## PhD Program on Experimental Medicine and Medical Biotechnologies
### XXXIII cycle

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**Background**

Multiple myeloma (MM) is an incurable malignancy of bone marrow (BM) plasma cells (PCs) that accounts for ≈10% of all hematological tumors.\(^1\) MM evolves from a clinically silent premalignant condition termed monoclonal gammopathy of undetermined significance (MGUS).\(^2\) A small subset of patients has an intermediate clinical phenotype between MGUS and MM, and they are referred to as having smoldering multiple myeloma (SMM).\(^3\) SMM is typically asymptomatic. The risk of progression from MGUS or SMM to overt MM is heterogeneous across patients and difficult to predict. Recently, diagnostic criteria for MM, and subsequently therapeutic intervention, have been revised, so to include even asymptomatic patients with certain clinical markers considered indicators of imminent progression.\(^4\) However, there is still an urgent need to identify clinical biomarkers that, during disease course, can accurately and dynamically identify MGUS and SMM patients who are at imminent risk to progress to overt MM. This need is supported by recent evidence suggesting a benefit in survival for high-risk SMM patients when treatment is started before malignant transformation of the disease takes place,\(^5\) consistent with the consensus that the pathway to cure cancer involves treating patients earlier, curing disease causation rather than symptomatology.

The purpose of this project is to evaluate, in patients with high-risk MGUS and SMM, if genetic mutations detected in circulating tumor DNA (ctDNA) at diagnosis or acquired during the course of the disease may represent dynamic biomarkers able to anticipate progression to overt MM.

**Rationale**

Approximately 2% of MGUS and 20% of SMM are at imminent risk of progression to overt MM after diagnosis. Clinical biomarkers accurately identifying them are still lacking.

The genetics of neoplastic PCs plays a role in priming the aggressive behavior of the disease. Although next-generation sequencing (NGS) has identified several gene mutations which may represent both novel prognostic markers and/or therapeutic targets in MM,\(^6\)\(^7\) the mutational landscape of MGUS and SMM and the impact of mutations on disease evolution are still largely unknown. The potential of mutational analysis during diagnostic work-up of PC dyscrasias at pre-malignant stages is restricted by the fact that purification of neoplastic PCs is cumbersome and sometimes unsuccessful due to the paucity of...
neoplastic cells in the BM, and that serial BM sampling is unfeasible in the clinical practice, thus preventing the molecular monitoring of the neoplastic clone and the identification of switch points on which the disease acquires high-risk genetic features. Furthermore, BM aspirate from a single anatomical site fails to capture the entire mutational repertoire of PCs, which can localize into multiple, genetically heterogeneous foci. On these bases, approaches that are complementary to the mutational analysis of BM biopsy are required in MGUS/SMM to gain information on the clinical relevance of mutations and translate them into clinical biomarkers. In this context, the analysis of circulating tumor DNA (ctDNA) is one promising possibility. ctDNA is shed into the peripheral blood (PB) by tumor cells and is representative of the whole tumor heterogeneity. It can be genotyped in sensitive and accurate manner, with the obvious advantage to avoid serial tumor biopsies for real-time monitoring of emerging mutations responsible for disease progression or drug resistance.

Hypotheses, aims and objectives

Our working hypotheses are that: i) mutations or their acquisition during disease course can be developed as dynamic biomarkers that anticipate progression to overt MM in MGUS and SMM patients; ii) ctDNA genotyping can accurately, dynamically and in real-time inform on tumor genetics and its evolution in MGUS and SMM patients.

In order to address our working hypotheses, the project will pursue the following aims:
1. Mutational analysis of MGUS and SMM at presentation by ctDNA genotyping;
2. Correlation of the mutations identified at baseline with clinical course and progression to overt MM;
3. Profiling of clonal evolution in MGUS and SMM by using ctDNA longitudinally collected during disease course;
4. Correlation of switch points in disease genetics evolution with progression to overt MM.

Methods

MGUS and SMM patients applying to the Complex Operative Unit of Hematology of the Fondazione IRCCS Ca’ Policlinico Milan, and staged at high-intermediate risk according to Mayo Clinic risk models will be enrolled in the study.

The mutational profiling will be carried out by means of a targeted sequencing strategy, focusing on a panel of 14 genes allowing the recovery of at least one mutation in \( \approx 70\% \) of patients with PC tumors, as already established in our laboratory in a panel of 30 MM patient at diagnosis. The gene panel will be analyzed in plasma cfDNA collected at baseline and longitudinally during disease course and, for comparative purposes to filter out polymorphisms, in normal genomic DNA from the paired granulocytes as source of germline material (if available). Since virtually all patients with high-risk MGUS and SMM undergo BM examination in clinical practice at diagnosis, whenever available genomic DNA from the paired CD138+ purified PCs from baseline BM aspiration will be also investigated to assess the accuracy.
of plasma cfDNA genotyping. Targeted ultra-deep-NGS will be performed by using the CAPP-seq approach. Multiplexed libraries will be sequenced by Illumina technology in order to obtain >2,000x coverage on >80% of the target region. VarScan2 will be used to call non-synonymous somatic mutations and a stringent bioinformatic pipeline will be applied to filter out sequencing errors. Mutational markers identified at diagnosis and in correspondence with switch points in clonal evolution will be correlated with clinical outcome of the patients.

**Projected achievements**

The research wish to demonstrate that ctDNA represents an accessible source of tumor DNA for the sensitive identification of genetic biomarkers associated with MM progression and that patients’ genotyping on the liquid biopsy is an effective and innovative approach for the early treatment of PC dyscrasias. Specifically, the proposed research will allow to:

- define the mutational landscape of MGUS and SMM;
- identify the mutational events and/or clonal evolution patterns that play a role in priming the aggressive behavior of the disease;
- based on gene mutations, derive molecular models for an accurate and dynamic identification during disease course of MGUS and SMM patients who are at an imminent risk of progression, thus warranting preventive interventions.

**References**

**Best publications**


**Most relevant publications for the project**


**Most recent publications**


Leukemia. 2017 Jan 5. doi: 10.1038/leu.2016.394. PMID: 28053325. [Epub ahead of print]


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<th>Project title</th>
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| Laboratory/Department | Laboratory of Clinical Pathology, Department of Biomedical Sciences for Health.  
|                | http://www.scibis.unimi.it/ecm/home/ricerca/laboratori/laboratorio-patologia-clinica |

**Introduction**

Obesity, mainly visceral obesity, is one of the leading risk factors for cardiovascular diseases (CVDs). Most of the obesity-related complications are known to be due to the ectopic accumulation of fat in tissues different from the adipose one, as liver and heart, a condition that promote organ damage and dysfunction [1].

The receptor for the advanced glycation end products (RAGE) is a multi-ligand molecule belonging to the immunoglobulin family. It has been initially described for its ability to bind the advanced glycation end products (AGEs) and to activate intracellular pathways that promote inflammation and oxidative stress which in turn induce organs damages [2-4]. Besides the cell surface form, RAGE exists also as a soluble plasma molecule (sRAGE) produced by cleavage of the membrane form or alternative splicing. Being a decoy receptor, sRAGE binds AGEs thus preventing their interaction with the membrane form and playing a role as a protective molecule [5]. In physiological conditions, RAGE is expressed at low levels in many different tissues, whereas in pathological conditions, from diabetes mellitus to cancer, kidney disease, sepsis and Alzheimer disease, its levels as strongly up-regulated [6, 7]. Differently, sRAGE levels are reduced in the same pathologies.

In the field of CVDs, many studies suggested that RAGE and its ligands are expressed at high levels in coronary artery disease, heart failure and ischemia. Moreover, RAGE antagonism has been proposed as a potential therapeutic strategy in the conditions [7]. RAGE may also promote adipocyte hypertrophy [8]. Relative to ectopic lipid accumulation, the AGES/RAGE pathway has been associate to hepatic steatosis till to the development of NASH [9,10]. Noidata are instead available relative to heart steatosis.

As obesity plays a major role in the onset and progression of different CVDs and also may promote lipid accumulation within the heart, our hypothesis is that RAGE could be a linker between obesity and obesity-related complications, as heart failure due to heart steatosis.

**Aim**

Aim of the present project is to explore the potential role of RAGE in promoting heart steatosis in obesity, also exploring the molecular mechanisms of such a relationship. This study will support clinical data, mainly of correlative nature obtained in the human [11,12], and may suggest potential targets to be further studied in the prevention/reduction of obesity-related cardiac complications.
**Target**

Our hypothesis is that in the presence of obesity, the accumulation of lipids in the heart may be mediated, at least in part, by RAGE. It is expected to observe an increase in lipid accumulation associated with high levels of cardiac RAGE and a simultaneous decrease in plasma levels of circulating sRAGE. Blood samples and heart tissues isolated from healthy, obese, obese and diabetic rats will be studied in terms of lipid accumulation, RAGE tissue expression, circulating sRAGE levels, activation/modify of specific genes involved in lipid metabolism.

**Materials and Methods**

Male Zucker rats will be used (Ministerial Authorization 325/2015PR of 2015/04/05, lasting 60 months). Rats will be stacked at room temperature (22±2 °C) and humidity (60±5%) constant, with a light-dark cycle of 12 hours each. Animals will have access to libitum to a standard-lipid standard diet (15%). For euthanasia, rats will be anesthetized with zoletyl (1.6 ml/kg) and sacrificed by cervical dislocation. Hearts and plasma samples will be harvested for histological, morphological and molecular studies. The degree of steatosis and the location of lipid accumulation will be evaluated through specific staining and flow citometry. The expression of RAGE will be evaluated both at the gene (Real Time RT-PCR) and protein level (Western Blot and IHC stainig). sRAGE and inflammatory parameters will be quantified by ELISA and multiparametric assays (Luminex Multiplex Assays). A specific PCR array for fatty acid metabolism (84 key genes) will be used to study the genes involved in regulating key enzymatic pathways of fatty acid metabolism.

**Preliminary data**

In two recent publications we described:
- the involvement of RAGE in the relationship between increased cardiac adiposity and metabolic dysfunction (Elena Dozio et al., Journal of Diabetes Research, Journal of Diabetes Research 2016 (2016), Article ID 2327341, 8 pages)
- The role of sRAGE as early cardiometabolic risk biomarker (Elena Dozio et al., Relationship between soluble receptor for advanced glycation end products (sRAGE), body composition and fat distribution in healthy women. of print]).

**Bibliography**


Best publications


Monocyte chemoattractant protein 1: a possible link between visceral adipose tissue-associated inflammation and subclinical echocardiographic abnormalities in uncomplicated obesity. Malavazos AE, Cereda E,

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### Project's Background Information

Cardiovascular (CV) and metabolic diseases are some of the major chronic diseases affecting populations worldwide and are associated with greater risk of disability, morbidity for other severe conditions, like cancer, and mortality (Catapano et al. 2016). The pathophysiology of CV and metabolic diseases is complex and multifactorial and includes a wide set of biomolecular determinants, including dyslipidemia, insulin resistance and visceral adipose dysfunction. Moreover, the current scientific interest in the role of gut microbiota in human health has emphasized its impact not only on gastrointestinal diseases, but also on chronic systemic diseases, like obesity, metabolic syndrome, dyslipidemia and CV diseases (Thushara et al., 2016; Pedersen et al., 2016). These observations are nowadays leading to 1. in-depth studies to evaluate the gut microbiota changes associated with CV and metabolic diseases, and 2. identification of potential approaches to correct such deranged gut microbiota by specific probiotics, prebiotics and postbiotics (Thushara et al., 2016; Yoo et al., 2016)). Interestingly, this laboratory has recently completed a clinical study with a probiotic (Bifidobacterium longum BB536; Al-Sheraji et al., 2015) in subjects with moderate hypecholesterolemia, observing a significant -26% reduction of circulating LDL cholesterol (Macchi et al., 2017). However, although some clinical data support this nutraceutical approach, more basic/translational research is needed to better clarify the details of the changes of gut microbiota in CV and metabolic diseases and to test potential novel treatment approaches.

### Project's Specific Goals

**The model system.** The zebrafish (*Danio Rerio*) is a well established vertebrate model system useful for the study of metabolically healthy and unhealthy obesity (Landgraf et al., 2017) as well as of dyslipidemia and atherosclerosis (Schlegel, 2016). Moreover, preliminary research started the study of zebrafish microbiome (Borrilli et al., 2016) as well as of the potential effects of the probiotic *Lactobacillus rhamnosus* on its glucose metabolism and obese phenotype (Falcinelli et al., 2017 and 2016).

**The specific goals.** To this aim, we propose to utilize the zebrafish model to:
1. evaluate the gut microbiota changes after inducing different patterns of dyslipidemia and obesity;
2. evaluate the effectiveness of treatment with specific probiotics in these pathological conditions.

**Research plan**

The Research plan will include the following 2 parts and the related tasks.

**Part A**
1. Set-up and phenotypical/genotypical characterization of a hypercholesterolemic/pro-atherogenic model of zebrafish
2. Analysis of gut microbiome changes (next generation sequencing)
3. Treatment with selected single or mix of specific probiotics; evaluation of the molecular and phenotypical effects of this treatment.

**Part B**
1. Set-up and phenotypical/genotypical characterization of a metabolically healthy and unhealthy obesity model of zebrafish
2. Analysis of gut microbiome changes (next generation sequencing)
3. Treatment with selected single or mix of specific probiotics; evaluation of the molecular and phenotypical effects of this treatment.

**Expected outcomes**

The project is expected to shed light on the gut microbiome changes associated to hypercholesterolemia/atherosclerosis and obesity with different degrees of severity, potentially identifying some specific probiotics that may be useful to counteract such diseases in humans.

**References**


Landgraf K et al. Short-term overfeeding of zebrafish with normal or high-fat diet as a model for the development of metabolically healthy versus unhealthy obesity. BMC Physiol. 2017 Mar 21;17(1):4.


Most relevant publications for the project


Chiara Pavanello, Carmen Lammi, Massimiliano Ruscica, Raffaella Bosisio, Giuliana Mombelli, Chiara Zanoni, Laura Calabresi. Cesare R.
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**Project title**: Developmental of zebrafish models for the study of congenital defects of the central regulation of reproduction (Congenital Hypogonadotropic Hypogonadism)

**Tutor**: Prof Luca Persani

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**Laboratory/Department**: Lab of Endocrine and Metabolic Research, Dept of Clinical Sciences and Community Health (c/o IRCCS Istituto Auxologico Italiano)

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**Project**

Congenital Hypogonadotropic Hypogonadism (CHH) is a largely unexplained disorder with a strong genetic component. More than 20 genes have been associated with its pathogenesis but the role played by these genes in the GnRH neuron development and activity as well as the mechanisms underlying the genetic variations are still elusive. The proposed project aims to take advantage of the zebrafish model to elucidate the role of some of these genes and the mechanisms underlying the genetic variations identified in the largest cohort of CHH patients ever collected worldwide (Bonomi, EJE 2017).

In recent years, the Lab under my supervision developed a strategy to clarify the pathogenesis of several endocrine diseases of genetic origin using several reporter zebrafish lines and techniques of genetic knock-down, such as the morpholinos and/or mutant fish lines created either by CRISPR/CAS9 or TALEN approach (Marelli et al, Thyroid 2017 or Mol Cell Endo 2016; Porazzi et al, Endocrinology 2012; Bassi et al, 2016; Vitale et al, Endo Rel Cancer 2014). Recently, we obtained the PROKR1b mutant fish, and we crossed it with the GnRH3 reporter line (in which the GnRH neurons are fluorescent). Here, I propose to exploit this zebrafish model in order to investigate:

- the phenotype of this mutant line, focusing on the reproductive defects and to understand the level of damage of the GnRH neurons (differentiation, migration, branching, GnRH synthesis and secretion);
- verify the pathogenic role of the PROKR2 mutations identified in the patients in rescue experiments of the phenotype of PROR1b-KO;
- possibly verify the role of novel genes that will be associated with CHH by Next-Generation-Sequencing approach in the families without mutations in the presently known candidate genes. We recently selected 15 families with such characteristics.

For the latter approach a stage of 4-6 months in a Lab abroad could also be organized.

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**Best publications**


**Project title**
HDAC8 and cohesins: "omics" analyses for the identification and functional validation of their targets using zebrafish (*Danio rerio*) and in vitro model systems.

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**Project**

**BACKGROUND AND RATIONALE:**
Recently, mutations in cohesins (RAD21, *SMC1A*, *SMC3*, *STAG2* and *NIPBL*) have been found in human myeloid malignancies such as in Acute Myeloid Leukemia (AML) (Mullenders et al., 2015). Mutations in members of the cohesin complex predominantly are frame-shift, missense and nonsense mutations all resulting in reduced or altered function of the affected protein (Xu et al., 2010). *Rad21*, *Smc1a*, *Stag2* and *Smc3* knockdown mouse models have been generated, showing altered stem cell homeostasis and myelopoiesis and, over time, a clinical picture similar to AML (Mullenders et al., 2015; Viny et al, 2015). Conversely, *HDAC8*, another member of the cohesin complex, has been demonstrated to exert the opposite function on AML progression in a specific subset of patients with the inversion of chromosome 16 (Qi et al., 2015). The inversion generates an aberrant fusion protein CMFβSMMHC (CM) that interacts with p53 and HDAC8. The activity of p53 was inhibited by HDAC8 as p53 remains in the deacetylated form and hematopoietic stem cells do not undergo apoptosis. HDAC8 inhibition, restores p53 acetylation and activity, allowing apoptosis specifically in inv(16) + CD34+ AML cells and abrogating AML propagation (Qi et al., 2015). Understanding the mechanism by which cohesins function in the balance between hematopoietic stem cell proliferation and differentiation toward myeloid compound, could aid in the identification of new interactors and in the development of new drugs specifically targeting cohesin haploinsufficient cells.

**PRELIMINARY RESULTS:**
Our previously described zebrafish models for *NIPBL* and *SMC1A* haploinsufficiency (Pistocchi et al., 2013, Fazio et al., 2016) have been investigated also for expression of specific hematopoietic transcription factors. The knockdown of *nipbl* and *smc1a* in zebrafish induces a left shift in myeloid precursors, suggesting that we created a suitable model to study the effects of cohesin haploinsufficiency in hematopoiesis and leukemia insurgence. We generated and characterized an *hdac8* new model for cohesin haploinsufficiency, with the injection of a specific antisense oligonucleotide morpholino (MO), and using HDAC8 inhibitors as PCI-34051 (Balasubramaian et al., 2008). In parallel, we have cloned the full length zebrafish *hdac8* mRNA and we are phenotypically and molecularly characterizing the overexpressed embryos.

**EXPERIMENTAL DESIGN:**
**Task 1**: Analysis of dysregulated expression of hematopoietic genes in zebrafish models for cohesin haploinsufficiency (nipblb-MO and smc1a-MO injected embryos). The whole transcriptional profile of hematopoietic stem cells HSCs sorted from the transgenic zebrafish line Tg(CD41:GFP) with nipblb and smc1a haploinsufficiency and controls, will be analysed with RNAseq techniques in collaboration with Prof. Cristina Battaglia (BIOMETRA Dept., Unimi). Bioinformatics analysis will be pursued in collaboration with Silvio Bicciato (University of Modena and Reggio).

**Task 2**: Analysis of dysregulated hematopoiesis and modifications in the HSCs acetylation profile of a zebrafish model with hdac8 haploinsufficiency or overexpression. We will use the zebrafish model for inhibition and overexpression of hdac8 to analyse post-translational modifications. The acetylation profile of HSCs sorted from the Tg(CD41:GFP) will be analysed with cell mass spectrometry in collaboration with Dr. Capitanio and Prof. Gelfi (Dept. Scienze Biomediche per la Salute, Unimi). Immunoblotting will be used to validate the levels of specific proteins. Proteomics approach will be supported by bioinformatic pathway analysis in order to obtain statistical information about the signalling pathway and biological networks in which differentially expressed proteins are involved.

**Task 3**: Analyses of cohesins dysregulated expression by qPCR experiments in blood samples derived from patients with myeloid malignancies.

**Task 4**: Pharmacological treatment of the promyelocytic HL-60 cell line with dysregulated cohesin expression. The expression of cohesins will be silenced with shRNA and the entire coding sequence of human HDAC8 will be inserted into a pcDNA3 expression vector to overexpress it.

**Best publications**


### Most relevant publications for the project


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<th>Project Title</th>
<th>WNT signaling inhibitors as promising diagnostic and prognostic serum markers of bone metastasis</th>
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| Tutor         | Emanuela Rita Galliera  
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**Background and rationale**

Bone turnover markers have been recently shown to be useful for the early diagnosis and monitoring of bone metastases [1,2], but the panel of diagnostic tools for metastasis detection still need to be improved. Recently, morphogenetic Wnt pathway have been described as an emerging pathway having a crucial role in bone carcinogenesis and metastases progression [3,4]. Wnt signaling have been shown not only to promote tumorigenesis, enhancing tumor growth and survival, but in particular to induce metastasis onset and progression in bone tissue [5-7]. In particular, Wnt signaling stimulate osteoblasts activity, leading to bone formation, while the endogenous negative regulators of Wnt signaling, Sclerostin and DKK-1, block osteoblasts activity and induce osteoclasts mediated bone resorption, leading to bone erosion [8-10].

**Hypothesis:**

We will test the hypothesis that the two main Wnt inhibitors, Sclerostin and DKK-1, display a good diagnostic and prognostic potential, in order to be applied to the routine their clinical use and, therefore, to improve the detection and the monitoring the follow up and the life expectation of patients displaying bone metastases.

The research will be performed in collaboration with Oncology Unit of the Hospital Policlinico san Donato (Dan Donato, Milan, Italy), composed by Dr. Domenico De Toma, Chief of Oncology Units, and Professor in the School of Medicine of Milan University and its staff. The Oncology Unit of the Hospital Policlinico san Donato is a keynote landmark for oncologic patients in Milan area, managing a large case number of tumor patients every year, both in emergency and in routine treatment of bone metastasis.

**Aims:**

- We will evaluate the potential diagnostic role of two main inhibitors of Wnt signaling, DKK1 and Sclerostin, in bone metastasis, originating from different kind of tumors. In order to evaluate the diagnostic potential of these two molecules, canonical marker of bone resorption and formation, as well as the fracture risk marker FGF23 will be measured and correlated with the serum level of DKK1 and Sclerostin.
• The amount of serum DKK1 and Sclerostin will be also compared to the number of metastatic sites in order to correlate the value of DKK1 and Sclerostin to metastatic spread.
• In order to define also the prognostic role of DKK1 and Sclerostin, these two markers will be evaluate in metastatic patients at different time point during metastases clinical monitoring.
• This approach would underline the clinical significance of these markers in their ability not only to identify metastatic patients from oncologic- non metastatic patients, but also to provide a new and not invasive tool to monitor the onset and the progression of bone metastases.

Experimental Design:
So far these WNT inhibitors have been examined mainly as new potential therapeutic targets [11], and there is little evidence suggesting their use as a diagnostic tool in cancer [12]. DKK1 and Sclerostin will be evaluated in a group of oncologic patients displaying bone metastases, as assessed by clinical evaluation, and compared to oncologic non metastatic patients. The amount of serum DKK1 and Sclerostin will be also compared to the number of metastatic sites in order to correlate the value of DKK1 and Sclerostin to metastatic spread.

We will also perform a longitudinal study, by measuring serum level of Sclerostin and DKK-1 in patients presenting bone metastasis at sequential time points (T0 : enrollment, T1: 6 month and T2: 12 months) according to clinical monitoring and treatment of bone metastases, correlating them to a panel of established metastatic bone turnover and metastasis markers, such as Survivin. This study will provide information about the prognostic value of DKK1 and Sclerostin and their ability to monitor the therapy effect on bone metabolism.

Since the metastases analyzed can be osteolytic or osteoblastic, in order to evaluate bone resorption and formation we will measure serum levels of TRAP5b and the two main bone-related metalloproteinases MMP-2 and MMP-9, as marker of bone resorption, and bone specific alkaline phosphatase (BAP) , osteocalcin (OC) as marker of bone formation, and FGF23 as marker of bone mineral metabolism and fracture risk.

The aim of the study is to assess the diagnostic potential of the two main Wnt inhibitors, Sclerostin and DKK-1, with a view to their clinical use to improve the detection and the monitoring the follow up and the life expectation of patients with bone metastases.

Expected results and relevance in the context of the state of the art
Since preliminary data of our group had already showed the diagnostic potential of Sclerostin on a small number of metastatic patients, the expected results on a larger population of metastatic patients displaying different primary tumors are a stronger correlation of Sclerostin and DKK-1 serum level with metastatic onset, in order to provide a reliable, accurate and non invasive in vitro diagnostic tool specifically tailored to the diagnosis of bone metastasis. In addition, the correlation of Sclerostin and DKK-1 serum level with bone
metastatic sites, which is a key factor in prognosis and therapy, will provide the diagnostic potential of these two biomarker not only to detect but also to quantify the metastatic spread. Moreover, the longitudinal study will show the prognostic value of these two markers, in order to improve the monitoring of the metastatic progression, providing a new panel of metastatic biomarkers.

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Best publications

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The rationale for this project stems from our previous works on the relevance of immune cells and their networks in human cancer (1-5). In the tumor microenvironment, immune cells, particularly macrophages, are appreciated for their plasticity and there is a strong interest on the environmental cues that can modulate their function in cancer. Also, recent works in the group have lead to appreciate how tissue distribution of immune cells in tumor tissues is an important feature that should be accurately evaluated to understand their function (3-5).

In this project we aim at exploring the biology of macrophages in cancer, by integrating molecular analyses to histopathological examinations.

Specifically, the project will develop along two main lines:

**Definition of macrophage immune networks and their prognostic function in human cancer.** Our long-standing collaboration with surgeons, clinicians, and pathologists grants us availability of paraffin-embedded tumor specimens to investigate immune networks in cancer patients. Macrophages (CD68+ cells) and other immune cells or tissue structures will be identified by IHC and their interactions will be analyzed by image segmentation algorithms (Matlab). The data obtained will be used to generate scores and categorize patients, to assess the prognostic value of the variables analyzed. The aim is to define whether specific immune signatures are associated to prognosis or response to therapy in cancer patients.

**Exploring macrophage immune networks in cancer preclinical models.** To mechanistically investigate the key networks identified as clinically important in human cancer, we will generate murine preclinical models of colorectal, pancreatic or lung cancer. The spatial and functional interaction between macrophages and other immune cells will be investigated by different experimental approaches, including gene targeting, whole-tissue imaging, in vivo imaging, multicolor flow cytometry, cells sorting and next generation sequencing, to reach a deep level of understanding of the molecular networks characterizing the biology of tumor-associated macrophages in vivo.

**References**

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iron release from osteoclasts in the pathogenesis of osteoporosis

Project title

Iron is an essential nutrient that is indispensable for various functions, such as oxygen transport, cell respiration and DNA synthesis. Both iron deficiency and iron excess can cause detrimental effects on cells and organ systems: in the former case, cell growth arrest and death; in the latter, toxicity due to an increase in reactive oxygen species (ROS), ultimately causing cell and tissue damage. Because of its dual role, iron level is tightly controlled by means of a network of proteins regulating its absorption, storage, recycling, and utilization both at the systemic and cellular level (1). One of the major proteins acting in iron homeostasis is ferroportin (Fpn), the sole cellular iron exporter present on the cell plasma membrane. Its binding to the liver-derived peptide hepcidin induces Fpn internalization and degradation, thereby inhibiting iron release into the plasma. This leads to retention of cellular iron and decreased levels of circulating iron. Alterations in these processes causes a variety of disorders associated with iron-deficiency or overload. The project focuses on osteoporosis, a multifactorial metabolic bone disease which affects mainly women, particularly during aging; since the elderly population is continuously increasing, the burden of the disease constitutes a global public health problem (2). The tight relationship between iron excess and osteoporosis is strongly supported by the association of bone loss and fractures with disorders characterized by iron overload, such as thalassemia and hereditary hemochromatosis. At present, convincing data indicate that increased osteoclasts activity may underlie the accelerated bone degradation observed in the presence of excess iron (3,4). The general purpose of the project is to understand the role of iron accumulation in cells involved in bone remodeling using proper animal models with iron overload localized to osteoclasts. To this end, in our project we will use the Fpn\textsuperscript{fl/fl};lysM-Cre mouse model recently developed in our laboratory, in which the Fpn gene is specifically inactivated in cells of the myeloid lineage, hence also in osteoclasts starting from their early progenitors. These mice will present an iron overload specifically in myeloid cells, and in particular in the osteoclasts for what pertains to the bone environment. The first aim of the project is to use histological and histomorphometric techniques in order to perform a careful characterization of the bone phenotype in Fpn\textsuperscript{fl/fl};lysM-Cre mice as compared to Fpn\textsuperscript{fl/fl} littermates, both in “basal” conditions at different ages and in pathological conditions, namely in the presence of ovariectomy-induced osteoporosis and in the presence of a bone fracture. Histological analysis of long bones as well as vertebrae will include Perl’s Prussian blue staining on non-decalcified sections, in order to confirm the expected iron
accumulation in the tissue in our mouse model. Moreover, osteoclast-specific markers will be used to confirm the accumulation of iron in these cells. Colocalization of ferritin (as revealed by immunohistochemistry) with osteoclast markers will further confirm that excess iron is stored in osteoclasts. Evaluation of serum parameters related to bone metabolism, including total calcium and phosphate, Tartrate-resistant acid phosphatase activity, parathyroid hormone, RANKL and osteoprotegerin, whose balance is crucial in bone homeostasis, type 1 collagen cross-linked C-telopeptide and osteocalcin (as bone turnover markers) will be quantified using dedicated ELISA assays. In parallel, hemoglobin and serological parameters related to iron metabolism, such as sideremia, transferrin saturation, ferritin (by ELISA) will be evaluated. Moreover, liver samples will be used to evaluate by Real-Time PCR analysis the mRNA levels of the iron regulators hepcidin and BMP6. Another aim of the project is to perform in vitro functional and expression studies in order to identify cell functions and molecular pathways affected by iron overload in osteoclasts. To this end, in vitro osteoclast differentiation assays from *Fpn*^{fl/fl};*lysM-Cre* splenocytes will be performed; generated osteoclasts will be counted and either fixed for cytochemical evaluations and immunostainings, or collected for RNA and protein extraction. The gene expression profile of the differentiated cells will be evaluated through microarray analysis and the results will be confirmed by immunoblot analysis of specific proteins. To test whether increased iron content in osteoclasts leads to ROS production (4), oxidative stress will be evaluated by flow cytometry using the cell permeant reagent DCFDA, a fluorogenic dye that measures ROS activity within the cell. Moreover, FACS analysis of Mitotracker-stained osteoclasts and determination of ATP levels will be used to evaluate the role of Fpn inactivation on mitochondrial biogenesis and activity. This research is expected to shed light on the molecular and cellular mechanisms elicited by iron overload in osteoclasts in the pathogenesis of osteoporosis. The expected results might have an important therapeutic relevance: in fact, if the hypothesis of a crucial pathogenetic role of osteoclast iron overload is confirmed, novel therapeutic strategies could be considered aimed at removing excess iron by means of chelating agents specifically targeting the bone compartment. In addition, the data might also add new insight into the osteoimmunological network and be relevant also to other diseases affecting both organ systems, such as arthritis.

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Iron is an essential nutrient that is indispensable for various functions, such as oxygen transport, cell respiration and DNA synthesis. Both iron deficiency and iron excess can cause detrimental effects on cells and organ systems: in the former case, cell growth arrest and death; in the latter, toxicity due to an increase in reactive oxygen species (ROS), ultimately causing cell and tissue damage. Because of its dual role, iron levels are tightly controlled by means of a network of proteins regulating its absorption, storage, recycling, and utilization both at the systemic and cellular level (1). One of the major proteins involved in iron homeostasis is ferroportin (Fpn), the sole iron exporter present on the cell plasma membrane. Its binding to the liver-derived peptide hepcidin induces Fpn internalization and degradation, thereby inhibiting iron release into the plasma. This leads to retention of cellular iron and decreased levels of circulating iron.

Iron can contribute to both tumor initiation and progression (2). Excess iron can lead to reactive oxygen species (ROS) formation and mutagenesis, as shown by the association between iron overload and cancer risk. Moreover, due to their generally elevated proliferative potential, cancer cells have a greater metabolic demand for iron than normal cells and hence express high levels of transferrin receptor (TfR1) to internalize transferrin-bound circulating iron. Indeed, iron chelators exert inhibitory effects on cell growth and have been considered for tumor therapy (2). Over the last years, several studies have shown that reprogramming of iron metabolism is a key function for a tumor cell. In particular, downregulation of both the iron storage protein ferritin and the iron exporter Fpn, together with increased TfR1 expression, leads to higher iron availability in a variety of cancer cells resulting in faster cell growth and adverse prognosis in cancer patients.

Since we have reported that the conditioned medium of M2 polarized macrophages, which show high Fpn expression and iron release, sustains faster growth of malignant and non malignant cell lines (3), the major aim of this project is to test in vivo the hypothesis that tumor associated macrophages (TAM), which acquire a M2-like phenotype and act as "protumoral macrophages" contributing to tumoral progression, support cancer growth also by increasing iron availability. Thus, we plan to evaluate in proper animal models whether the lack of macrophage Fpn affects tumor growth. To this end, we will use the Fpn\textsuperscript{fl/fl};lysM-Cre mouse model recently developed in our laboratory, in which the Fpn gene is specifically inactivated in cells of the myeloid lineage. These mice present iron retention and accumulation in macrophages.
Experimentally, we will evaluate the growth and the characteristics of chemically-induced tumours in $Fpn^{fl/fl}$;lysM-Cre mice and their floxed $Fpn^{fl/fl}$ littermate controls. According to our working hypothesis, the animals with Fpn deficiency in macrophages are expected to develop significantly less tumours. Moreover, a delay in the appearance of tumours and a reduced tumour growth rate are expected. In parallel, we will verify that the changes in iron handling observed in human M2 macrophages (3), such as high Fpn expression, are also present in murine TAM.

To induce fibrosarcomas, $Fpn^{fl/fl}$-lysM-Cre mice and their floxed littermates $Fpn^{fl/fl}$ will be injected subcutaneously with 3-methylcholanthrene. For mammary tumorigenesis, DMBA will be administered by gavage for 6 weeks. The growth rate of solid tumours will be evaluated twice weekly by recording two perpendicular diameters of the tumour mass with vernier caliper. At predetermined time points, animals will be sacrificed and the tumour explanted, weighed and subject to macro and microscopical analysis of tumoral mass and adjacent normal tissue; for metastatizing tumours the number and weight of metastasis in target organs will be evaluated.

In addition to histological and histochemical techniques aimed at a careful characterization of the tumors, in vitro analysis on different cell types separated from tumor specimens will be performed. The expression of genes related to iron metabolism and to carcinogenicity will be evaluated at the protein and mRNA levels. This research is expected to shed light on the molecular and cellular mechanisms elicited by microenvironmental iron deprivation in the pathogenesis of cancer. The expected results might have therapeutic relevance: in fact, if the hypothesis of a pathogenetic role of TAM iron release is confirmed, novel therapeutic strategies interfering with macrophage iron release in the tumor compartment could be considered.

References

Best publications
4) Transferrin receptor induction by hypoxia: HIF-1 mediated transcriptional activation and cell-specific post-transcriptional regulation.
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<td><strong>Project title</strong></td>
<td>Enhancing the success of acute leukemia stem cell eradication: targeting Wnt signaling by porcupine inhibitors</td>
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<td>Laboratorio di Genetica Molecolare&lt;br&gt;Dipartimento di Scienze della Salute- DISS&lt;br&gt;Ospedale San Paolo</td>
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Despite the involvement of aberrant Wnt signaling in a variety of human leukemias (1,2), relatively little is known about the role of Wnt receptor components in leukemia pathogenesis. In the present project, focusing our attention on the major locus associated in hematopoiesis to the regenerative function (3) and dysergulated in leukemia (4). We recently provided evidences for a new recurrent rearrangement involving the WNT10B locus within intron 1 (WNT10B<sup>R</sup>). Moreover, we demonstrated the consequent expression of a non-physiological transcript variant (WNT10B<sup>IVS1</sup>), retaining 77 nucleotide of IVS1 and lacking exon1, thus we analyzed the significance in AML (5).

Although we confirmed higher expression for FZD4 in AML (4,6), it is still not known which FZD receptor(s) bind WNT10B. The MOLT4 lymphoblastic leukemia cell line was selected for functional studies since it expresses the WNT10B<sup>IVS1</sup> allele, in order to visualize interactions we’ll use proximity ligation assay (PLA).

Aims of this proposed project are:

i.) study at single cell level the impact of porcupine inhibitors (7) on the components of Wnt pathway induced by the WNT10B<sup>IVS1</sup> in MOLT4 cell line and primary leukemic samples;

ii.) evaluate whether the inhibition of WNTs release by N-palmitoyltransferase PORCN inhibitor (i.e. WNT974) represents a useful therapeutic strategy to enhance the effectiveness of pharmacological agents, providing the rational for the clinical development of Wnt/β-catenin inhibitors in Acute leukemia therapy.

iii.) On the basis of the encouraging recent results, we are generating a zebrafish transgenic line expressing wnt10b in a temporal inducible fashion, specifically in the hematopoietic stem cells. To restrict the expression of the transgene in the hematopoietic territories, the zebrafish runx1 promoter has been selected. Runx1 is expressed in all definitive hematopoiesis sites in mammals and zebrafish, as also demonstrated through the construction of a transgenic runx1:EGFP zebrafish line, in which EGFP expression marks all sites of runx1 expression during development and adulthood.

We have generated a construct, named pBluescript II SK_runx1P2_wnt10b+polyA_L200_R150, in which wnt10b coding sequence is under the control of the runx1 promoter that will allow to drive wnt10b expression in definitive hematopoietic tissue, as embryonic AGM and the adult kidney. The transgene is flanked by
transposable elements in order to dramatically increase the rate of transgenesis. This transgenic model will be used to test the effectiveness of Wnt signaling inhibition on aberrant myelopoiesis.

**References**


**Best publications (4)**


**Most relevant publications for the project**


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**Most recent publications**


   Corrigendum: Scientific Reports 2017 Apr 26;7:46788. doi: 10.1038/srep46788


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<th><strong>Project title</strong></th>
<th>Variable expressivity in Noonan syndrome and Neurofibromatosis type 1: identification of pathogenetic mechanisms with perspectives of a personalized medicine</th>
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Single gene disorders are expected to be inherited on the basis of Mendelian laws, actually different factors can make difficult the prediction of the transmission risk. Variable expressivity of phenotypic traits increases the complexity of the interpretation of a specific phenotype transmission and cannot be explained entirely by a gene or allelic alteration (1). While the identification of gene variants that affect phenotypes is rapidly progressing, the determination of factors and mechanisms involved in variation of expression traits requires both more complex experimental designs and methodologies. Noonan syndrome (NS) and Neurofibromatosis type 1 (NF1) are RASopathies with autosomal dominant inheritance (2), characterized by genetic heterogeneity and variable expressivity. The remarkable genetic heterogeneity due to either mutations in one of the RAS/MAPK pathway genes or CNV encompassing NS genes cannot completely explain the variable expressivity of this condition. The genotype-phenotype correlation is even more complex in 20-30% of patients for whom the pathogenetic mutation remains unknown. (2, 3,4). Thus new NS genes are expected to be identified. As far as NF1 most mutations are truncating variant, while the subgroup NF1 microdeletion syndrome, showing a complex phenotype, is characterized by a deletion of NF1 and 20-30 flanking genes. Moreover a further subtype of neurofibromatosis, the spinal neurofibromatosis, typically characterized by neurofibromas on the spinal nerve roots, could be associated to NF1 missense mutations, but at now the etiology is unknown (5, 6). Variable expressivity pinpoints in NS and NF1 the problem of undiagnosed potential transmitter patients showing a mild or subclinical phenotype that are diagnosed only after the birth of an affected infant with a more severe phenotype. Both genetic and epigenetic mechanisms should be considered to unravel the impact of variable expressivity on the transmission of a specific trait. We think that the dosage of the mutated allele may have an effect on the phenotype severity as well as the presence of variants in more than one NS gene. Interestingly, the role of genetic variants in inter-individual differences of the gene expression levels pinpoints an important class of DNA polymorphisms, the expression Quantitative Trait Loci (eQTLs) that affect the expression level of genes (1, 7). cis-eQTLs causing differential allelic expression (DAE) may lead to the overexpression of either the mutant or the wild-type allele in affected heterozygous individuals, influencing either the penetrance or the variable expressivity of the disease.
More recently, we reported on the position effect on genes flanking a deletion in an NF1 microdeletion patient, indicating that the complex phenotype could be given not only by the deleted, but also by the remaining genes, probably deregulated by means of epigenetic mechanisms (8). On the basis of the above premises the aims of the proposed PhD project are:

a) to search for new NS genes and new genetic variants in NS patients with a negative genetic screening and in patients with a more severe clinical phenotype compared to affected relatives by targeted next generation sequencing

b) to study the effects of DAE in vitro inducing the rescue of the phenotype by RNA interference, and in zebrafish model generating zebrafish mutant knock-in lines by using the CRISPR/Cas9 system.

c) to define the position effect in NF1 microdeletion syndrome by studying the chromatin structures with specific techniques.

d) to identify the etiology of spinal NF in a large cohort of patients through the characterization of genetic lesions with different genomics techniques.

The study of the mechanisms implicated in NS and NF1 genetic heterogeneity will address a genotype-phenotype correlation and provide new insights on these diseases, opening new perspective for the development of a personalized medicine.

References


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Background and rationale. Despite advances in chemotherapy and the advent of immunotherapy based on PD-1/PD-L1 checkpoint inhibitor antibodies, advanced lung cancer remains an incurable disease. Therefore, researchers are investigating the tumor microenvironment for less invasive and more effective methods of treating lung cancer.

A resident lung microbiota has been described recently; versus the intestinal tract, the bacterial density in airways is modest, and its composition has been examined primarily by genomic approaches (1, 2). As in the gut, in which certain microbial species have been related to the induction of Tregs (3), also the commensal bacteria that colonize the respiratory tract regulate pulmonary immunity, modulating immunosuppressive populations (4, 5). Recently, the bacterial composition of lung microbiota was associated with discrete local host immune responses based on analysis of immune genes expression in bronchoalveolar lavage (BAL) fluid in HIV-infected patients (6), revealing that bacteria in the lower airway influenced immune tolerance in the lung microenvironment.

Although less studied, an association between lung bacterial taxa and cancer has been also reported. A study on sputum samples of patients with lung cancer showed fewer bacterial operational taxonomic units (OTUs) in cancer patients as compared to control (7). Interestingly, a relation between the stage of cancer and microbiota composition has been also observed and an high abundance of commensal Legionella was detected in subjects who developed metastases (8).

Considering the role of microorganisms that colonize the respiratory tract in regulating pulmonary immunity, these data suggest that colonization of lung with certain microbial species might play a role in promoting tumor development by sustaining an immune “tolerance” state or alternatively that tumor itself could subvert immune response and local immunity against cancer by modifying the lung microbiota.

We and others have demonstrated that aerosolization is an efficient and non-invasive method of delivering molecules, such as antibiotics, antibodies, cytokines and Toll-like receptor agonists, to the lung (9-12). Thus, we hypothesize that nebulization of antibiotics or probiotics to influence pulmonary microbiota is a novel approach for locally subverting tolerance in the lung and promoting immunosurveillance against lung cancer and metastases.

Aims. In this project we aim:
• To examine whether antibiotics or probiotic aerosolization reduces immune tolerance, promotes immune activation in the lung;
• To analyze whether the composition of lung microbiota affects immunological control of tumor implantation/growth in preclinical models of experimental and spontaneous metastases;
• To identify a relationship between specific bacterial taxa, the local immune microenvironment and the prognosis of patients with lung cancer.
• To evaluate the effects of antibiotics and probiotics on response to anti-PD-1 immunotherapy in preclinical models
• To investigate the correlation between specific bacterial taxa and the response to checkpoint immunotherapy in lung cancer patients.

Impact on Cancer. This project will advances our understanding of the factors that impact tumor growth in the lung. Few studies have examined the influence of lung microbiota on lung cancer and none has manipulated it to subvert immune tolerance and establish local immunity against cancer. If immune responses can be activated locally by aerosolization of antibiotics or probiotics, this novel and non-invasive strategy can be extended to immunotherapies for lung cancer.

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Best publications


Most relevant publications for the project


Most recent publications

**Project title**: Investigation of dendritic cells (DC) in the tumor microenvironment for DC-tailored immunotherapy of human glioma

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http://www.unimi.it/chiedove/cv/ENG/silvia_dellabella.pdf

**Background**: Immunotherapy of glioma is a promising approach for improving the poor prognosis provided by standard of care treatment of these patients. The modest improvement of patient survival yielded so far by immunotherapeutic trials suggest deleterious immunosuppressive action of glioma on dendritic cells (DCs), which are crucial initiators and stimulators on anti-tumor immune responses. The functional specialization of distinct DC subsets and the complex molecular pathways regulating DC activatory/tolerogenic profile are key determinants in effective anti-tumor responses.

Glioma heterogeneity is a hot topic in neuro-oncology, as it greatly influences tumor aggressiveness and responses to treatment. Intratumor heterogeneity likely is the key to understanding treatment failure.

**Hypothesis**: DCs are present in glioma microenvironment and have a tumor-associated tolerogenic phenotype. They are differently affected in different patients and in different areas of the same tumor, according to the high heterogeneity of this cancer. The tolerogenic profile of glioma-infiltrating DCs hampers the anti-tumor activity of effector mechanisms activated by immunotherapeutic treatments. Peripheral blood DCs (pbDCs) mirror tumor-infiltrating DCs and can be used as circulating biomarkers.

**Aims**: In order to improve our ability to plan immunotherapeutic interventions that specifically target tumor-induced DC defects in glioma patients, this project will investigate the molecular mechanisms involved in DC tolerogenicity and the relevance of DCs in glioma progression and response to therapy.

**Experimental design**: Prospective study on 50 glioma patients (GBM and lower-grade glioma type I-III) undergoing surgical resection, followed by either standard therapy, or immunotherapy (some patients will be enrolled in clinical trials that will be active at Humanitas Clinical Center during project running). Intratumoral DCs will be characterized at baseline according to tumor grade and intratumoral heterogeneity (sampling...
from heterogenous regions, as detected by combined PET/MR imaging), by flow cytometry, transcriptomics (RNAseq), immunohistochemistry, functional assays (allostimulatory activity). pbDCs will be characterized at baseline and every 3 months during treatment and follow-up.

**Expected results:**
The project will put on test the following working hypotheses:
1) distinct subsets of steady-state and inflammatory DCs are present in glioma microenvironment and have a tolerogenic phenotype
2) glioma heterogeneity is associated with heterogeneity of infiltrating DCs
3) distinct molecular mechanisms sustain the tolerogenic properties of infiltrating DCs and their definition is a key element for planning immunotherapeutic strategies that specifically target tumor-induced DC defects
4) infiltrating DC subsets and their tolerogenic profile have a functional relevance in disease progression and response to treatment
5) the characterization of pbDCs can provide circulating biomarkers for patient follow-up and can unreveal the mechanisms underlying success or failure of standard and immunotherapeutic treatments of glioma patients.

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<td>Crosignani A, Riva A, Della Bella S.</td>
<td>Analysis of peripheral blood dendritic cells as a non-invasive tool in the follow-up of patients with chronic hepatitis C. World J Gastroenterol 2016, 22: 1393-1404.</td>
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**Most recent publications**

- Crosignani A, Riva A, Della Bella S. Analysis of peripheral blood dendritic cells as a non-invasive tool in the follow-up of patients with chronic hepatitis C. World J Gastroenterol 2016, 22: 1393-1404.
<table>
<thead>
<tr>
<th>Project title</th>
<th>Exploiting computational biology to identify Mecp2-dependant pathways of therapeutic relevance</th>
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<tr>
<td>Tutor</td>
<td>Nicoletta Landsberger</td>
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<tr>
<td>E-mail</td>
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<tr>
<td>Laboratory/Department</td>
<td>Laboratory of molecular and cellular biology applied to neurodevelopmental diseases / Dept. Medical Biotechnology and Experimental Medicine. <a href="http://www.facebook.com/Laboratori-Landsberger-183337865199935/">www.facebook.com/Laboratori-Landsberger-183337865199935/</a></td>
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**Project**

Mutations in *MECP2* cause a broad spectrum of neuropsychiatric disorders including Rett syndrome (RTT), a devastating neurological disease that because of its incidence (1:10,000 female births) represents the most common genetic cause of severe intellectual disability in girls worldwide. Most of the RTT-associated mutations are believed to cause the phenotype as a result of moderate to severe loss-of-function of the protein. However, the existence of an *MECP2* duplication syndrome suggest that protein or gene replacement therapies might not represent a valid therapeutic approach in the immediate future. No treatment is available to combat the primary pathology for any of the *MECP2*-opathies. Indeed, patients are treated only to ameliorate secondary phenotypes. It has, however, been established that phenotypic rescue is possible in *MeCP2* deficient mice upon reactivation of the endogenous *Mecp2* gene, therefore raising the possibility of therapeutic intervention\(^1,2\). Initial studies have supported an exclusive neuronal role for MeCP2 in RTT; however, recent data suggest that glial cells play a major role in the disease. Importantly, selective re-expression of *Mecp2* in astrocytes of *Mecp2* null mice re-established the normal neuronal dendritic morphology, improved locomotion, anxiety and respiratory abnormalities and prolonged lifespan\(^3\). While these studies prove that RTT is characterized by impairments in both neuronal and astroglial cells and support the possibility of targeting astrocytes as complementary strategy for improving the RTT condition, it still remains obscure which key molecules in astrocytes affect neuronal structure and function. Literature data and our preliminary results indicate that *Mecp2* deletion causes some molecular defects in astrocytes, that could affect astrocytic signalling, cell division and neuronal support functions. In particular, the regulation of glutamate homeostasis, exerted by the activity of glutamate transporters, could be affected by MeCP2 deletion, thus influencing synaptic functions\(^4\). Clinical and experimental evidence indicate a defect glutamate metabolism in RTT, although results are still controversial\(^5,6\). Further, it remains unknown the contribution of glutamate homeostasis on *Mecp2*-related neuronal defects. Since *MECP2* encodes a multifunctional protein with several roles in the regulation of gene expression\(^1\), RTT is believed to be mainly caused by perturbations in gene transcription\(^7\). MeCP2 regulates thousands of genes in the mature brain and it is assumed that most expression changes induced by its loss affect neurons. However, we still have to disclose which genes/molecular pathways are mainly
associated with MeCP2 deficiency and very little is known about the changes occurring in Mecp2 deficient astrocytes.

The major goals of this project are: i) to dissect the transcriptional consequences of Mecp2 deletion during astrocytic development and adult stage in purified astrocytes from mouse cortex; ii) to identify candidate genes for novel therapeutic strategies; iii) to test the therapeutic efficacy of the two most promising strategies selected by a 'drug repositioning' approach; iv) to test whether restoring glutamate transport in astrocytes could benefit neurons and improve behavioral phenotype in Mecp2 KO animal models.

Aim1. Exploiting computational biology to identify Mecp2-dependent pathways in astrocytes.

Mecp2 regulates thousands of genes in the mature brain. However, very few genes have consistently shown altered expression after MeCP2 deficiency and very little is known about the changes occurring in astrocytes. By using innovative strategies based on the concept of 'systems biology', we will generate a transcriptomic data to identify genes with pharmacological relevance for treating RTT. A short list of candidate targets and drugs will be selected for experimental validation. Understanding whether drugs approved for other diseases can be used to treat RTT (drug repositioning) will be a crucial aim of this effort.

1A. Preparation of astrocytes from cortex of WT and Mecp2 KO mice and gene expression profile.

The student will sort astrocytes by MACS Mylenyi kit from cerebral cortex of WT and Mecp2 KO mice at two different developmental ages (P7 and P30), reflecting a different maturation step in gliogenesis and gene expression profiling will be performed through RNASEq. The bioinformatics analysis of the produced data will occur with the essential collaboration of prof. G. Pavesi (University of Milan).

1B. Computational identification of pharmacologically relevant crossroads for the treatment of RTT. (performed in collaboration with prof. F. Di Cunto, University of Turin). We plan to exploit the gene expression data to identify new potential target genes, whose modulation is likely to counteract the effects produced by Mecp2 loss. Among them, we will pay particular attention to those candidates that are known targets of approved or experimental drugs, thus implementing a ‘drug repositioning’ strategy. This approach is especially interesting because it may bypass the need for the huge financial resources necessary to perform phase-one and phase-two clinical trials on new molecules, allowing to move directly from preclinical models to patients. Since we aim at finding novel therapies, in this project we will focus on the most promising drugs associated with the top scoring hits obtained from the list of candidate targets. If no suitable drug can be selected, we will test the rescuing capacity of viral vectors expressing a selected mRNA or RNAi of the top candidate targets. We aim at comparing 2 compounds (or virus). Through qPCR, WB or IHC, the student will confirm in vivo the deregulation of selected drug targets on cortical astrocytes derived from WT and Mecp2 KO mice at P7 and P30. Since pharmacological
experiments will be initially performed on cultured astrocytes, the latter will be also used for validation of candidate genes.

**Aim 2. Testing novel therapeutic approaches.**

Our preliminary results and previous data indicate an alteration in glutamate transport in Mecp2 KO astrocytes; the student will thus test the effect of rescuing it both on Mecp2 KO neurons and in animal models. Further, he/she will investigate the therapeutic validity of the two most promising drugs/approaches selected from aim 1.

**2A. Evaluating the therapeutic potential of rescuing glutamate transport.**

The student will complete the molecular characterization of Mecp2 KO cortical astrocytes, focusing the attention on glutamate homeostasis. By WB analysis, he/she will analyze *in vitro* and *in vivo* the protein expression of the molecular determinants governing the glutamate turnover. He/she will also compare the glutamate clearance capability of WT and Mecp2 KO astrocytes. Since we have already found in Mecp2 KO astrocytes a significant decrease in a relevant gene, the benefits of increasing its expression will be tested by two approaches. In the first one, astrocytes will be infected with a lentivirus carrying the gene under a GFAP promoter; in the second one, astrocytes will be treated with a drug which has been demonstrated to increase the expression of this gene by a transcriptional mechanism. The efficacy of both approaches in reverting glutamate transporter's expression and glutamate clearance capability will be measured. Then, considering the *non-cell autonomous* effect exerted by astrocytes on neurons, it will be tested whether restoring the selected gene expression in astrocytes could improve morphological (axonal length, dendritic branching, nuclear area) and functional (by Ca²⁺ imaging experiments) alterations in neuronal cells. In case of encouraging *in vitro* results, a preclinical study will test the therapeutic efficacy of the drug in Mecp2 animal models.

**2B. Evaluating the therapeutic potential of the most promising drugs deduced from aim 1.**

Having selected the two most promising drugs, their efficacy will be evaluated in *in vitro* cultured Mecp2 KO astrocytes and neurons, and in case of positive results in a preclinical study. At first, the student will test whether the selected drugs can revert or significantly amend the molecular defect(s) in Mecp2 KO astrocytes that was/were identified through bioinformatics and that represent the rationale for testing the selected drugs. Then, he/she will investigate the capability of these novel strategies to improve both morphological and functional alterations in neurons. In the presence of positive *in vitro* results, the therapeutic potential of one of the selected molecules will be evaluated in a preclinical study.

**References**

Best publications


Most relevant publications for the project


Most recent publications


### Project title
Role of LSD1 in aging- and stress- dependent epigenetic drift leading to depression and anxiety disorders

### Tutor
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### Laboratory/Department
Laboratory of Neuroepigenetics, Department of Medical Biotechnology and Translational Medicine

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By 2050, for the first time in history, the number of elders in the world will exceed the number of the young (UN, 2002) warranting new molecular and clinical studies aimed at improving health in older adults. Aging is manifested by a gradual decline of normal physiological functions in a time-dependent manner. Epigenetic alterations represent one crucial mechanism behind the deteriorated cellular functions observed during aging and in age-related disorders. Frailty is an age-related diseases described as loss of ability to adapt to environmental stress because of diminished functional reserves. In this perspective, stressful events, because of their impact on the Hypothalamic–Pituitary–Adrenal (HPA) axis, promote chronic inflammatory state likely contributing to the onset of Frailty. In addition, we and others have shown that stress directly impact on brain structure, inducing loss of hippocampal neurons and causing hippocampal atrophy. These structural effects underlie memory deficit and promote the onset of depression, also representing two critical Frailty traits. Relevantly, also the healthy aging brain undergoes a modest structural decline, raising the possibility that stressing an aging brain could result in pathological brain modifications that could stand at the basis of Frailty or foster related mood alterations including depression.

The project includes clinical and neuro-epigenetic approaches aimed at understanding the molecular and neuroanatomical bases of Frailty and its associated mood disorders. The aims of the project include (i) the identification of epigenetic hallmarks of Frailty in post-mortem frail hippocampi: (ii) the preclinical evaluation of a pharmacological approach aimed at resetting pathologic stress-induced chromatin modifications to alleviate Frailty symptomatology. (iii) in collaboration with clinicians from the Fondazione IRCCS Ca’ Granda Ospedale Maggiore Policlinico expert in human brain imaging, the project will also include the development of a valuable new human neuroimaging-based Frailty diagnosis to understand the molecular and neuroanatomical bases of Frailty and its associated mood disorders.

Furthermore, in the frame of promoting a proactive stream of information between scientific research and the society, the project will include the challenging aim to set up a sensitization plan directed to raise public awareness about the importance of individual participation in post-mortem human brain donation, in collaboration with expert from the Istituto Superiore di Sanità and the Azienda Ospedaliera di Reggio Emilia Santa Maria Nuova. The long-term potential of this initiative is to generate a nucleation center for the
creation of the first Italian Brain Bank, which will represent a crucial tool to link preclinical finding to clinical brain research in the Frailty field and also aimed at improving brain research toward a better mental health at any age.

We recently showed how the epigenetic enzyme Lysine-specific demethylase 1 (LSD1) represents a unique example of molecular transducer of stressful stimuli, able to modify chromatin in response to stress. Indeed, in mice in response to psychosocial stress, LSD1 increases its repressive activity causing heterochromatinization of plasticity gene targets. Such increase of LSD1 repressive activity has an intrinsic negative implication, since brain circuitry aplasticity represents the substrate of psychiatric disorders and cognitive deficits. Strikingly, LSD1 also increases its repressive activity during aging, in both rodents and humans indicating that stress and aging can converge to increase LSD1 repressive function, likely fostering the brain structural modification and the onset of age-related pathologies.

We will test the central hypothesis that synergistic convergence of stress and aging leads to LSD1-mediated heterochromatinization and transcriptional repression of the Immediate Early Genes (IEGs) egr1 and c-fos, affecting neuronal morphology in a way that can change hippocampal structure and relative volumes. To this purpose, we will perform molecular analysis in frozen post-mortem human hippocampi from frail patients and in rodent model of stress and aging. The aim of this projects is to validate the chromatin modifier LSD1 as a novel pharmacological target to treat age-dependent cognitive decline and depression related to pathological aging and Frailty.

REFERENCES:


Best publications


Most relevant publications for the project


Most recent publications


<table>
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<tr>
<th>Project title</th>
<th>Regulation of adaptive immunity by gut microbiota</th>
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<tr>
<td>Tutor</td>
<td>Fabio Grassi</td>
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<tr>
<td>E-mail</td>
<td><a href="mailto:fabio.grassi@unimi.it">fabio.grassi@unimi.it</a></td>
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The human gastrointestinal (GI) tract is a complex ecological niche, in which all the three domains of life (Archaea, Bacteria and Eukarya) and Viruses co-exist in close association with the host. This complex microbial community, referred to as the gut microbiota, has co-evolved with the host in a mutualistic relationship that influences many physiological functions such as host energy metabolism, development and function of the immune system. The subtle equilibrium between the gut microbiota and the host is a key element in human health. In fact, alterations in the composition of the microbial community structure, termed as “dysbiosis”, have been associated to an increasing number of medical conditions such as metabolic disorders (e.g. diabetes, obesity), blood pressure alteration and heart disease, autoimmunity. Different studies in mice and humans have demonstrated that obesity is associated with changes in microbiota’s diversity and abundance. Furthermore, it has been suggested that intestinal dysbiosis has a causal role in the development of obesity and insulin resistance. Indeed, faecal microbiota transfer (FMT) from conventional to germ-free (GF) mice results in significant increase in body-fat content and insulin resistance that are associated to inflammation and macrophage accumulation in adipose tissue. The gut microbiota encodes a more versatile metabolome than the host and a well-balanced microbiota is a necessary requirement for stable functional metabolic interactions with the host. Since the immune system and the gut microbiota start developing together at birth, it has been hypothesized that their co-evolution maintains and selects mutualistic or symbiotic microorganisms within the GI niche. This early co-existence is necessary to avoid undesirable reactions against the healthy gut microbiota. The intestine and its associated immunological components have to deal with a number, in some cases dichotomous, tasks. Apart from all the functions related to digestion and absorption of nutrients, the intestine has to be tolerant towards mutualistic/commensal microorganisms and to keep control over pathobionts (i.e. those resident microbes with pathogenic potential), preventing microbial overgrowth and invasion of the epithelial intestinal barrier. In turn, the gut microbiota has to modulate and regulate several aspects of host’s immune system towards tolerance rather than responsiveness. Central in this homeostatic relationship is the local production of immunoglobulin A (IgA), which is the most copious Ig isotype produced by the human immune system. IgA interaction with the polymeric Ig receptor (pIgR) and luminal secretion guarantee mucosal protection by entrapping microorganism
in the mucus, neutralizing invading pathogens and microbial inflammatory compounds. T cell dependent high affinity IgA is critical in maintaining intestinal homeostasis and efficient mucosal defence by limiting translocation of pathobionts from the gut lumen into the organism. Conversely, T-independent IgA responses are sufficient for maintaining a beneficial microbiota by avoiding translocation of commensals through the mucus layer. Peyer’s patches (PPs) are the secondary lymphoid organs within the ileal mucosa, where T cell dependent IgA responses originate. Most lymphocytes localized in PPs inhabit germinal centers (GCs), where Tfh cells interact with B cells and facilitate B cell proliferation, induction of activation-induced (cytidine) deaminase (AID) with consequent Ig class switch recombination (CSR), somatic hyper mutation (SHM) and affinity maturation. Since Tfh cells in PPs are essential for GC reaction and IgA affinity maturation, they play a critical role in the modulation of the structure and function of intestinal microbial communities.

Adenosine triphosphate (ATP) is a ubiquitous extracellular messenger, which activates purinergic receptors in the plasma membranes of eukaryotic cells termed P2 receptors. P2X 1-7 receptors are ATP-gated cation channels, whereas P2Y1, 2, 4, 6, 11-14 are guanine nucleotide-binding protein (G protein)-coupled receptors (GPCRs), which bind also ADP, UDP, UTP or UDP-glucose. ATP released during tissue damage acts as danger associated molecular pattern (DAMP) for cells of the innate immune system through stimulation of P2 receptors. P2rx7 is a signature gene of effector T cell subsets. Sustained P2X7 activation leads to the formation of a pore permeable to molecules up to 900 Da and cell death. We have shown that P2rx7 is selectively and highly expressed in Tfh cells of PPs. Extracellular ATP was detected in the supernatant of in vitro cultured intestinal commensals. We addressed whether ATP derived from bacteria could contribute to endoluminal ATP in the small intestine. In mice from our specific pathogen free (SPF) facility, we detected micromolar concentrations of ATP in the ileum. In contrast, in GF mice ATP was barely detectable, indicating that mucosal colonization is important in determining the endoluminal ATP concentration. Notably, the analysis of fluids (e.g. urine, bile and serum) from other epithelial or endothelial layered sterile organs (or almost sterile, like bladder) did not reveal detectable ATP. The concentrations of ATP we detected in the ileum were sufficient to trigger pore formation and Tfh cell death in a P2X7 dependent fashion. In fact, deletion of P2rx7 in these cells resulted in resistance to cell death induced by intestinal ATP, enhanced GC reaction in PPs and increased faecal IgA levels. These results indicate that ATP is a bacterial metabolite, which modulates adaptive IgA response to ensure physiological mucosal colonization. Furthermore, the lack of P2X7 mediated control of Tfh cells results in alterations of the gut microbiota and dysregulated metabolic homeostasis consistent with the central role of sIgA in regulating host-microbiota interactions as well as host metabolism and physiology.
Motivated by increasing evidences of the role of the gut microbiota in the complex interactions connecting the immune system and host metabolism, we asked whether deregulated secretory IgA responses due to enhanced T follicular helper cells activity in the Peyer’s patches because of defective ATP-gated P2X7 receptor signalling contributes to impaired glucose homeostasis and metabolic abnormalities in dysmetabolic subjects. Based on our preclinical model, we hypothesize that the ATP/P2X7 axis might be exploited as a therapeutic target in dysmetabolic conditions. In this project proposal, we aim at identifying microbial and metabolic signatures typical of subjects carrying hypoactive P2X7 for the design of a microbiome-based therapy for treatment of metabolic disorders. To achieve this goal we will apply an interdisciplinary approach exploiting metagenomics, metabolomics, immunology and computational biology as well as a vast and unique clinical cohort of thoroughly characterized subjects. The advancement in knowledge on the physiopathology of metabolic alterations provided within this project proposal will likely contribute the basis for the development of new, next-generation therapeutic approaches that, by restoring the metabolic and microbial equilibrium of the gastrointestinal tract, will improve the quality of life of subjects suffering of dysmetabolic conditions.

**Best publications**


**Most relevant publications for the project**


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Most recent publications


Project title: Obesity and inflammation: understanding the relevance and pathophysiology of lactate

Tutor: Massimiliano Ruscica

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Laboratory/Department: Pharmacological and Biomolecular Sciences

**Background:** Obesity – clinically defined by BMI >30 kg/m² – is placing an ever increasing burden on public health worldwide. Individuals with obesity are at increased risk of several health conditions including type 2 diabetes mellitus, dyslipidemia, liver steatosis, hypertension, cardiovascular disease, osteoarthritis, as well as various forms of cancer. As with any chronic disease that affects large populations, the pathophysiology of obesity and, in broader perspective, of eating disorders is extremely complex, including, but not limited to, genetic predisposition, environmental changes associated with lifestyle, and individual preferences. Indeed, feeding rhythmicity, social environment, physical inactivity, and long-term stress are reported to promote obesity. There is a deep link between chronic inflammation and obesity and fat deposition in the adipose tissue during aging (Bjorndal et al., 2011) which is often related to increased expression of adipokines (leptin, chemerin, resistin), pro-inflammatory molecules (tumour necrosis factor (TNF)-α, interleukin (IL)-1, and IL-6), and the decrease of the anti-inflammatory cytokine, adiponectin, and the expression of myokines, that affect the immune system. Indeed, adipose tissue is infiltrated with activated immune cells (including T cells, macrophages, and dendritic cells) which in turn promotes the overproduction of proinflammatory cytokines including TNF, IL-6, resistin and monocyte chemotactic protein-1 (MCP-1/CCL2). A complex network of soluble mediators derived from immune cells and adipocytes, perpetuate the infiltration of leukocytes in adipose tissue. Lactate is a major metabolite of glucose metabolism in adipose tissue, particularly in enlarged adipocytes of obese rodents and humans. There are evidence that lactate itself function as an intrinsic inflammatory mediator that leads to increase IL-17A production by T cells and macrophages, resulting in the promotion of chronic inflammation (Yabu et al., 2011), typical condition of obesity, and that lactate stimulates VEGF production by endothelial cells leading to enhanced migration and resulting in lactate-induced angiogenesis independently of O2 conditions, perhaps causing the continuing massive recruitment of lymphocytic cells that perpetuate the inflammatory state in the adipose tissue of obese subjects.

**Aim:** The overall gap that this project aims to fill is to unravel how lactate transporters impact lymphocyte T cells trafficking in adipose tissue and control adaptive immune responses in obesity.

**Objectives:** First year: To investigate the impact of lactate-mediated control of T cell migration and functions during diet induced obesity chronic inflammation via the use of T cell specific conditional
knockout (KO) murine model of the lactate transporters, Slc16a1. Analysis of the data
Second year: Evaluation of metabolic changes (ie, mitochondrial activity); characterization of immune populations. Analysis of the data
Third year: Translate the relevance of Slc16a1 transporter in humans by investigating the impact of single nucleotide polymorphisms of Slc16a1 gene in relation to metabolic features and disease outcome in a cohort of obese patients (BMI ≥40). Analysis of the data and final report.

Best publications

Most relevant publications for the project


<table>
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<tr>
<th><strong>Project title</strong></th>
<th>PHENOTYPIC AND EPIGENETIC CHARACTERIZATION OF MYCOSIS FUNGOIDES AND SEZARY SYNDROME AT DIAGNOSIS AND DURING THE DISEASE COURSE: SEPARATE ENTITIES OR TWO STAGES OF THE SAME DISEASE?</th>
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<tr>
<td><strong>Tutor</strong></td>
<td>Prof. Francesco Onida</td>
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<td><strong>E-mail</strong></td>
<td><a href="mailto:francesco.onida@unimi.it">francesco.onida@unimi.it</a></td>
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<td><strong>Laboratory/Department</strong></td>
<td>Laboratorio di Istotipatologia Cutanea / Dipartimento di Medicina Interna Fondazione IRCCS Ca’ Granda Ospedale Maggiore Policlinico – Università degli Studi di Milano</td>
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<td><strong>Project</strong></td>
<td>Mycosis fungoides (MF) and Sézary syndrome (SS) are the two commonest form of cutaneous T-cell lymphoma (CTCL). MF is characterized by localized and indolent erythematous patches and plaques which can evolve after many years in tumor stage or leukemic disease; instead, SS is characterized by erythroderma, presence of circulating malignant T-cells in the peripheral blood, and generalized lymphadenopathy. In the past, SS and MF were considered a disease continuum, but recent phenotypical and genetic studies have been supported the idea that they are distinct diseases. In fact, in early MF malignant T cells have a resident memory T phenotype and express a Th1 profile, supporting the indolent and localized clinical course. As disease progresses and tumor burden increases, a more Th2 skewed cytokine profile is found, enhancing the tumors ability to evade the host immune response. In SS, malignant T cells have predominantly a Th2 profile, even if T regulatory (Treg) and Th17 profiles have been reported. Sézary cells could have two memory phenotypes: T central memory (TCM), associated with high proliferation potential and erythroderma, and migratory memory T cells (TMM), associated with discrete lesions with ill-defined borders. Considering that exact pathogenesis of SS and MF is still unknown and diagnosis and therapy in “atypical cases” are often a challenge, the overall aim of this project is to characterize by phenotypic and epigenetic analyses cases of both MF and SS, in order to better define the relationship between these two malignancies; in particular, the project should focus on the analysis of patients with a high tumor burden in the blood (B2 stage) but localized MF lesions in the skin and SS patients who relapse with typical early MF after systemic therapies. The study of these particular clinical presentations should help to elucidate if they are clinical variants of SS or an evidence of plasticity of malignant T-cells coming from differences in the microenvironment in the skin and in the blood. By allowing the acquisition of new insights into the pathogenesis of MF and SS progression, this study may allow to optimize the selection of treatment strategies during the disease course. As a matter of facts, after systemic therapies including also allogeneic stem cell transplantation, SS may relapse with the original T-cell clone population but with a clinical picture of MF. In these cases, definition of the link between MF and previous SS can lead to the identification of markers which may help to better evaluate prognosis of patients</td>
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with SS and MF. In addition, outlining the relevance of high tumor burden (B2) in the contest of the strict definition of SS, permits to redefine the criteria for diagnosis of SS which now exclude some clinical variants, still orphan of a univocal diagnosis.

Best publications


Most relevant publications for the project (5)


Most recent publications

(6)


**Project title**  | Biomarkers of rheumatic diseases through the study of DNA methylation, proteomics, and autoantibodies  
---|---  
**Tutor**  | Carlo Francesco Selmi  
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**Laboratory/Department**  | Autoimmunity and Metabolism  
Division of Rheumatology and Clinical Immunology  
Humanitas Research Hospital  
**Project**  
Autoimmune diseases are chronic diseases that affect over 5% of the European population, with significant impact on quality of life and socioeconomic costs. Autoimmune diseases become manifest after a long preclinical period, during which identifying subjects at risk may lead to earlier and more effective treatment and limit disability. Autoantibodies are present in most patients with autoimmune diseases, as systemic sclerosis patients are virtually all positive for antinuclear antibodies (ANA), and anti-centromere (ACA), anti-topoisomerase I (anti-Scl70), and anti-RNA polymerase III antibodies identify patient subgroups, but no reliable biomarker can predict SSc susceptibility and internal organ involvement. Moreover, most chronic inflammatory diseases, i.e. psoriatic arthritis (PsA), are seronegative, or no known autoantibody can be identified. Furthermore, up to 15% of healthy subjects are also positive for ANA, therefore other biomarkers are needed to allow an early diagnosis, disease stratification and prognosis in autoimmune and chronic inflammatory diseases.  
**Objective.** To identify biomarkers for autoimmune diseases, in particular systemic sclerosis (SSc) and psoriatic arthritis (PsA), using different the most advanced techniques as well as using unique study designs.  
**Methods.** We will enroll SSc and PsA patients followed at the Unit of Rheumatology and Clinical Immunology at Humanitas Research Hospital, Rozzano (MI). Demographic and clinical data will be collected as well as blood samples every 6 months. As controls, we will enroll psoriasis and Raynaud’s phenomenon patients and healthy subjects matched for age and sex. Blood samples will include: serum analysis for known and unknown autoantibodies using radioimmunoprecipitation and proteomic analysis using SOMAscan platform at SomaLogic, Inc. (Boulder, CO, USA), whole blood DNA methylation analysis (Infinium MethylationEPIC BeadChip) and transcriptome profile (Illumina TruSeq Stranded mRNA kit).  
**Best publications**  
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Recent advances in 3D culture technology allow embryonic and adult mammalian stem cells to generate organoids in vivo, which reflect the key structural and functional properties of organs they originate (Clevers, 2016).

3D organoids represent a powerful tool to study important human physiological and pathological processes, to predict drug response in a personalized manner and to be used for regenerative medicine and gene therapy. In this context, organoids become a fascinating approach to investigate complex processes like tumor growth and development, resembling the in vivo mechanisms.

Immune system plays a pivotal role in immune-surveillance processes that result in tumor limitation and elimination. Indeed, the vast majorities of solid tumor are immunogenic and recruit different types of immune system cells, which are able to kill the tumor in their site. However in many cases, tumor cells can evade the immune surveillance using different strategies (e.g. loss of tumor antigens expression and or recruitment of immune cells with immunosuppressive functions). CD4+ T regulatory cells (Treg), physiologically engaged in the maintenance of immunological self-tolerance and immune homeostasis, are able to inhibit anti-tumor immune responses of effector cells and are found at high frequencies in various types of cancer. A recent transcriptional analysis performed in my laboratory (De Simone et al., 2016) revealed that tumor-infiltrating Tregs, isolated from CRC (colorectal cancer) and NSCLC (non-small cell lung cancer) patients, expressed a unique specific gene signature, correlated with patients survival. In line with our findings, non-lymphoid tissues infiltrating Tregs can exhibit specific phenotypes and transcriptional profile involved in glucose metabolism, tissue repair and muscle regeneration, far from their well-established suppressive roles (Cipolletta et al., 2012).

The molecular and epigenetic characterization of model normal and CRC derived organoids, together with the co-cultured Tregs will be performed through multiple computational approaches, which include bulk (ChIP-seq, ATAC-seq) and single-cells (scRNA-seq and scATAC-seq) approaches. Bioinformatics analysis and integration of these datasets will lead to a detailed molecular description of tumor Treg cells and to the potential discovery of novel diagnostic and prognostic markers and therapeutic targets with higher efficacy and specificity and reduced side-effects.

The epigenomics analysis will allow us to define chromatin states
configuration and to identify key regulatory elements. The cell-type specific chromatin configuration will be defined by a de novo chromatin state discovery approach that integrates publicly available and in-house produced data, using tools such as ChromHMM (Ernst & Kellis, 2012). Moreover, we will provide a more detailed characterization of key regulatory elements performing a differential occupancy analysis using tools such as DiffBind (Ross-Innes et al., 2012). The intersection of distinct histone markers and chromatin accessibility features, previously identified as differential in one condition, will allow the identification of candidate functional element (i.e. promoter or enhancer regions) which will be further investigated. We will exploit a fully automated analysis pipeline for single-cell transcriptomic data, which includes the use of several different unsupervised algorithms to investigate the complexity of input data and dimensionality reduction procedures. In particular, Principal Component Analysis (PCA), t-distributed Stochastic Neighbour Embedding (t-SNE) and clustering technique are commonly adopted for exploratory data analysis and for the unsupervised classification of cellular sub-populations of biological relevance. Differential expression analysis is then applied for the identification of ‘signature’ genes specifically expressed in each sub-populations of cells. Enrichment and semantic analysis will be used to gain insights into the functional role of candidate genes identified with the integration of transcriptomic and epigenomic data.

Best publications


Most relevant publications for the project


Most recent publications

http://doi.org/10.1016/j.jaci.2016.11.045

http://doi.org/10.1186/s13075-017-1305-1

http://doi.org/10.1038/nsmb.3392
<table>
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<tr>
<th>Project title</th>
<th>Epigenetic homeostatic mechanism in neuronal adaptation and metaplasticity to environmental stimuli</th>
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<tr>
<td>Tutor</td>
<td>Elena Battaglioli</td>
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<tr>
<td>Laboratory/Department</td>
<td>Laboratory of Neuroepigenetics, Department of Medical Biotechnology and Translational Medicine</td>
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The aim of this project is to unravel the molecular machinery regulating the epigenome of neuronal cells and impacting brain maturation and morphology, leading to adaptive but also maladaptive forms of plasticity which are often related to neurological and psychiatric disorders\(^1\)\(^-\)\(^3\).

In particular we are interested in deciphering new molecular pathways of synapse to nucleus cross-talk instrumental to coupling the perception of stimuli with their molecular effects on neuron physiology\(^4\).

Indeed, an important body of literature indicates that maladaptive neuronal plasticity underlie among other pathologies, also the onset of human depression, a burden affecting 300 million people worldwide\(^5\). Major depression is often associated at the neuronal level with aberrant glutamate concentrations at the synaptic space\(^6\), \(^7\). New and promising therapeutic strategies ground on selective NMDA receptor blockers such as ketamine to treat depressive symptoms hindering the calcium channel permeability of these glutamate receptors\(^8\). On the other hand, a possible alternative strategy can derive from a deep dissection of neuron-specific glutamate transduction pathways, ultimately leading to the identification of alternative, novel pharmacological targets. Indeed, glutamate is responsible through a plaethora of receptors-related signaling pathways to promote long-term synaptic plasticities such as LTP and LTD, two processes that requires stable modifications of synapse-related transcriptome and proteome\(^9\). These transcriptional modulations can be achieved and stabilized via plastic chromatin changes. In other words, the neuronal epigenome represents itself a promising alternative target to buffer the promoting effects of glutamate on the induction of maladaptive forms of neuronal plasticity leading to mental disorders\(^10\)-\(^12\). Remarkably, preliminary data from our lab show that the epigenetic enzyme Lysine-specific Demethylase 1 (LSD1) plays a dual role in the cross-talk between glutamate receptors at the synapse and transcription at the nucleus. Our group discovered that LSD1 is instrumental in transducing environmental stimuli under the form of inherent neuronal plasticity, but also representing a target whose function is directly modulated by these same challenges active in desensitizing neuronal glutamate response\(^4\). LSD1 is a highly specific flavin-dependent demethylase that acts as a transcriptional corepressor removing mono- and di- methyl groups from lysine 4 of histone H3 in concert with CoREST and HDAC1/2\(^13\).

This project aims at clarifying the extent to which LSD1 represents a nodal factor to discriminate among different NMDAR signal...
transduction pathways, further unveiling if it is ultimately involved in the modulation of LTP/LTD probability. We perform an extensive evaluation of the involvement of LSD1 in orchestrating IEG transcriptional repression instrumental to blocking LTP thus favoring LTD formation thus participating in the core of neuronal functions such as the information processing underlying learning memory and emotional behavior. All these features are degraded and debased in human depression as well as others psychiatric disorders of the central nervous system representing the foremost processes of pathogenesis. We will exploit murine knock out models and in particular neuroLSD1KO mice where LSD1 function is enhanced through the loss of its dominant negative neurospecific splicing isoform. These mice have already shown molecular uncoupling of stress and inherent stress-dependent transcriptional induction of plasticity genes in the hippocampus, emerging on a deficient establishment of anxiety-like phenotype. We will perform 1) behavioral and pharmacological assays, chromatin-based epigenetic assessments, production of in vivo models of stress-related depression 2) ex-vivo organotypic hippocampal cultures, for structural, transcriptomic and epigenetic analysis 3) electrophysiological analysis. This experimental approach will eventually lead to the demonstration that LSD1 is involved in choosing the fate of the stimulus induced neuronal plasticity, representing a relevant factor to target aberrant plastic consolidation at the base of psychiatric disorders.

REFERENCES
**Best publications**


**Most relevant publications for the project**


Most recent publications


