS. In contrast, CN high tumors consist of serous carcinomas and some high-grade endometrioid carcinomas. MSI-high tumors, which include low-grade and high-grade endometrioid carcinomas, have mismatch repair (MMR) protein defects superimposed on the mutations seen in CN low tumors. And finally, POLE-mutant tumors are defined by hotspot mutations in the exonuclease domain of DNA polymerase epsilon (POLE). POLE-mutant tumors have dramatically better progression-free survival, regardless of histotype
and grade, whereas copy-number high tumors have the poorest outcome. The largest
group (copy number low and MSI-high tumors) have an intermediate prognosis.

The objective of the study is to characterize the molecular subtypes of the recurrent earlystage
low-grade EC, to define a subset of patients that might benefit from adjuvant
treatment and one where clinical follow-up could be omitted.

Objectives: To assess the molecular characterization of apparent early-stage low-grade
endometrial cancer that experience recurrence.

Specific aim: To identify the molecular subtypes (according to the genomics-based clinical
classifier for endometrial cancer) of stage IA G1 endometrioid endometrial cancer that experience recurrence.

Study design: A propensity match case-control comparative analysis (1:2) between patients who experienced recurrence vs. those with uneventful follow-up (at least three years follow up).

Intervention: Tumor DNA analysis, Proactive Molecular Risk Classifier for Endometrial Cancer (ProMisE), to define the molecular classification of recurrent low-grade EC (case), and uneventful propensity match group (control). Specifically, immunohistochemistry to identify the presence or absence of mismatch repair (MMR) proteins sequencing for polymerase-ε (POLE) exonuclease domain mutations (POLE EDMs), and IHC for tumor protein 53 (p53) (wild type vs. null/missense mutations; p53 wt and p53 abn, respectively).

Study population: Patients who underwent hysterectomy with o without retroperitoneal staging (pelvic +/- para-aortic lymphadenectomy or sentinel lymph node) for apparent early-stage

endometrial cancer at Del Ponte Hospital between 01/01/2000 and 31/12/2018.
Inclusion Criteria: Endometrioid histotype; Grading: G1; Myometrial invasion < 50% at final pathology.
Exclusion Criteria: Positive node (either macrometastasis or low volume metastasis) at final pathology
Variables and Outcomes:
Demographic characteristics: age at surgery: continuous and categorized (es: <50; >50); BMI: continuous and categorized (es: <30; >30)
Tumor characteristics: Final histology; Grading; LVSI;
Surgical characteristics: Approach: open; minimally invasive; lymph node status evaluation (lymphadenectomy/sentinel lymph node biopsy)
Adjuvant treatment: External Beam Radiation therapy / Brachytherapy / Chemotherapy
Oncological outcomes: Recurrence; site of recurrence; Death; Disease-free survival (months); Disease-specific survival (months); Overall survivalshs)