University of Modena and Reggio Emilia

PhD COURSE OF CLINICAL AND EXPERIMENTAL MEDICINE


# PhD DAY 2019

## Abstracts

**April 11-12 (9:30 a.m.)**
**Meeting Room – Civil Hospital of Baggiovara**
**(1355 Giardini street, Baggiovara - Modena)**

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## PhD School in Clinical and Experimental Medicine

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**PhD School in Clinical and Experimental Medicine** @PhDSchool... · Feb 15
#Frailty measured with 37-item frailty index is inversely associated with both immune-activation, depicted by CD4/CD8 ratio and immune-deficit, depicted by CD4 count in people living with #HIV. Full text available on: journals.plos.org/plosone/articl... @UNIMORE_univ @PLOSONE

**The dynamic association between Frailty, CD4 and..**
Objective To investigate the association between current CD4+ T-cell count and CD4/CD8+ ratio with severity of frailty among people aging with HIV. Methods Cross-se..
journals.plos.org

3 6

**PhD School in Clinical and Experimental Medicine** @PhDSchoolC... · Feb 9
The 2017 edition of Italian guidelines for the use of antiretrovirals is the first attempt to join #HIV and #geriatric care management addressing clinical needs of older adults living with #HIV. Just published in @JFrailtyAging, check it out! ncbi.nlm.nih.gov/pubmed/30734825 @UNIMORE_univ

2 5

**PhD School in Clinical and Experimental Medicine** @PhDSchoolC... · Feb 8
Just published our clinical trial regarding the efficacy of a self-administred decongestion therapy in women with breast cancer-related arm lymphedema. I'm very happy, proud and thankful to my colleagues and to my Institution @Ausl_RE @IsabelCampanini

**Efficacy of self-administered complex decongestiv..**
Purpose After surgery, breast cancer-related lymphedema (BCRL) is a frequent chronic condition. The complex decongestive therapy (CDT) delivered by phy...
link.springer.com

1 2 6

PhD School in Clinical and Experimental Medicine Retweeted

**Azienda USL Modena** @Ausl_modena · Feb 4
#worldcancerday2019 : fondamentale l'importanza dei programmi di screening. A questo link, quelli attivi in provincia di Modena #prevenzione #collodellutero, #mammella, #colonretto:

**COME FARE PER SCREENING ONCOLOGICI**
ausl.mo.it

The International Doctorate School in Clinical and Experimental Medicine (CEM) is organized by the Department of Biomedical, Metabolic and Neural Sciences in collaboration with other Departments of the University of Modena and Reggio Emilia and is under the direction of Prof. Giuseppe Biagini.

The educational program and research opportunities are directed towards the acquisition of skills required for basic and clinical research at Universities, public or private Research Institutes, and Hospitals. A Faculty of internationally recognized professors is responsible for the educational activities and takes part into the organization of the doctoral program.

From 2018 (cycle XXXIV) onwards the PhD Course of "Clinical and Experimental Medicine" is organized in 3 curricula:

*Nanomedicine, Medicinal and Pharmaceutical Sciences*
*Translational Medicine*
*Health Sciences*

From cycle XXIX to XXXIII the PhD Course of "Clinical and Experimental Medicine" was organized in 3 curricula:

*Medicinal and Pharmaceutical Sciences*
*Translational Medicine*
*Health Sciences*

From cycle XXV to XXVIII The Doctorate School of "Clinical and Experimental Medicine" was organized in 5 curricula/thematic areas:

*Oncology*
*Public Health*
*Cellular and Molecular Pathophysiology*
*Clinical, Genetic and Molecular Medicine*
*Surgery*

# XXXII cycle

***Dr. Angelo Territo***

*CEM Curriculum: Translational Medicine*

*Tutor: Prof. Giampaolo Bianchi*

*Co-Tutor: Prof. Salvatore Micali, Dr. Alberto Breda*

# PROSPECTIVE COMPARATIVE STUDY ON ROBOT-ASSISTED VS OPEN KIDNEY TRANSPLANTATION: TREND TO LESS PERIOPERATIVE INFLAMMATORY RESPONSE AND SIMILAR FUNCTIONAL RESULTS

**Background**

Open kidney transplantation (OKT) is the standard approach in kidney transplantation (KT), but robot-assisted kidney transplantation (RAKT) has been recently standardized, demonstrating to be a feasible, reproducible, and safe technique that offers surgical advantages thanks to the greater degree of freedom in movements and enhanced visualization of the surgical field. In addition, RAKT decreases the complication rate, hospital stay, and postoperative pain and improves aesthetic results.

Any surgical procedure generates a cascade of reactions stimulated by the release of pro-inflammatory cytokines, resulting in a postoperative Systemic Inflammatory Response Syndrome (SIRS). Different studies have reported that minimally invasive surgery allows to reduce SIRS substantially, due to small incisions, less tissue manipulation and less bleeding.

**Objectives**

The aim of this study is to prospectively quantify and compare the inflammatory response and the functional results of OKT vs RAKT.

**Methods**

We prospectively compared the inflammatory response between standard OKT and RAKT. 30 patients underwent pre-emptive KT between January and December 2018 (15 RAKT, 15 OKT). Blood levels of inflammatory markers (NGAL, CRP, IL-6) were measured at several time points: T0 (preoperative/baseline), T1 (post op h. 1), T2 (post op h. 6), T3 (post op h. 12), T4 (post-op h. 24), T5 (post-op day 2), T6 (post-op day 3) and T7 (post-op day 5) after KT. Serum creatinine and

estimated glomerular filtration rate (eGFR) were evaluated at post-op days 1, 3 and 7. A point-to-point analysis was performed, the differences in clinical variables between RAKT and OKT were evaluated using the unpaired t-test or non-parametric Mann Whitney U test.

**Results**

IL-6 and CRP significantly increased in both groups after surgery compared with baseline (×9 and ×15 respectively, all $p<0.01$). There was a significant difference in the mean level of IL-6 at T1 and T3 in the advantage of RAKT ($p<0.01$). Significant differences in the mean level of CPR were found at T3 and T5 in the advantage of RAKT ($p<0.01$). Compared to baseline NGAL significantly decreased in both RAKT ($p<0.01$) and OKT ($p<0.01$). Significant differences in the mean level of NGAL were found at T3, T4 and T6 in the advantage of RAKT. Serum creatinine and eGFR at postoperative days 1, 3 and 7 were similar in RAKT and OKT (all $p>0.05$).

**Conclusions**

This is the first reported prospective study to evaluate and compare the inflammatory response of RAKT versus OKT. RAKT trended to induce a lower inflammatory response compared with OKT. Short term functional outcomes were similar in RAKT vs OKT. Therefore, these data objectively demonstrated that the robotic approach seems to be less invasive than the conventional OKT, representing an attractive and promising alternative to the open approach.

**Figure 1. A** Point-to-point serum concentration of IL-6 at T0 to T7 in the RAKT and OKT patient groups. **B** Peak concentrations of IL-6 in the two groups. Graphs show mean values with 95% confidence intervals

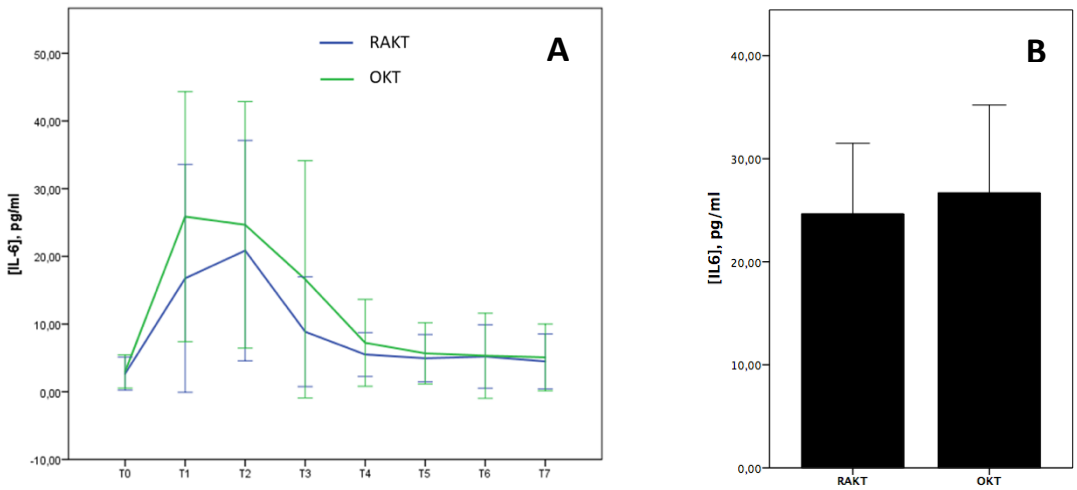


**Figure 2.** **A** Point-to-point serum concentration of CRP at T0 to T7 in the RAKT and OKT patient groups. **B** Peak concentrations of CRP in the two groups. Graphs show mean values with 95% confidence intervals

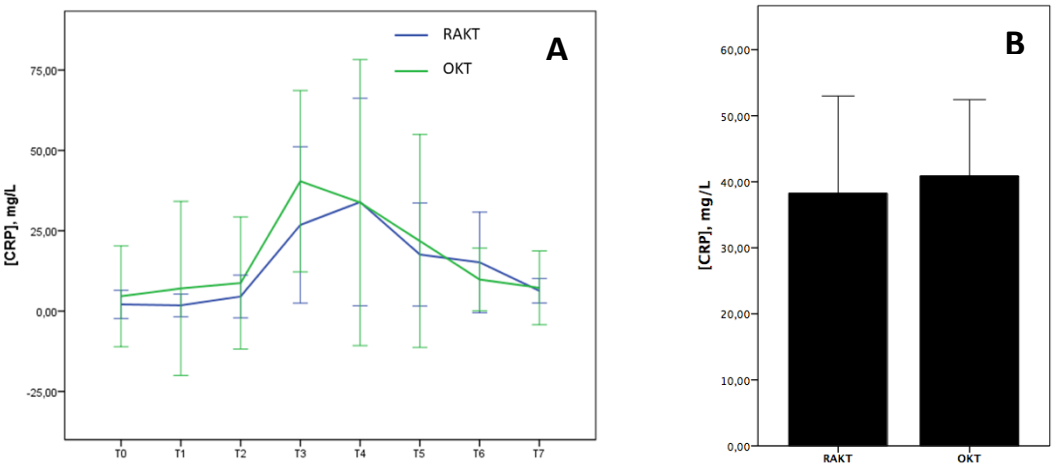
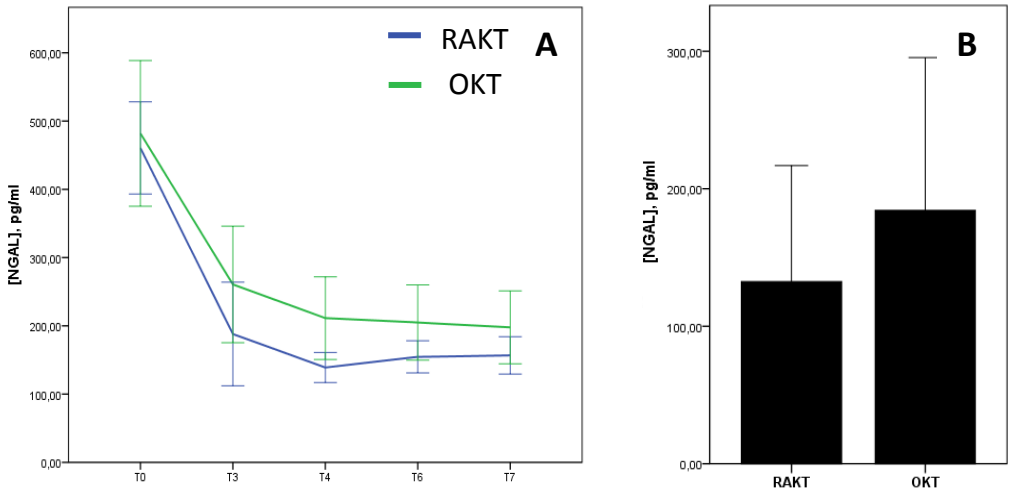



**Figure 3.** **A** Point-to-point serum concentration of NGAL at T0, T3, T4, T6 and T7 in the RAKT and OKT patient groups. **B** Peak concentrations of NGAL in the two groups. Graphs show mean values with 95% confidence intervals.



***Dr. Ilaria Giovannacci***

*CEM Curriculum: Translational Medicine*

*Tutor: Prof. Cristina Magnoni*

*Co-Tutor: Prof. Marco Meleti*

# AUTO-FLUORESCENCE AS A DIAGNOSTIC TOOL FOR ORAL EPITHELIAL DYSPLASIA AND CARCINOMA: A SET OF CLINICAL AND HISTHOPATHOLOGICAL STUDIES

## Background

AutoFluorescence (AF) is a peculiar visual property of some tissues directly associated to the concentration and distribution of specific fluorophores, namely molecules that can absorb and re-emit specific light wavelenghts. Malignant and potentially malignant changes may therefore result in AF variations (hypo- and hyper-fluorescence), such a feature being potentially useful for diagnostic purposes.

## Objectives

This PhD program is focused on the study of fluorescence of the oral mucosa, jawbones and skin. Objectives are:

- investigate the correlation between degree of AF and histopathological features of oral mucosa, in order to assess the usefulness of AF in the diagnosis of malignant and potentially malignant lesions: a pilot study has previously been presented. Here we report an update based on the inclusion of more cases (114).
- search the correlation between AF alterations and histopathological characteristics: the results of a histopathological study carried out on a model of oral carcinomas is reported.
- develop a tool able to evaluate AF degree in a quantitative (objective) mode: an experimental setup with a spectrophotometer has been developed. Ethical committee approval was granted for a study on the skin.

**Methods**

Clinical study

One hundred and fourteen lesions suspicious for oral epithelial dysplasia (OED) and oral squamous cell carcinoma (OSCC) were included in the present evaluation. A system emitting 400-460 nm light (VELscopeVx - LED Medical Diagnostics Inc., Barnaby, Canada) was used to assess AF. Each specimen was classified as normo-/hypo-/hyper-/ fluorescent and the histopathological pattern was analyzed. With regard to the histological diagnosis, lesions were graded as having: no dysplasia; dysplasia (mild or moderate) and carcinoma (in situ, micro-invasive, or invasive SCC and verrucosa carcinoma (VC).

Statistical analysis was performed through the IBM-SPSS statistical package v.22. Inferences about the groups have been performed using t test for independent data and the equivalent nonparametric Kruskal-Wallis test.

Histopathological study

Twenty oral lesions with histological diagnosis of SCC or VC were included in the present evaluation. All lesions were evaluated with regard to AF features before biopsy. Eight histological categories were investigated, in order to identify which histopathological features are possibly related to the pattern of AF: a) mean length of the entire epithelium (MLE); b) mean length of the keratin layer (MLK); c) mean length of the epithelium without taking into account the overlying keratin; d) overall area of the epithelium (OAE); e) mean depth of inflammatory infiltration (MDI); f) overall area of blood vessels (OAV); g) mean area of blood vessels (MAV) and h) mean diameter of blood vessels (MDV). Evaluations were performed through the Nikon NIS-Elements software® (3.0 version).

Data analysis was performed using IBM-SPSS statistical package v.22. Both parametric (Student's t-test) and non-parametric tests (Mann-Whitney U-test) were used. The results were considered statistically significant for a p-value of less than 5% ($p < 0.05$).

**Results**

Clinical study

A strong statistically significant association between histological alteration (OED or carcinoma) and AF alteration considering both hypo- and hyper-fluorescence was highlighted ($p=0.005$).

A statistically significant difference in the AF alteration related to diagnosis, with progressing value from no dysplasia to dysplasia and then carcinoma was demonstrated. (No dysplasia: altered AF in

42.9% of cases; Mild/Moderate dysplasia: altered AF in 78.9% of cases; In situ Ca/OSCC: altered AF in 100% of cases - $p<0.001$).

Histopathological study

Mean MLE within the group of hypofluorescent lesions was 513 µm while it was 790 µm among hyperfluorescent lesions. A trend toward significance was observed through the Student's test (p: 0.079), indicating that hyperfluorescent carcinomas have a ticker epithelium. Among hypofluorescent lesions, mean MLK was 41.3 µm while it was 197 µm for hyperfluorescent cancers. Either parametric and non-parametric tests highlighted a strong statistical association between MLK and typology of AF ($p < 0.001$, for all tests). Analysis of MLE, MLK and OAV through binomial logistic regression showed that, when the three variables are pooled, they have a prediction value of 100% with regard to the typology of fluorescence (accuracy, sensitivity and specificity of 1.00). However, were analyzed singularly, no one of such variables has a statistic significant association as regard to the prediction of fluorence typology.

**Conclusions**

The clinical study demonstrated that AF alteration is statistically related to histological alteration. So, not only hypo-fluorescence, but also hyper-fluorescence should be taken into consideration, especially if it is associated with anamnestic and clinical suspicious features.

The histopathological study shows that the most important indicator of AF alteration is keratin. A model taking into consideration MLE, MLK and OAV may potentially indicate the type of AF alteration in 100% of cases.

***Dr. Eleonora Truzzi***

*CEM Curriculum: Medicinal and Pharmaceutical Sciences*

*Tutor: Prof. Eliana Grazia Leo*

**DRUG DELIVERY STRATEGIES FOR GERANIOL NOSE-TO-BRAIN ADMINISTRATION**

**Background**

Geraniol (GER) is a natural compound with demonstrated anti-oxidant and neuroprotective activities in neurodegenerative disorders, such as Parkinson's Disease. GER activity is however impaired by its fast metabolism following oral administration, with a half-life of about 12 minutes. To overcame this limitation, nose-to-brain administration is a promising strategy because it allows the direct delivery of drugs into the brain and avoids the blood-brain barrier and the first-pass metabolism. However, GER is a highly volatile compound with an irritant effect on the mucosae, so an appropriate delivery system is required for its administration by this route.

**Objectives**

The aim of the project was the development of different drug delivery strategies for the nose-to-brain delivery of GER. The first strategy consisted in developing GER loaded lipid/polymeric nanoparticles and cyclodextrins (CDs) inclusion complexes. The second strategy was the production of a GER prodrug by its conjugation with the ursodeoxycholic acid (GER-UDCA) and the encapsulation of this prodrug into lipid/polymeric nanoparticles.

**Methods**

For the encapsulation of GER, in its free liquid form, different carriers were designed starting from varied materials such as lipids (Compritol, Gelucire, Isopropyl myristate, triacetin), polymers (PLGA and Gelatine) and CDs (β-CD and HP-β-CD), obtaining GER-loaded nanoparticles (GER-NPs) or GER-CDs complexes. For the second strategy, PLGA and Compritol were employed to obtain GER-UDCA loaded polymer nanoparticles (GER-UDCA-NPs) and solid lipid nanoparticles (GER-UDCA-SLNs), respectively. All the formulations obtained were freeze-dried and characterized regarding size and size homogeneity by dynamic light scattering, drug loading, encapsulation efficiency and *in vitro*

drug release by HPLC analysis. The inclusion GER-CDs complexes were also characterized in regard to morphology by electron microscopy, infrared (IR) spectroscopy, CHN analysis, differential scanning calorimeter (DSC) and thermogravimetry (TG). GER-UDCA loaded carriers were characterized about *in vitro* prodrug hydrolysis in liver homogenate. Moreover, for GER-UDCA-SLNs, i*n vivo* pharmacokinetic profile in the cerebrospinal fluid (CSF) were obtained in male rats.

**Results**

Freshly prepared GER-NPs were around 150-200 nm, homogenous in size and with a high encapsulation efficiency. GER-NPs were freeze-dried in order to increase formulation stability. While no modification in the size was observed, the drug loading became negligible, probably owing to the volatility of the drug. Only in the case of the inclusion complexes composed by β-CD/HP-β-CD, the encapsulation efficiency remained high (about 80%). Results obtained by DSC, TG, CHN and IR spectroscopy showed that both the inclusion complexes were successfully formed.
Regarding GER-UDCA carriers, both SLNs and NPs demonstrated to be spherical in shape, with homogeneous size of about 120-180 nm. After freeze-drying, drug loading was 6% and 12% and encapsulation efficiency 89.3% and 60.1% for SLNs and NPs, respectively. Data regarding *in vitro* GER-UDCA release from the carriers evidenced a higher dissolution rate than the free drug, probably due to the increase of surface contact between the drug and the release medium. The *in vitro* hydrolysis study of GER-UDCA demonstrated that lipid carriers (GER-UDCA-SLNs) were suitable for *in vivo* nose-to-brain administration, which was compared to the oral administration of free GER. GER-UDCA was detected in CSF within 180 min with an AUC value of 736 ± 28 µM·min, while GER was detected in CSF within 60 min with an AUC value of 473 ± 23 µM·min.

**Conclusions**

GER was efficiently incorporated into lipid or polymeric-based nanoparticle suspensions. However, after freeze-drying, no formulation resulted able to stably encapsulate the GER. On the contrary, inclusion complexes obtained using the two different CDs resulted stable even after freeze-drying. *In vivo* pharmacokinetics studies and mucosal histopathology are ongoing to evaluate the *in vivo* performance of these formulation via nose-to-brain.
The newly synthesized prodrug GER-UDCA proved to be a valid alternative for GER delivery via nose-to-brain after encapsulation into SLNs. In fact, the nose-to brain administration of GER-UDCA-SLNs leads to a markedly prolonged (about 2 hours) half-life of GER in CFS in comparison of

GER orally administered. Moreover, taking into account that the oral dose of GER was about 100 times higher than the intranasally administered GER as conjugate in the prodrug, the AUC value in CSF of rats was significantly higher ($P < 0.001$).

***Dr. Claudia Omarini***

*CEM Curriculum: Translational Medicine*

*Tutor: Dr. Federico Piacentini*

**MUTATIONAL PROFILE OF HER2 POSITIVE EARLY BREAST CANCER TREATED WITH NEOADJUVANT CHEMOTHERAPY**

**Background**

Breast cancer (BC) with amplification and/or overexpression of Human Epidermal growth factor Receptor 2 (HER2+) oncogene are about 15% of the BC diagnosis. Poor prognostic clinical features and aggressive behavior characterize HER2+ tumors. Neoadjuvant systemic therapy (NST) is a treatment option in patients with early-stage HER2+ BC. Tumor response to NST well correlates with survival. In particular, pathological complete response (pCR) significantly predicts long-term outcomes. Results from neoadjuvant trials suggest that HER2+ BC subtype is a heterogeneous group including tumors with different treatment sensitivity and prognosis. To date, the main challenge remains the identification of mutational profile able to predict treatment sensitivity prior any intervention.

**Objectives**

The aim of the study is to investigate the mechanisms of treatment resistance/sensitivity in a sample of HER2+ BC patients treated with NST. We compare the mutational profile of HER2+ BCs that achieved pCR to those with residual disease after NST. Moreover, we want to identify treatment-induced mutation on the surgical specimens of patients with residual tumor after primary systemic treatments.

**Methods**

Next-generation sequencing (NGS) methodology is used to analyze genetic status of 22 cancer-related genes on tumor tissue from both primary BC biopsy and surgical specimens in women with residual disease after NST. NGS is a high-throughput methodology able to performed gene expression profiling, chromosome counting, detection of epigenetic changes and molecular analysis. In particular, we analyze the status of the following genes: EGFR, ALK, ERBB2, ERBB4,

FGFR1, FGFR2, FGFR3, MET, DDR2, KRAS, PIK3CA, BRAF, AKT1, PTEN, NRAS, MAP2K1, STK11, NOTCH1,CTNNB1, SMAD4, FBXW7, TP53. An exploratory analysis in terms of treatment outcome, survival outcomes and single gene mutation will be carry out.

**Results**

Overall, we identified 571 patients treated with neoadjuvant systemic chemotherapy who underwent surgery at Modena Cancer Center. 209 of them had HER2 positive stage I-III BC. 199 patients were suitable for our analysis. Patient and tumour characteristics and treatment information were collected. Standard biological parameters *(*Ki67, nuclear grade, hormone receptors and HER2 status*)* were correlated to pCR. Globally, pCR was achieved in 66 patients (33%), mainly in hormone receptor negative HER2+ BC. To date, PI3KCA was found to be the gene with main mutations. The evaluation of mutational gene profile on all the samples is ongoing as well as the correlation between gene mutations, survival outcomes and treatment sensibility.

**Conclusions**

Gene expression analysis, performed until now, identify some gene mutations potentially predictive of treatment resistance. Further analysis are ongoing.

***Dr. Antonio Quotadamo***

*CEM Curriculum: Medicinal and Pharmaceutical Sciences*

*Tutor: Prof. Maria Paola Costi*

## NEU-5708 LEADS TO CANDIDATE MEDICINAL CHEMISTRY OPTIMIZATION

### Background

One of my PhD project regard the Neglected tropical diseases (NTDs), a collection of 20 infectious diseases that are a leading cause of morbidity and mortality, affecting over 1 billion people worldwide. Parasites of the family of Trypanosomatidae are agents of serious human diseases, including African sleeping sickness, Chagas disease and Leishmaniasis. Yet the current therapeutics show increasing cases of treatment failure, have low efficacy, and severe side effects [1,2]. As such, new treatments are urgently needed that meet the Target Product Profiles (TPP) for the respective diseases. However, these diseases suffer from a "translation innovation gap" where drugs rarely progress from in vitro drug screening to the preclinical phase.

In order to continue my research, I have reached the laboratory of Prof. Pollastri at Northeastern University in Boston. The starting points provided for this project have been identified by Drugs for Neglected Diseases Initiative (DNDi) via the NTD Drug Discovery Booster project a global consortium of pharmaceutical companies like as AstraZeneca plc., Celgene and Merck [3]. We propose to fill this gap in the preclinical pipeline, integrating academic drug discovery efforts with industry and public-private partnerships. This will be achieved through the identification of compounds that are effective *in vivo* models of the respective diseases and that adhere to the TPPs. In collaboration with DNDi, our approach relies on phenotypic screening which has the greatest success rate for delivering first-in-class chemotherapeutics, even during a period of time where the focus was on target-based drug discovery.

### Objectives

The goal of this project is to identify compounds that demonstrate proof-of-concept disease-modifying effects in the animal models of the respective disease, such that they become the focus of the candidate-seeking medicinal chemistry campaign. The specific aim of the last year of my PhD project was to identify lead-to-candidate medicinal chemistry optimization using structural

changes that are tolerated in terms of biological potency/selectivity but that also confer an improved ADME profiles.

**Methods**

Compounds synthesis: Different compounds have been processed by means of specific reaction steps, using conventional or microwave-assisted synthesis by comparing total reaction time and percentage yield. The single intermediates and the final compounds were purified by crystallization or chromatographic techniques. The chromatography process was carried out either with ISOLERA® or using the flash column chromatography, with regards to the purification complexity of the newly obtained molecular candidates. The compounds were fully characterized through Nuclear Magnetic Resonance (NMR) spectroscopy, mass spectral techniques (Ion Trap LC-MS). NMR spectra (1D-NMR: 1H and13C-NMR; 2D-NMR: COSY, HSQC, and HMQC) were recorded on a Bruker Advance 600 MHz WB spectrometer.

Biological evaluation: Antiparasitic assays and *in vivo* efficacy model towards *Trypanosoma brucei*, *Leishmania major* and *T. cruzi* were performed at DNDi via WuXi or London School of Hygiene and Tropical Medicine. Pharmacokinetic properties were evaluated at Celgene and/or AstraZeneca.

**Results**

The hit series have been identified through iterative screening and testing cycles of multiple pharmaceutical company libraries. Based on this approach, I focused on the design and synthesis using NEU-5708 as a starting point with $EC_{50}$ against L. infantum and T. cruzi of 2.6 µM and 0.73 µM respectively. The synthesis of these imidazo[1,2-*a*]pyridine derivatives was achived using my optimized version of the Groebke-Blackburn-Bienaymé multicomponent reaction reaching a quantitative yield by microwaves chemistry from substituited 2-aminopyridines, aromatic aldehydes and isociantes moiety obtained in different approaches. We plan two phases of medicinal chemistry optimization: SAR scoping (which is directed at establishing the limitations of the hit chemotype), and potency/properties optimization (operating within the allowable SAR scope). The primary goal is to improve the biological potency was obtained.

**Conclusions**

Supported by the extensive set of assessments described, medicinal chemistry design and synthesis are performing in my period abroad at Northeastern University. This process will closely

follow a state-of-the-art industrial optimization program. All the synthesized compounds will be tested against the parasites and in order to ensure compounds with the greatest degree of 'drug-likeness' AstraZeneca and DNDi will be provide in silico ADME properties. Among the 20 compounds already tested, some of them proved to have a good *in vitro* antiparasitic activity with no cytotoxicity. The most promising anti-trypanosoma compounds were selected in order to improve potency and cellular selectivity and for study the activity *in vivo*, assessing the physicochemical properties (solubility, metabolic stability, cLogP and measured LogD), with an eye towards achieving *in vivo* exposure and efficacy.

**REFERENCES** [1] M. P. Barrett et al., Br. Med. Bull. 104 (2012) 175–196. [2] Botta, M. (2018). Neglected diseases: Extensive space for modern drug discovery. [3] https://www.dndi.org/diseases-projects/open-innovation/drug-discovery-booster.

***Dr. Annalisa Guida***

*CEM Curriculum: Translational Medicine*

*Tutor: Prof. Massimo Federico*

## DECIPHERING IMMUNE RESPONSE TO CHECKPOINT INHIBITORS AND FINDING NOVEL BIOMARKERS IN METASTATIC RENAL-CELL CARCINOMA

### Background

Nivolumab represents the new second-line treatment for metastatic renal cell carcinoma (mRCC). This drug is a fully human IgG4 against PD-1 and his role is to inhibits programmed death-1 (PD-1)/PD-1 ligand 1 (PD-L1) immune checkpoint. In the majority of patients, this drug is able to restore the patient's tumour-specific T-cell-mediated response thus improving both overall survival and objective response rate. However, a lack of clinical response occurs in a number of patients, which varies according to the drug itself, the underlying disease, and other factors, hence raising questions about how to predict and increase the number of patients who receive long-term clinical benefit from immune checkpoint therapy. Unlike traditional cancer therapies, checkpoint inhibitors act primarily on cells of the immune system. The requirement for the immune system as a mediator of the drug's activity suggests that the balance of positive and negative regulators of the immune response at the time of therapy may be critical for therapy efficacy. Among these regulators, cytokines, chemokines, and other soluble factors regulate the survival, activity, and location of immune cells and thus represent potential players in determining drug efficacy. Of particular interest are soluble factors involved in the recruitment and regulation of effector T cells, the frequency of different subsets of regulatory T cells and the ratio between effector T cells and regulatory T cells.

### Objectives

The main aim of this project is to identify immune and serum biomarkers that are modulated in patients with metastatic renal cell carcinoma during and treated with immune checkpoint inhibitors and that can discriminate patients who most likely benefit from such therapy.

**Methods**

This is a prospective, longitudinal, interventional study on patients with mRCC who will receive Nivolumab in standard clinical practice. The project investigates changes in main immune parameters in patients with mRCC treated with nivolumab by analysing blood samples at baseline and after 1, 2, 3, 6 and eventually 12 months. Thirty mL of blood were collected and peripheral blood mononuclear cells (PBMC) were isolated according to standard procedures. PBMC were stored in liquid nitrogen. Then, PBMC were thawed according to standard procedures and stained with a viability probe and the following antibodies recognizing: CD3, CD4, CD8, CD25, CD127, FoxP3, ICOS, CXCXR6, CXCR3, CD95, CD45RA, CCR7, CD95, HLA-DR, CD38, CD28, CD27, CD71, CD87, CD39, TIM3, TIGIT, CCR4, Glycoforin, PD-1/IgG4, CD57, KI-67. This 28-color multicolour flow cytometry panel was set up in collaboration with Dr. Enrico Lugli (Humanitas, Milan). Samples were acquired by using a BD Symphony flow cytometer. Compensation was set using single stained controls and gating strategy was checked by using FMO. Data analysis was performed using FlowJo 9.6 under Mac OSX.

**Results**

From January 2016 until October 2018 we enrolled 21 patients. The median age was 60 years (33-79). The majority of patients had clear cell histology (90%). Nivolumab was given as second-line therapy in 57% of patients, as third line therapy in 29% of cases. According with International Metastatic Renal Cell Carcinoma Database Consortium Score (IMDC score) 72% of patients were in the intermediate prognostic risk group and 14% in poor risk. With a median follow-up of 14 months (min: 2 max: 31), 6-months and 12-months survival rate were 74% (95%CI 48-88) and 47% (95%CI 22-68), respectively. Median progression-free survival (PFS) was 4.2 months (95% 3-10). Disease control was achieved in 8 patients (40%), defined responder (R). At time of analysis treatment was ongoing in 4 patients. Despite small population size, no significant differences in clinical features were observed between R and non-R. Preliminary data on PBMC show that Ki-67, a marker of cell proliferation, is increased after 15 days of therapy in some patients. Accordingly, the expression of HLA-DR and CD38 are increased.

**Conclusions**

Reactivation of the immune system is one of the main goals of nivolumab. We expect to identify easily measurable immune biomarkers that predict the responsiveness to nivolumab. Finding

novel biomarkers that predict the response to therapy with nivolumab and monitor its efficacy can be of great benefit for the success of treatment not only to increase the number of patients who assume this therapy but also to identify those who have to change treatment without losing time so allowing an optimal allocation of economic resources**.** Longer follow up is required to assess preliminary immunological data.

***Dr. Natalia Oddone***

*CEM Curriculum: Medical and Pharmaceutical Sciences*

*Tutor: Prof. Giovanni Tosi*

*Co-Tutor: Prof. Barbara Ruozi*

**ROS-RESPONSIVE POLYMERS: INNOVATIVE DDS FOR THE TREATMENT OF BRAIN DISEASES**

**Background**

Nowadays, several Drug Delivery Systems (DDS) improving the bioavailability and specificity of several drugs, have been developed. Frequently, the drug loaded into DDS should be released in a modulated manner accordingly to the preparation project, and the release should be site specific, to reduce side effects and increase therapeutic efficacy[1]. The design of DDS that release drugs in response to a specific endogenous stimulus, known as "Smart" DDS, could solve these issues. A possible trigger to promote drug release from properly designed DDS could be the exploitation of variation in ROS species concentration. High levels of ROS are common in cancer, inflammatory diseases and neurodegenerative diseases[2,3], and therefore polymer sensible to these species could be synthesized and used to prepare DDS. Particularly, linkers containing the ROS-responsive moiety Thioketal (TK), due to their easy synthesis and biocompatibility, represent good candidates as linking groups for the design of ROS-responsive DDS[4].

**Objectives**

The overall goal of this PhD is to apply ROS reacting and biocompatible molecules, namely Thioketal (TK) linkers, for design of ROS-responsive DDS for Glioblastoma (Project-1) and Alzheimer's disease (AD) (Project-2). The first objective of this third year of PhD, regarding Project-1, is to complete the proof of concept studies with model methoxypolyethylene glycol (mPEG) fluorescent prodrugs: mPEG-TK-Cy5 (ROS-responsive prodrug) and mPEG-Cy5 (non-ROS-responsive prodrug), by studying comparatively their uptake and Cy5 release *in vitro* on rat C6 glioblastoma cell line. The second objective is to study the *in vitro* cytotoxicity of ROS-responsive and non-ROS-responsive prodrugs with Melphalan (MPH): mPEG-TK-MPH and mPEG-MPH, respectively, which were synthesized and characterized during the second year, on C6

glioblastoma cell line and DI TNC1 astrocyte cell line (control of normal cells). In order to improve cell penetration and thus enhance the cytotoxicity of MPH prodrugs, the third objective will be to prepare and characterize polymer micelles from mPEG-TK-MPH and mPEG-MPH, with subsequent *in vitro* cytotoxicity assessment on human U87MG glioblastoma cell line as well as glioblastoma patient-derived cells. To simulate the conditions encountered clinically in tumors, these studies will be performed under normoxic and hypoxic conditions. The last objective will be to evaluate the antitumor efficacy of mPEG-TK-MPH and mPEG-MPH micelles *in vivo,* in a human to mouse model of glioblastoma (obtained by the injection of human U87MG glioblastoma cells). Concerning project-2, the objective during this third year, is to synthesize and characterize the ROS-responsive co-polymer with poly lactic-co-glycolic acid (PLGA): PLGA-TK-PLGA for the subsequent preparation of ROS-responsive PLGA NPs, planned for the encapsulation of anti-AD hydrophobic drugs.

**Methods**

**1. *In vitro* uptake and Cy5 release from mPEG-TK-Cy5 and mPEG-Cy5 on rat C6 glioblastoma cell line.** C6 cells were 72 h-incubated with mPEG-TK-Cy5 and mPEG-Cy5 at equivalent Cy5 concentrations of 0,9 and 4,5 µg/mL. At the end of the incubation time, cells were fixed, and early endosomes labelled by immunocytochemistry with Rab5 primary antibody and secondary antibody coupled to Alexa488, respectively. After cell nuclei counterstaining with DAPI, cells were analyzed by confocal laser microscope (Zeiss LSM710).

**2. *In vitro* mPEG-TK-MPH and mPEG-MPH cytotoxicity on C6 glioblastoma and DI TNC1 astrocyte cell lines.** The cytotoxicity studies with MPH prodrugs were performed by treating C6 glioblastoma or DI TNC1 astrocyte cell lines with mPEG-TK-MPH and mPEG-MPH at an MPH equivalent concentration of 11 µM for 48 hours. Through an apparatus which measures cell attachment (parameter of cell viability), namely xCELLigence® RTCA DP[5], curves of cell index as a function of time, were obtained for each treatment.

**3. Synthesis and characterization of PLGA-TK-PLGA.** PLGA-TK-PLGA co-polymer was prepared starting from PLGA-TK-COOH, which was synthesized and characterized in the second year, and PLGA-NH2. The co-polymer synthesis was performed through EDC/NHS coupling of PLGA-TK-COOH with PLGA-NH2 at a molar ratio of 1,19: 1, respectively. The characterization of the co-polymer

was performed through GPC (G7800A Agilent 1260 Infinity GPC/SEC Multi Detector Suite) analysis of MW distribution.

**Results**

In order to test if Cy5 from mPEG-TK-Cy5 prodrug can be released in response to the intrinsic ROS levels present on cancer cells, C6 glioblastoma cells were incubated with mPEG-TK-Cy5 and mPEG-Cy5 prodrugs at two equivalent Cy5 concentrations. Confocal microscopy studies showed that both prodrugs were internalized by cells. Differences on Cy5 fluorescence localization inside cells could be clearly noticed at the higher Cy5 equivalent concentration (4,5 μg/mL). While Cy5 from mPEG-Cy5 was clustered distributed inside endocytic vesicles, Cy5 signal from mPEG-TK-Cy5 was mainly disperse through the cytoplasm. These results suggest that Cy5, triggered by the intrinsic ROS levels on glioblastoma cells, was detached from mPEG-TK-Cy5 with subsequent diffusion into the cytoplasm. On the contrary, on mPEG-Cy5 incubated cells, Cy5 could not be detached upon ROS on these cells. Taken together, these qualitative results, validate the use of TK-based polymers for the design of active prodrugs against glioblastoma.

To evaluate the in vitro antitumor efficacy of mPEG-TK-MPH and mPEG-MPH prodrugs, cytotoxicity studies on C6 glioblastoma cells were performed. When these cells were treated with either of the prodrugs or free MPH, a significant inhibition on cell growth in comparison to control group, was observed. Furthermore, significant higher inhibition on cell growth was also observed between mPEG-TK-MPH and mPEG-MPH treatments, while free MPH was significantly more cytotoxic in comparison to either of them. On the other hand, on normal astrocyte cells, treatment with free MPH also inhibited cell growth, even in a greater extent in comparison to glioblastoma cells. Noteworthy, the prodrugs did not inhibit cell growth on these cells, indicating their selectivity to cancer cells over normal cells.

As a means to design ROS responsive PLGA NPs for the delivery of anti-AD hydrophobic drugs, TK-technology was applied for the synthesis of PLGA-TK-PLGA co-polymer which was then characterized by GPC. A mean MW of 23,9 KDa, which was shifted respect to starting homopolymers: PLGA-TK-COOH (19,5 KDa) and PLGA-NH2 (18,1 KDa), was calculated using a polystyrene MW standard curve. Further characterization studies of this co-polymer are currently ongoing.

## Conclusions

We were able to validate the ROS responsiveness of mPEG-TK-Cy5 in vitro on C6 glioblastoma cells. On the other hand, we also demonstrated that, mPEG-TK-MPH, showed to be more cytotoxic than mPEG-MPH on these glioblastoma cells, being not cytotoxic on DI TNC1 astrocyte cells. We conclude that, due to their ability to specifically deliver drugs upon ROS stimulus with increased effectivity and selectivity for cancer over normal cells, the application of TK-technology in the design of prodrugs, constitutes a promising approach for future therapeutics against Glioblastoma.

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# MELANOMA DYNAMICS STUDIED BY NON-INVASIVE IMAGING TECHNIQUES – NOVEL TECHNOLOGY FOR MELANOMA DIAGNOSIS AND TUMOR AGGRESSIVENESS-EVALUATION AND COST-EFFECTIVENESS ESTIMATION IN THE PERSPECTIVE OF TWO DIFFERENT HEALTH CARE SYSTEMS: THE ITALIAN NATIONAL HEALTH SERVICE AND THE BRAZILIAN UNIQUE HEALTH SYSTEM (UHS)

## Background

Brazil is a large country presenting 92% of its extension in the intertropical zone. It also has a vast sea cost, which is an incentive to the sun exposure through the whole year. Regarding skin cancer, non-melanoma is the most frequent cancer in Brazil and presents high cure rates if diagnosed and treated early; whereas melanoma presents the worst prognosis being responsible for 95% of deaths. Early diagnosis of melanoma plays an important role in patient survival, since tumor excision may be done early, avoiding costs and consequences that could negatively impact the Brazilian Health Unique System. Reflectance confocal microscopy (RCM) is a noninvasive imaging technique, and which has already proved to be able to diagnose and correlate the morphology of the atypical cells with melanoma behavior. Pellacani et al demonstrated RCM reduced in 2/3 the number of benign lesions excised, and concluded that the use of RCM could lead to a saving of 260,000 Euros / 1 million inhabitants examined.

## Objectives

To analyze the cost-effectiveness of RCM in the screening of suspected melanocytic lesions in the Brazilian UHS, based on the economic analysis using the Decision Tree Modeling model - this design allows us to analyze the costs and consequences (costs and effectiveness or benefits of the alternatives in long term) of the incorporation of a new technology to aid in the early diagnosis of melanoma.

**Methods**

In the base-case, the scenarios to be modeled will be formed by a hypothetical cohort of 1.000 patients / year, diagnosed with advanced stage melanoma (stages III and IV) and screened for the need or not of excision of those lesions. It will be based on this cohort that the budgetary impact will be realized, for a time horizon of 10 years, and a discount rate of 3% will be applied. In the reference scenario, the diagnosis of melanoma and the evaluation for the need of excision or not is based on the identification of dermoscopic criteria associated with the histopathological evaluation of the lesion. In the alternative scenario, we suggest the RCM evaluation of the doubtful melanocytic lesions as a way of optimizing the diagnostic efficacy and accuracy of melanoma. The perspective of the analysis of the decision tree will be the Brazilian UHS, and the time horizon will also be 10 years. Costs in both scenarios will be estimated by micro-costing techniques, based on consultations with the BRASINDICE and the Table of Procedures Management System (SIGTAP), and will consider only the direct medical costs including the total cost of ownership of the technologies used in each of the scenarios of the base case, which will be verified directly with the manufacturers or distributors / representatives of the equipment in Brazil. In order to estimate the outcomes probabilities and effectiveness, a systematic review will be carried out, in accordance with a Scientific-PTC Technical Opinion, as recommended by the Methodological Guideline for Technical and Scientific Opinions, of the Brazilian Network for the Evaluation of Healthcare Technologies. Only the parametric uncertainties, which are those related to the parameters assumed and imputed in the model, will be treated. For this, probabilistic sensitivity analyzes will be performed, based on second order Monte Carlo simulations (10,000 simulations). The TreeAge® software will be used to perform the Markov modeling, to calculate the net monetary benefit and to perform the deterministic and probabilistic sensitivity analyzes, and to plot the graphs.

**Results**

Still in process.

**Conclusions**

To be done.

***Dr. Simone Vitiello***

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## MOLECULAR ASPECTS IN THE ACTIVE / INACTIVE EQUILIBRIUM OF HUMAN THYMIDILATE SYNTHASE

### Background

Human thymidylate synthase (hTS) relevance as an antitumoral drug target has been thoroughly documented in scientific literature. The folate dependent enzyme catalyzes the methylation of 2'-deoxyuridine – 5' – monophosphate (dUMP) to 2' – deoxythymidine – 5' – monophosphate (dTMP), using a methyl group given by a cofactor, the $N_5N_{10}$methylentethrahydrofolate (mTHF).

Structure has been resolved via X-ray diffraction, highlighting two monomers linked together to form the unit enzyme. In an aqueous solution, the monomeric and dimeric forms of the enzyme are at equilibrium. Structurally different from all analogous enzymes, the human thymidylate synthase sports a rather unique feature: the ability to switch between an active state and an inactive one. The active and inactive conformers have been resolved via X-ray diffraction: structure comparison among the two has produced important elements to understand such behaviour. Current literature agrees in considering two main differences, which identify each conformer. First, the orientation of loop 181 – 197, containing the Cys193 residue, responsible for the catalytic activity; second the loop 107-128, regularly defined in the active conformer, while disordered in the inactive one.

While it has been established how some molecules may affect such equilibrium, acting as "effectors" favoring one of the two conformers, current literature shows however little to none information regarding how the transition process occurs, its molecular properties, mechanisms and conditions. It is widely accepted for example, that dUMP acts as an effector towards the active conformer, while phosphate ions shift the balance towards the inactive one. Drawbacks of the current pharmacological treatment – among the most important, protein over expression leading to pharmacoresistance – call for new molecular targets to be identified and inhibited by novel molecules. Being able to obtain information regarding the structure of a certain target as well as

the molecular details of its interactions with an inhibitor may constitute a huge input in the drug development process.

**Objectives**

The long-term object of the current thesis work delves into the characterization of the molecular aspects underlining the hTS active – inactive equilibrium and the switch from one conformer to the other. Such characterization is being performed by considering different thermodynamic and kinetic observables associated with binding to effectors and the subsequent conformational switch.

One of the main objectives of last year was to assess if it was possible to purify hTS in a phosphate free buffer. Such a modification was deemed necessary as the purification buffer of election contains phosphate, one of the effectors of hTS. Furthermore, the dialysis process previously employed had some technical difficulties which lead to a purified protein loss. Another rather important objective was the analysis of the catalytic activity of the purified protein within the new buffer. The importance of this objective highlighted, if any, differences in the behaviour of the enzyme between the two buffers. Further objectives regarded the analysis of the enzyme – effector interaction in the new buffer in terms of both Isothermal titration calorimetry and spectrofluorometry.

**Methods**

hTS wild type (hTSwt) was expressed in competent BL21(DE3) cells following an established protocol. Enzyme purification has been achieved by means of a MPLC system (AKTA Prime, Amersham Industries), in a new, phosphate free medium, which eliminated the necessity of dialysis. Functionality of the newly purified protein was assessed by means of a spectrophotometric kinetic assay (Varian Cary 100 UV – Vis Spectrophotometer, Jasco V-730 UV – Vis Spectrophotometer). $K_m$ and $K_{cat}$ values of the hTSwt eluted in the phosphate free buffer has been obtained via an established protocol (Jasco V – 730 UV – Vis Spectrophotometer) and confronted to the wild type enzyme in the standard buffer. Isothermal Titration Calorimetry (MicroCal VP-ITC Microcalorimeter) and Spectrofluorometry (Horiba – Jobin Yvon Fluoromax 3) have been employed in order to follow the changes in the emission properties of important amino acidic residues – Trp and Tyr - in different concentrations of both effectors and any thermodynamic changes due to binding or structural rearrangement, respectively. Current work

regards the analysis of the spectrofluorometry data in regards of the contributes of Trp and Tyr and how their emission contributions evolve with increasing concentrations of the effectors. Concentrations in spectrofluorometry have been chosen based on those reached in the ITC. This was a mean to observe the same behaviour of the titration with effectors with two different points of view – one based on thermodynamics, the other on spectrofluorometry.

**Results**

The purification process of hTSwt in the new buffer required minor adjustments to the buffer pH, but overall results of the first batch of purification showed that the enzyme in the new buffer offers catalytic features comparable with those in the election buffer. $K_m$ and $K_{cat}$ have been calculated over the new buffer and found to be comparable with those of the enzyme in the election buffer. Confronting spectrofluorometry data of hTSwt exposed to increasing concentrations of sulphates / phosphates in both buffers exhibited some marginal differences, although the shape of the emission spectrum of Trp in the two buffers may underline more data than at a glance. ITC data issues whilst titrating with $P_i$/$S_i$ have been resolved by simply adapting the injection system to the signal intensity of the instrument. In other words, it was possible to maintain the working concentration by reducing the injection volume and increasing the amount of injections per experiment.

**Conclusions**

In conclusion, the thesis project aims at defining molecular aspects of the active – inactive switch of the human thymidylate synthase, especially by analyzing both single and multiple interactions from an energetic and a structural standpoint. Direct purification of hTSwt in a phosphate free buffer can be considered a direct improvement from dialysis. Such process proved hard to integrate in a purification protocol for two reasons. First, it led to a purified protein loss, second, it required additional time to allow proper buffer change in the purified sample, and special conditions have been ensured so that the enzyme would not precipitate in the process. Direct purification of the enzyme in a phosphate free environment eliminated such issues, granting comparable catalytic performances to the enzyme in solution within the election buffer. Spectrofluorometry data of both $P_i$ / no $P_i$ buffer hTSwt enzyme in the different titrations has been obtained from initial experiments. Data will require further analysis – peak deconvolution for instance will be used to determine if there is any contribution of Tyr to the Trp emission spectra,

which may hold interesting structural information –. Success in obtaining readable ITC data holds importance as it allows to obtain a thermodynamic point of view of the same exact process as in spectrofluorometry. Reducing injection volume has proven valuable as starting point to evolve quality of data at the cost, however, of time needed for each experiment. Such extension requires attentive care in ambient temperature control during the whole experimental procedure. There is importance in improving working conditions in such sense, as the thermodynamics of the process may hold important meaning in comprehending how the process works. Additional work will regard determination of the MEM – Maximum Entropy Method – as a mean to analyze TCSPC – Time Correlated Single Photon Counting – data, mutant purification in a phosphate free buffer and the usage of ANS – 8-Anilinonaphthalene-1-sulfonic acid – as a probe to investigate the presence of water pockets. Such experiments may be the first step towards the identification of molecular flags of active – inactive hTS in solution, as well as a step forward to the understanding of the complex molecular works underlying the active – inactive transition process.

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# XXXIII cycle

***Dr. Silvia Tanzi***

*CEM Curriculum: Translational Medicine*

*Tutor: Prof. S.Luminari*

**EARLY PALLIATIVE CARE IN HEMATOLOGIC ADVANCED PATIENTS**

**Background**

Integration of palliative care (PC) into standard oncology care has been established by the literature to be essential and it serves as a guideline all over the world. Oncological patients do not differ from hematological patients in the deterioration of several Quality of life (QoL)' dimensions. Despite this acknowledgement, hematological patients do not access palliative care services, or access only occurs in last days of their life.

**Objectives**

The aim of my PhD project is to pilot and evaluate a new integration model between Palliative care and standard hematological care in an Italian hospital.

**Methods**

My project can be interpreted as a Phase 0-II, according to the MRC Framework for the assessment of the complex interventions. Within this framework, the study can be described as follows:

- a systematic literature review on hematological patients and palliative care was done. This review provides the theoretical background and the historical comparison of the intervention (phase 0, so called *the theory*)
- the intervention: a feasibility mix method study is ongoing; a group of hematological patients (the intervention arm) receives integrated hematological care and palliative care services throughout the course of the predictive last active treatment. The intervention is compared with the standard care for hematological patients (phase I-II. *Modelling and exploratory trial*).

**Results**

Phase 0: Systematic review was conducted. Search terms were early palliative care or simultaneous care and hematologic or onco-hematologic patients. Of 233 studies 13 were included. Results were grouped into population, study design, type of intervention, outcomes/indicators and type of comparison. Population is different from one study to another including all hematologic patients or disease specific hematologic patients, in different treatment's stage and disease 'stage, inpatients and/or outpatients. The majority of the studies are retrospective, with just 2 studies randomized control trial. Palliative care Interventions also differ, from just a consultation of a Palliative care service to a more comprehensive service with mobile team, hospital beds and home care service. We divided outcomes in two categories: about efficacy on symptoms control or about Impact of palliative care intervention on aggressiveness measures in end of life and access to palliative care. Palliative care service showed an efficacy on improvement pain, depressive mood and symptom control in hematologic patients. Supportive programs improved significantly the DNR documentation in hematologic patients and Hospice referral.

Phase I-II. Patients' recruitment started in November 2018. Expected results will be the description of new feasible integration between palliative and hematological care for advanced patients. Important preliminary results on the efficacy in QoL's dimensions will be collected, such as qualitative data on patients, involved caregivers and professionals.

***Dr. Aida Meto***

*CEM Curriculum: Health Sciences*

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*Co-Tutor: Dr. Eva Pericolini*

# REDUCING MICROBIAL PLAQUE ON ORTHODONTIC CLEAR ALIGNERS BY CUPRAL:

# AN *EX VIVO* PILOT STUDY

## Background

Recently (1st PhD year), we demonstrated the efficacy of Cupral, a copper-calcium hydroxide-based compound, against microorganisms commonly harbored in the oral cavity; particularly, both planktonic cell growth and biofilm production are profoundly affected (Meto et al.; *Dental Materials Journal*, in press).

Multi-bracket orthodontic treatment enhances the risk of device-associated microbial plaque and in turn oral infections (Harradine NW, *J Orthod*. 2003). Recently, the introduction of new devices, such as clear aligners, allows teeth alignment without fixed brackets, minimizing microbial risk, dental trauma or apical resorptions. No information exists on the relevance of such orthodontic aligners as a focal point of adhesion/colonization by oral cavity microorganisms.

## Objectives

The aim of this study was to investigate the ability of Cupral to remove microbial plaque naturally produced onto orthodontic clear aligners used by healthy individuals.

## Methods

A commercially available dental paste, named Cupral, based on copper-calcium hydroxide, was used. A healthy volunteer was enrolled (female, 32 years old), undergoing orthodontic treatment using thermoplastic clear aligners (Nuvola®, Rome/Italy). Aligners were worn for two-weeks and then forwarded to the lab; during that time, regular hygiene had been performed. Three sets of aligners were examined along 1-year analysis. Microbial plaque was measured prior and following

exposure to Cupral by conventional and confocal microscopy; also, colony forming units (CFU) assay was performed.

**Results**

Confocal microscopy revealed the presence of abundant plaque, irregularly distributed onto the aligner surface; following Cupral treatment, a drastic decrease occurred in plaque thickness and matrix presence. As assessed by CFU assay, total microbial load approached $10^9$ CFU/aligner, with slight differences in aerobiosis and anaerobiosis culture conditions; several macroscopically different types of colonies were detected and 6 prevalent species were identified by MALDI-TOF. Following Cupral treatment, microbial load dropped to undetectable levels, irrespectively of the conditions considered.

**Conclusions**

Exposure of clear aligners to Cupral results in the elimination of contaminating microorganisms; the antimicrobial activity is retained up to 1.25% concentration.

Overall, our data open to a novel use of Cupral, a copper-calcium hydroxide-based compound commonly used in clinical endodontic practice, in daily hygiene practices with promising results.

***Dr. Michele De Maria***

*CEM Curriculum: Translational Medicine*

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*Co-Tutor: Dr. Domenico Merlo*

## ANALYSIS OF INTRAOCULAR INFLAMMATION AFTER EYE SURGERY: NON-STEROIDAL ANTI-INFLAMMATORY DRUGS VERSUS CORTICOSTEROIDS

### Background

The past decades have witnessed a surge in the scenario of eye surgery because of the continuous development of new surgical techniques, more sophisticated instrumentations, and innovative eye drugs and delivery systems. However, managing intraocular inflammation and preventing its complications are topics of debate among ophthalmologists worldwide. It is well known that uncontrolled inflammation after eye surgery increases the risk of post-operative complications. Several studies have shown the positive effect of corticosteroids, and non-steroidal anti-inflammatory drugs (NSAID) eye drops, administered alone or in combination, for the treatment and prevention of intraocular inflammation, especially after cataract surgery.

Despite a large number of published papers on this topic, no widely accepted guidelines for the post-operative use of these drugs have been published. Still, scientific evidence is much needed to establish a better therapeutic regimen to increase postoperative outcomes and reduce complications.

### Objectives

The project aims to enhance the current knowledge regarding topical NSAIDs versus standard treatment with topical corticosteroids after eye surgery. The main objectives of this project are:

- to evaluate the comparative effectiveness of topical monotherapy with NSAIDs versus corticosteroids in controlling intraocular inflammation after uncomplicated cataract surgery (Bromfenac vs. Dexamethasone Study - *BVD Study*, *Preoperative NSAID Treatment Study – PreTreat Study*);
- to adopt the laser flare photometry (LFP) to obtain a quantitative and non-invasive assessment of the anterior chamber inflammation (*BVD Study*, *PreTreat Study*);

- to review the method of analysis of anterior chamber inflammation in order to shed the light on the necessity of quantitative and comparable data especially in the setting of clinical trials.

**Methods**

The *BVD study* is a phase IV, single center, randomized, active-control, parallel design, open-label trial comparing Bromfenac 0.09% solution and Dexamethasone 0.1% suspension in patients that underwent uncomplicated cataract surgery. Patients with a senile cataract and no other ocular comorbidities who underwent uneventful phacoemulsification were randomized 1:1 to receive dexamethasone 0.1% ophthalmic suspension or bromfenac ophthalmic solution 0.09% for two weeks. All patients underwent a complete ophthalmological evaluation on the day before surgery and postoperatively at day 1, 3, 7, 9, 11, 14, and 30. The LFP was used to objectively quantify anterior chamber inflammation and optical coherence tomography (OCT) to measure macular thickness.

After 6 months from surgery, all patients were recalled to perform the visual acuity assessment, the LFP and the OCT to analyze the anterior chamber inflammation and the macular thickness in a long-term follow-up.

**Results**

Seventy-six patients (37 in Dexamethasone group and 39 in Bromfenac group) with senile cataract and no other ocular comorbidities have been enrolled. Dexamethasone was not superior to bromfenac to reduce inflammation to the preoperative level. In both treatment groups, the mean LFP value increased the day after surgery and progressively decreased after starting the topical therapies, with no return at baseline at day 30. There was no statistically significant difference at all time points between dexamethasone and bromfenac groups in terms of mean laser flare. Visual acuity improved steadily after surgery in both groups. The mean macular thickness was higher in the dexamethasone group one month after surgery (293.51 ± 61.55 vs. 274.82 ± 28.17 microns; p = 0.0467). The proportion of patients with central macular thickness >300 microns at day 30 did not differ between treatment groups.

After 6 months, the proportion of patients with a laser flare value back to baseline was 43.24% (n=16) and 43.59% (n=17) in dexamethasone and bromfenac respectively (chi-square = 0.04, p =

0.97). The proportion of patients who developed a cystoid macular edema CME was 13.5% (n=5) and 0% (n=0) in Dexamethasone and Bromfenac group respectively (chi-square = 5.64, p=0.0175).

**Conclusion**

Bromfenac and Dexamethasone eye drops appear equally effective in reducing anterior chamber inflammation measured by laser flare after uncomplicated phacoemulsification. The presence of a mean LFP value higher than the baseline at 6 months revealed a faint inflammatory process that lasts several months after surgery and may be one of the possible causes for a late-onset CME. However, the long term OCT analysis revealed a better proficiency of bromfenac in reducing the risk of cystoid macular edema both in the short- and long-term.

***Dr. Mariarosa Maiorana***

*CEM Curriculum: Translational Medicine*

*Tutor: Dr. Francesco Iannuzzella*

**ROCK Study: A Registry study Of Cancer-associated Kidney disease**

**Background**

Onco-nephrology is a new and evolving subspecialized area in Nephrology that deals with kidney diseases in cancer patients.

Chronic kidney disease (CKD), acute kidney injury (AKI) and cancer are connected in several ways. Not only can cancer lead to the development of CKD but also presence of CKD can be associated with cancer. Although the overall incidence and prevalence of CKD among cancer patients are still uncertain, there is growing evidence to suggest that the risk is high and still increasing.

The improvement in the survival rates of neoplastic patients due to the new chemotherapeutic agents, including biological drugs, has increased the number of patients who develop renal disease due to neoplasia.

However, there are no definitive and solid data on the frequency of AKI and CKD in neoplastic patients in relation to the stage of renal disease and the characteristics of progression.

**Objectives**

The aim of the study is to estimate the prevalence and incidence of CKD and the incidence rate of AKI in patients included in the Cancer Registry of the province of Reggio Emilia from 1 January 2016 to 31 December 2016; in addition, to evaluate the effect of acute kidney injury on clinical outcomes of mortality and progression of nephropathy.

Furthermore, to set up an onco-nephrological bundle both for research and diagnostic-therapeutic applications and to identify a specialized nephrological counseling intervention for high-risk patients.

**Methods**

We made a monocentric, observational, retrospective study collecting data from patients included in the Cancer Registry of the province of Reggio Emilia from 1 January 2016 to 31 December 2016.
Inclusion criteria were: - ppatients included in the Cancer Registry with diagnosis of cancer; - age > 18 years. Exclusion criteria were: age < 18 years; - diagnosis of chronic myeloproliferative disorders and myelodysplastic syndromes; - diagnosis of non-melanomatous skin cancers.
For each patient, we collected the following data: sex, age, ethnicity, weight, serum creatinine and urea, haemoglobin, type of cancer, possible metastases, anticancer drugs prescribed, hospitalizations.
Clinical data were used to defined prevalence and incidence of CKD based on stage of kidney disease, type of cancer and risk of progression of kidney disease; to defined the incidence rate of AKI in oncological patients with previous normal renal function and its effects on mortality.

**Expected results**

The application of measures to reduce the risk of AKI, especially in high-risk patients, would reduce its incidence, the risk of progression of CKD and improve the outcomes of hospitalized patients with cancer.
The risk factors, confirmed by prospective studies, could be used to create a predictive score to identify high risk patients for developing AKI.
Furthermore, data obtained from the study will be evaluated for epidemiological purposes.

***Dr. Francesco Venturelli***

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## NEW TOOLS FOR HPV-BASED CERVICAL CANCER SCREENING

### Background

The new challenge in cervical cancer prevention is the reduction of over-diagnosis and over-treatment. These phenomena may increase with the introduction of the HPV test. Indeed, since the HPV-DNA test is less specific than Pap-test, we need evidence-based guidelines defining the best management of HPV positive women and how to use available biomarkers. We need triage tests to reduce colposcopy referral of HPV-DNA positive women, and we need appropriate follow up strategies for women who had a colposcopy and received a treatment for a high-grade cervical intraepithelial neoplasia (CIN2 or CIN3).

### Objectives

- To assess the accuracy of biomarkers (HPV E6/E7 mRNA and p16/Ki67) as test of triage in HPV-DNA based screening protocols.
- To assess the prognostic value of biomarkers (HPV E6/E7 mRNA and p16/Ki67) for the identification of regressive lesions.
- To update guidelines for the follow up of women treated for CIN2 and CIN3 with evidence-based recommendations.

### Methods

Reggio Emilia is coordinating the follow up and the analyses of the New Technologies in Cervical Cancer 2 (NTCC2) double testing accuracy. NTCC2 aims to measure the accuracy of mRNA and p16/Ki67 and their negative predictive value for CIN2 or more severe lesions (CIN2+). The project recruited women who were invited for a new screening round based on HPV-DNA test within the

screening programs (i.e. aged 25-59). HPV-DNA positive women were tested for cytology, E6/E7 mRNA and p16/Ki67. Women with ASC-US or more severe (ASC-US+) cytology were referred to colposcopy whereas women with negative cytology were randomized to immediate colposcopy or to 1-year follow up by HPV-DNA test. All women referred to 1-year control were tested for HPV-DNA, mRNA and p16. Women negative to all tests were referred to 4-year follow up. Regardless of mRNA and p16 results, HPV-DNA positive women were referred to colposcopy. The final endpoint of the study is the confirmed CIN2+. All the lesions found during the 5.5-year follow up will be included in the endpoint. A preliminary analysis on data from 3 of the 5 recruiting centres was performed.

Preliminary analyses on the cross-sectional accuracy and the role of mRNA as predictor of CIN2+ and infection persistence were performed.

Regarding the third objective, as part of the Italian Group for Cervical Cancer Screening (GISCi), we started an evidence-based revision process of protocols for the follow up of women treated for CIN2 and CIN3.

**Results**

To date, the recruitment phase of NTCC2 and the 12-month follow up has been completed including 41,127 women. Preliminary analyses included 33,388 women, of which 2354 (7.1%) were HPV-DNA positive. The results of both mRNA and cytology were available for 2333 HPV-DNA positive women: 1617 (69%) were mRNA positive and 651 (28%) were cytology positive. Cumulatively, 125 CIN2+ (63 CIN2 and 62 CIN3) were found considering the first colposcopy performed at baseline or 12 months follow up.

The sensitivity was 96% (120/125; 95%CI 91-99) and 69% (86/125; 95%CI 60-77) for mRNA and cytology, respectively.

The estimated Positive Predictive Value (PPV) was 10% for mRNA (120/1215; 95%CI 8-12) and 15% for cytology (86/581; 95%CI 12-18).

Among the 788 HPV-DNA positive/cytology negative women randomized to immediate colposcopy, 22 CIN2+ (14 CIN2 and 8 CIN3) were detected (2.8%), while among the 894 randomized to 1 year HPV re-testing, 754 completed the follow up and 17 CIN2+ (10 CIN2 and 7 CIN3) were found (2.3%)(RR 0.81;95% CI 0.43-1.52).

The 5 mRNA negative CIN2+ were found after immediate colposcopy; of these 2 (1 CIN2 and 1 CIN3) were cytology positive and 3 (all CIN2) were cytology negative.

Finally, evidence from the literature on the identified PICOs were retrieved, assessed and synthetized in Summary of Findings and Evidence-to-Decision tables on the GRADEpro online tool. This will be the base for the discussion and appraisal process with the Panel members in plenary sessions to formulate recommendations.

**Conclusions**

E6/E7 mRNA assay would refer to immediate colposcopy more than two third of HPVDNA-positive women, but its sensitivity is very high and could allow longer interval for retesting.

Similar detection of CIN2+ after immediate colposcopy and 1-year HPV retesting suggests limited regression, if any, of CIN2+ in one year.

Quick regression of mRNA negative CIN2+ could explain the lack of mRNA negative CIN2+ observed in the retesting arm if confirmed on larger scale.

The data collection on the 12 months follow up from all the five recruiting centres is ending.

Updated GISCi guidelines are expected to be published by the end of 2019.

Complete analysis on baseline and 12-month follow up will be available in the beginning of 2020.

Data on the 5-year follow up will be available by the end of 2021.

***Dr. Giulia Besutti***

*CEM Curriculum: Translational Medicine*

*Tutor: Dr. Paolo Giorgi Rossi*

*Co-Tutor: Prof. Guido Ligabue*

# EFFICIENCY AND APPLICABILITY OF INTERNATIONAL GUIDELINES FOR NON-ALCOHOLIC FATTY LIVER DISEASE ASSESSMENT IN HIGH RISK PATIENTS AND ACCURACY OF IMAGING TESTS IN THE DIAGNOSIS OF NON-ALCOHOLIC STEATOHEPATITIS AND FIBROSIS

## Background

Patients with type 2 diabetes mellitus (T2DM) have a high prevalence of Non-Alcoholic Fatty Liver Disease (NAFLD) and Non-Alcoholic Steatohepatitis (NASH), which can progress to fibrosis, cirrhosis, and hepatocellular carcinoma. International societies for the study of the liver proposed recommendations for the diagnosis and treatment of NAFLD, not yet validated in high-risk patients.

Liver biopsy is the gold standard for NAFLD assessment. Given the upcoming introduction of new therapies, the lack of a non-invasive tool to identify patients who need treatment is a central issue. New ultrasound (US) and magnetic resonance (MR) techniques have shown promising results in diagnosing NASH and fibrosis.

## Objectives

**AIM 1:** Preliminarily, a quality assessment of NAFLD guidelines was conducted. The primary objective is to evaluate guideline efficiency in diagnosing NASH/fibrosis among high-risk patients, by calculating the positive predictive value of specialist referral (PPV1) and liver biopsy (PPV2). Secondary objectives are to evaluate: 1) PPV1 and PPV2 in subgroups with different criteria for referral; 2) burden of clinical examinations generated by the recommendations; 3) number of subjects excluded for other liver conditions; 4) adherence to referrals.

**AIM 2:** Preliminarily, we conducted a systematic review on the accuracy of imaging tests in NASH diagnosis. The primary objective is to evaluate sensitivity and specificity of imaging methods (US and MR) in the diagnosis of NASH/fibrosis in high-risk NAFLD patients, using liver biopsy as the reference. Secondary objectives are to evaluate the association of imaging biomarkers with

histologic NAFLD biomarkers (steatosis, activity and fibrosis), clinical and anthropometric characteristics of the included patients.

**Methods**

**AIM 1:** The AGREE2 tool was used to evaluate the quality of the existing NAFLD guidelines. In an ongoing pilot observational study, T2DM patients are stratified in different risk categories based on fatty liver index (FLI), liver enzymes and NAFLD fibrosis score (NFS). As proposed by the guidelines, higher risk patients are referred for US and, if liver steatosis is confirmed, for hepatologist evaluation. If other liver disease and other causes of steatosis are excluded, the hepatologist may refer patients for liver biopsy. An expert pathologist evaluates and scores steatosis grade, NASH and fibrosis. To evaluate guideline efficiency, PPV1 - PPV2 for NASH/fibrosis diagnosis, and the burden of clinical examinations generated by the recommendations will be calculated.

**AIM 2:** We included in the systematic review cross-sectional/cohort studies of NAFLD patients comparing imaging with histology. MEDLINE, Scopus, EMBASE and Cochrane databases were searched. Studies were screened on title/abstract and assessed for eligibility on full-text. Risk of bias was assessed using QUADAS-2 tool. In an ongoing prospective study, high-risk NAFLD patients referred for liver biopsy undergo: 1) US including US-fatty liver indicator and Shear Wave Elastography, 2) multiparametric liver MR without contrast administration, obtaining multiple biomarkers. Sensitivity and specificity of different imaging tests, alone and in combination, for NASH and fibrosis diagnosis will be calculated, using liver histology as the reference.

**Results**

**AIM 1:** According to the AGREE II criteria, American and European guidelines had the highest scores. In the last 3 months, guidelines have been applied to 95 T2DM patients (35% females, 63.2±11.1 y/o) at the first access to the Reggio Emilia diabetology clinic. Of them, 12 were aged >75 y/o and 7 had other chronic liver disease/causes of steatosis. Of the remaining 76 patients, 36 had incomplete serum markers to calculate risk and will be re-evaluated, 13 needed no further referrals, 27 performed liver US (18 for elevated liver enzymes and 9 for high FLI/NFS). Of these 27 patients, 23 had liver steatosis confirmed and were referred to the hepatologist. Among the 12 patients already evaluated by the hepatologist, 5 were referred for liver biopsy.

**AIM 2:** In the systematic review on imaging diagnosis of NASH, of the 641 records screened, 61 were included in a scoping review, and 30 with accuracy results in data synthesis. Imaging techniques included: US and MR elastography and not-elastographic techniques, computed tomography and scintigraphy. Histological NASH definition was heterogeneous. In 28/30 studies, no prespecified threshold was used (high risk of bias). AUROCs were up to 0.82 for transient elastography, 0.90 for US-based elastography, 0.93 for MR elastography, 0.82 for US scores, 1.00 for MR spectroscopy, 0.91 for MR imaging techniques. Results derived mostly from single studies without independent prospective validation.

**Conclusions and future perspectives**

Even if in a small preliminary analysis, the application of recommendations to a selected cohort of high-risk patients is generating a high burden of hepatologist evaluations and liver biopsies. Future results (AIM 1) will help assessing the feasibility of recommendations and will allow the evaluation of different scenarios according to changes in the referral criteria. Our systematic review demonstrated that there is currently insufficient evidence to support the use of imaging to diagnose NASH. More studies are needed on US and MR elastography and non-elastographic techniques, the most promising, harmless and feasible methods. With our study (AIM 2) we expect to contribute in the identification of the combination of imaging tests which is accurate enough to potentially replace histologic evaluation.

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## EUROPEAN COHORTS OF OLDER HIV ADULTS: POPPY, AGEHIV, GEPPO, COBRA AND FUNCFRAIL

### Background

The recent and rapid demographic changes affecting people living with HIV (PLWH) produced a subset of older adults demanding a prompt response both in clinical practice and research setting. The unmet medical needs of this emerging population could be addressed with existing large observational HIV cohort studies, but unluckily older adults are rarely represented in these cohorts. Therefore, the scientific community had to properly design studies that include older people living with HIV (OALWH); aged more than 50 years, or geriatric PLWH, aged 65 years or more, in order to explore the interaction between aging and HIV itself, antiretroviral therapy (ART) and non-infectious co-morbidities (NICM).

Choosing between aging (50+) and geriatric (65+) cohorts may represent a trap, but also a possibility to measure different outcomes and obtain different evidence. HIV aging cohorts (50+) are individuals who tend to accentuate age-related NICM in comparison to the general population, but might be still too young to experience a significant burden of geriatric syndromes and frailty. Geriatric PLWH (65+) represent a heterogeneous population almost equally distributed between people aging with HIV and people who acquired HIV at an older age. Designing a geriatric HIV cohort with HIV-negative controls allows to compare clinical presentations using the comprehensive geriatric assessment (CGA) having the opportunity to consider HIV as one of the many determinants of frailty.

### Objectives

The aim is to describe ongoing aging HIV cohorts that include older (>50 years) or geriatric (>65 years) PLWH and present the key results obtained in those studies.

**Methods**

In Europe, there are ongoing cohorts that comprise OALWH or geriatric PLWH: AGEhIV, POPPY, GEPPO, FUNCFRAIL and COBRA. The AGEhIV cohort study from the Netherlands started in 2010 and was the first in enrolling OALWH, including people above the age of 45. Consecutively, in 2013 POPPY study was established in the UK and Ireland, addressing people being at least 50 years old, but also PLWH below 50 years as controls. FUNCFRAIL, the Spanish cohort initiated in 2018, follows the same age criteria and has started recruitment in May 2018. The Italian GEPPO cohort from 2015 is the only study that enrols people above the age of 65, traditionally considered the geriatric age. All the studies except FUNCFRAIL have at least one control group. All the cohorts share a prospective observational study design and are multicentre, with the only exception for AGEhIV. While all studies enrol OALWH that meet age criteria, POPPY only require as inclusion criteria a sexual route of acquisition of HIV. All these cohorts share similar study aims such as prevalence of NICM, the impact of age(ing) on ART efficacy, drug-drug interactions between ART and NICM therapies, incidence of and risk factors for geriatric syndromes. All studies also share similar tools to assess outcomes - blood and urine tests, detailed medical history, questionnaires, DEXA scan. Although all studies collect data regarding geriatric syndromes, only GEPPO and FUNCFRAIL incorporate a CGA.

**Results**

A few studies have shown that PLWH had higher levels of immune activation, regulatory T-cells, PD-1 expressing CD4+ cells and shorter telomeres. Several studies explored prevalence, risk factors and management of single NICM in OALWH and geriatric PLWH. Moreover, in a recently published study of a two-independent dataset of POPPY and AGEhIV cohorts that included more than 1500 OALWH, it was observed that NICM tend to occur in non-random patterns. Two studies examined the overall prevalence of NICM and MM between OALWH and HIV-negative controls. The AGEhIV study showed that OALWH (n=540) had a notably higher mean number of NICM compared to matched HIV-negative individuals (n=524) - 1.3 (±1.14) vs 1.0 (±0.95); $p<0.001$; with a greater prevalence of more than one NICM (69.4% vs 61.8%; $p=0.009$). The GEPPO cohort compared the prevalence of and risk factors for NICM in 1258 geriatric PLWH and 315 HIV-negative controls. The prevalence for NICMs was the same in the two groups except for dyslipidaemia which was more frequent in geriatric PLWH.

**Conclusions**

The already present demographic transition affecting PLWH are not paralleled by significant changes in clinical management. Existing aging HIV cohorts are pointing out unmet medical needs of OALWH but are still not representative of the entire European HIV aging epidemic. In order to better describe aging trajectories in this population, it is essential to collect and measure immunological biomarkers. Moreover, there are no studies designed to detect best ART strategies in this population and various outcomes that go beyond the viro-immunological success, such as frailty, geriatric syndromes, physical function, disability, quality of life and healthy aging are still not routinely part of aging cohorts. Results from such designed aging HIV observational studies will also inspire randomized clinical trials. Also, results from aging cohorts with outcomes that go beyond the undetectability will pave the way to health care providers to encounter unmet needs of OALWH.

*The full paper is available on: https://link.springer.com/article/10.1007/s41999-019-00170-8*

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# NEUROPLASTICITY IN CHOLINERGIC PROJECTIONS FROM HORIZONTAL LIMB OF DIAGONAL BAND-MAGNOCELLULAR PREOPTIC NUCLEUS COMPLEX TO BASOLATERAL AMYGDALOID NUCLEUS IN THE MODEL OF TEMPORAL LOBE EPILEPSY BY KAINIC ACID IN RATS

## Background

Epilepsy is a group of brain diseases characterized by spontaneous recurrent seizures, affecting seventy million people around the world. Temporal lobe epilepsy (TLE) is the most prevalent type epilepsy type (~75%) in adults. The model of TLE post kainic acid-induced status epilepticus (SE) in rats mimics many aspects of this disease in humans. Although huge advances in neuroplastic alterations underlying epilepsy and its comorbidities were made in the last five years, much of such mechanisms are to be enlightened. Recent studies from our collaborators reported for the first time neuroplastic alterations in septo-hippocampal and mesopontine-thalamic cholinergic systems. Therefore, we aimed to investigate whether remodeling is also present in other cholinergic projections from basal forebrain in the kainate model of TLE.

## Objective

Given the remarkable shrinkage of basolateral amygdaloid nucleus (BLA) and its strong immunostaining for vesicular acetylcholine transporter (VAChT), we assessed the horizontal limb of diagonal band/magnocellular preoptic nucleus (HDB/MCPO) cholinergic projections to BLA in TLE model of post-SE induced by kainic acid in rats.

## Methods

For this purpose, adult male Sprague-Dawley rats were treated with either kainic acid (25 mg/kg i.p., KA group, n = 5) or the same amount of vehicle (saline i.p., Ctrl group, n = 6). Rats were monitored for Racine's stage ≥3 spontaneous seizures by means of video-EEG 8 h/day every other day, starting from14$^{th}$ day post-SE. After 7 weeks, rats were deeply anesthetized with ketamine 90 mg/kg i.p., and transcardially perfused with 4% PFA in PBS pH 7.4. The brains were removed, post-

fixated for 24 h, embedded with sucrose 30%, cut into 40 µm sections with vibratome, and stored in glycerol-PBS at -20 ºC until use. A standard immunostaining procedure was carried out with polyclonal primary antibodies against vesicular acetylcholine transporter (VAChT). After mounted, slices were visualized in an AxioScope microscope equipped with an AxioCam MRc5 digital camera (both Zeiss, Germany). For varicosity density evaluation, using 100X objective lens, 3 non-overlapping images were taken from basolateral amygdaloid nucleus (BLA), bilaterally, in 3-4 sections per animal. Images were analyzed by a macro tool from ImageJ software: a 1274 $\mu m^2$ counting frame was applied on each image, measuring the number and cross-sectional area of each varicosity between 0.03 and 1.59 $\mu m^2$ within the frame. Results of each animal were normalized for an arbitrary area of 30.000 $\mu m^2$. From that, a frequency histogram was plotted by grouping varicosities in bins of 0.04 $\mu m^2$. Using 100X objective lens, images were taken from approximately 30 neurons from HDB/MCPO across at least 4 sections per animal. After scale calibration, length and width of neuronal perikarya were taken by the ImageJ software. Also, the same sections were visualized under an Olympus BX-53 microscope equipped with a computer-controlled 3D motorized stage with the Stereo Investigator (SI) software (Williston, USA). For measurement of BLA volumes and estimation of cholinergic neuronal numbers in HDB/MCPO, the Cavallieri reconstructor and the Optical fractionator stereological protocols from the SI were respectively used unilaterally in 6-8 and 4-6 slices per animal. Statistics was carried out by two-tailed parametric Student's T test in Prism 6 software.

**Results**

Although the density of varicosities in BLA was not significantly altered, a trend of frequency increasing observed in the first three frequency bins in the kainate group might represent a mild sprouting of thin cholinergic fibers. The volumes of BLA, as measured by Cavalieri reconstructor tool of SI, showed a significant volume loss, about 40%, in kainate treated animals (1.498 ± 0.12; 1.09152 ± 0.37; expressed as Mean ± SEM, in $mm^3$, for Ctrl and KA groups, respectively), $p$ = 0.0342. The estimation of volume of cholinergic cell bodies in HDB/MCPO evidenced significant smaller cells in the kainate treated group (241.53 ± 53.59; 80.44 ± 3.54, as Mean ± SEM, in $\mu m^3$, for Ctrl and KA groups, respectively), $p$ = 0.0238. The estimations of total numbers of cholinergic neurons in HDB/MCPO complex in control and kainate treated animals are to be completed soon.

**Conclusion**

Our results points that an important remodeling of cholinergic pathways to amygdaloid body takes place during the epileptogenic process in the model of TLE induced by kainic acid in rats. Those neuroplastic alterations might potentially contribute to epilepsy and its comorbidities. In spite of the loss of volume and of susceptible cell populations in amygdaloid complex have been longly described in rodent models of TLE, to the best of our knowledge, the present report is the first assessment of the state of cholinergic projections from HDB/MCPO to BLA in this model.

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**DEEP CHARACTERIZATION OF BIO-MOLECULAR PATTERNS IN METASTATIC COLORECTAL CANCER: AN EXPERIMENTAL ANALYSIS ON A SURGICAL COHORT**

**Background**

Metastases from CRC are the leading cause of cancer-related mortality and are present in approximately 25% of patients at diagnosis. The liver is the most common site of metastases from CRC and about 50% of patients will develop hepatic metastases during the course of disease. The lung is the second most frequently involved site in metastatic disease and affects 10% - 15% of patients with CRC at diagnosis.

Rewarding survival rates have been reported after resective surgery of isolated hepatic or pulmonary metastases (25% - 58% and 32% - 68% at 5 years, respectively). Currently, there are few data on long-term results in colorectal cancer patients who underwent surgery for both hepatic and pulmonary metastases after colorectal resection. In such patients, the standard of treatment is still under debate. However, one of the main issues regards of the lack of bio-molecular factor to be considered when stratifying the long-term prognosis and personalizing the treatment of such patients.

Therefore, surgical resection of both hepatic and pulmonary metastases is nowadays recommended based only on clinical aspects, while genetic/bio-molecular aspects involved in colorectal tumor carcinogenesis have been poorly investigated.

**Objectives**

*AIM 1:* We will analyze the long-term results (overall survival and progression-free survival) of a large surgical series of patients undergoing surgery for both hepatic and pulmonary metastases after colorectal cancer resection, in order to identify every clinical, pathological or surgical variable able to influence long-term outcomes.

*AIM 2:* We will perform a lab analysis of the surgical specimens, consisting of deep genetic sequencing with the double aim of exploring the bio-molecular alterations in both metastatic sites, and comparing them with the bio-molecular profile of primary colorectal cancer.

**Methods**

*AIM 1:* A retrospective analysis of clinical, pathological, surgical and follow-up information will be performed using a dedicate anonymous database. All consecutive patients who underwent surgery for hepatic or pulmonary colorectal metastases in two Institutions (Presidio Ospedaliero Arcispedale Santa Maria Nuova di Reggio Emilia – Azienda Unità Sanitaria Locale-IRCCS di Reggio Emilia and the Catholic University of the Sacred Heart in Rome) from January 1993 to June 2015 will be enrolled in the present study. Patient, treatment, and outcomes variables were analyzed using Log-rank, Cox regression, and Kaplan-Meier methods.

*AIM 2:* We will collect specimens of primary site (colorectal cancer) and metastatic site (liver and lung) at the lab of Promoting Center (Azienda Unità Sanitaria Locale-IRCCS di Reggio Emilia). From matched primary and metastatic lesions, DNA extraction from formalin fixed paraffin embedded (FFPE) tissues will be performed using Maxwell nucleic acid extractor (Promega). NGS approach will be used to sequence hot spot regions within a panel of 56 genes whose alteration has been shown to be clinically relevant in several cancers. Thus, NGS libraries will be prepared using Myriapod ® NGS-IL 56G Onco panel (Diatech pharmacogenetics srl, Jesi, Italy). Sequencing runs will be performed by MiSeq platform (Illumina) on v2 cartridge (2x151). Primary and secondary analyses of NGS data will be performed by Myripod NGS data analysis software (NG 900-SV, Diatech pharmacogenetics srl, Jesi, Italy). Mutations will be considered reliable with a minimum frequency of 5% and a minimum coverage of 500X. Focusing on functional modifications we will exclude from the final list all non-coding mutations and considered only variants responsible for a missense, frameshift, deletion or start/stop gain. Frequency distribution of identified mutations and comparison between primary and metastatic lesions will be performed using Fisher's exact test and generalized linear models. All statistical analyses will be performed using R Software v 3.5.1.

**Results and expected results**

*AIM 1:* Liver turned out as the first site of metastasis in 75% patients, lung in 13% patients, and both sites in

12% patients. Multiple hepatic metastases were detected in 67% patients and pulmonary metastases in 31% patients. Two hundred eighteen surgical interventions were performed (mean 2.56 for each patient). Overall survival (OS) rates at 3-, 5-, and 10-year follow-up from colorectal resection were 94, 79, and 38% respectively. Median OS was 8.31 years. Survival turned out significantly longer for patients with disease-free interval (DFI) exceeding 1 year between first metastasectomy and diagnosis of second metastases and in patients affected by metachronous pulmonary metastases.

*AIM 2:* We will explore the complexity of the genetic profile of metastatic colorectal cancer, by investigating bio-molecular alterations of 56 genes directly involved in cancerogenesis. Moreover, by comparing the genetic profiles of metastatic sites and primary tumor, we will investigate the association between genetic asset and cancerogenetic process. These speculative results could be the proof of principles for future pre-clinical or experimental analyses on this issue.

**Conclusions**

*AIM1:* Surgical resection of both hepatic and pulmonary metastases of CRC represents a safe and effective

treatment. It might lead to rewarding long-term survival rates in high selected patients. Shorter DFIs between first metastasectomy and diagnosis of second metastases can determine worse prognoses. In addition, poor outcomes could be predicted also for patients affected by synchronously detected pulmonary CRC metastases, although further confirmatory analyses are strongly required.

*AIM2:* Bio-molecular analyzes are still ongoing. No conclusion is currently available.

# XXXIV cycle

***Dr. Salihanur Darici***

*CEM Curriculum: Nanomedicine, Medicinal and Pharmaceutical Sciences*

*Tutor: Prof. Sandra Marmiroli*

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# COMBINATION OF PI3K/mTOR INHIBITORS WITH STANDARD CHEMOTHERAPEUTIC AGENTS TO OVERCOME RESISTANCE RESULTING FROM THE PROTECTIVE ROLE OF THE BONE MARROW ENVIRONMENT IN ACUTE LEUKAEMIA

## Background

Acute myeloid leukaemia (AML) is a heterogeneous malignant haematopoietic disorder, characterized by the accumulation of immature blast cells in the bone marrow and peripheral blood. Despite the characterization of several putative therapeutic targets, standard AML therapy still relies on the use of chemotherapeutic agents that often leads to therapy resistance and subsequent relapse, driving an unquestionable need of new therapeutic options. Thus, in addition to novel cytotoxic agents able to restore the sensitivity of AML cells to chemotherapy, epigenetic modifiers of transcription and cell cycle regulation are increasingly the focus of research. One of the most promising research areas in oncology is the development of small molecule therapeutics based on inhibition of the PI3K/AKT/mTOR pathway, a master regulator of most cellular processes including differentiation, proliferation, survival and metabolism, as well as of leukemic transformation. However, hitherto targeting key effector molecules of this pathway failed to demonstrate durable therapeutic effects, mainly owing to the onset of compensatory signalling leading to relapse. On the other hand, mounting evidence highlights the significant contribution of the bone marrow environment to leukemogenesis and drug resistance. As such, it is understood that the bone marrow niche serves as a sanctuary for quiescent leukaemia stem cells, promoting growth and survival. One possible approach to overcome resistance promoted by the bone marrow niche is to combine standard chemotherapy with targeted inhibition.

## Objectives

1. Describe the molecular characteristics, as well as the phosphoproteomic profile and energy metabolism of AML cell lines and primary cells.

2. Based on 1) evaluate the potential of combination therapy consisting of standard chemotherapeutic agents and second-generation PI3K/mTOR inhibitors as an alternative therapeutic approach.
3. Identify key features of bone marrow environment that modulate the response to PI3K/mTOR inhibitors.

**Methods**

In this study, preclinical models including fully characterized cell lines, primary patient cells and patient-derived xenografts (PDX) will be used. First, optimal drug combinations consisting of dual PI3K/mTOR inhibitors and standard chemotherapeutic agents (cytarabine and etoposide) will be determined by means of cytotoxicity assays. Specific inhibition (e.g. phosphorylated AKT) will be confirmed by Western blot. Next, cell models will be characterized by next-genome sequencing, reverse phase protein array (RPPA) and Seahorse assay (energy metabolism); and the ability of combined treatments to modulate this profile will be studied. To address the contribution of the bone marrow environment, aforementioned conditions will additionally be evaluated in hypoxic coculture with human stromal cell line Hs5. Obtained result will be confirmed using a murine PDX model, demonstrating the clinical relevance of our studies. Moreover, cytokine profiling will be performed to identify key components within the coculture model that contribute to drug resistance that could potentially be targeted. Finally, according to the obtained results, attractive combinations of signalling inhibition and standard chemotherapy will be proposed to improve patient-specific stratified strategies.

**Expected results**

We expect that by selecting drug combinations based on the mutational, phosphoproteomic and metabolic profile, we will demonstrate critical features toward precision medicine in AML. Moreover, by dissecting the interplay between leukaemia cells and the surrounding bone marrow environment, we expect to improve our current understanding of mechanisms of therapy resistance and potentially identify novel therapeutic targets.

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**THE COMORBILITY BETWEEN METABOLIC SYNDROME AND ANXIOUS-DEPRESSIVE SYMPTOMS: THE ROLE OF DIET AND PERSONALITY TRAITS ACCORDING TO A PSYCHO-NEURO-IMMUNO-ENDOCRINOLOGICAL (PNEI) PERSPECTIVE**

**Background**

In Western societies, cardiometabolic diseases and psychiatric disorders are among the leading causes of morbidity and disability and their association has been investigated in recent years in the perspective of interdisciplinary psycho-neuro-immuno-endocrinological (PNEI) integration.

Recent evidence suggests the association between Metabolic Syndrome (MetS), a set of cardiovascular risk factors, and anxious-depressive symptomatology, in outpatients, suggesting that associations are many but often underdiagnosed and undertreated.

Both MetS and anxiety-depressive symptomatology could be related to high levels of psychosocial stress and to an impaired quality of life, through still unclear mechanisms, albeit chronic and subclinical inflammation has been suggested as a possible link.

Dietary habits could also represent a possible mediator of this association. However, little information is available on the relationship between dietary intake and mood symptoms in patients with MetS.

**Objectives**

Primary aim is to evaluate the association between modifiable lifestyle factors (nutrition, stress level and quality of life) and symptoms of anxiety and depression in outpatients with MetS.

Secondary aim is to assess the possible role of chronic and subclinical inflammation as a mediator of these conditions.

**Methods**

In this cross-sectional study, outpatients of both gender, aged between 18 and 80 years, referred to a Nutritional clinic in Modena University hospital for assessment will be enrolled.

Patients will be excluded if they have been diagnosed with disorders or prescribed with medications primarily affecting immune or inflammatory system activity, such as anti-inflammatory drugs. Moreover, patients affected by the following conditions will be excluded because their potential with the major focus of the research: prior stroke, heart attack or other cardiovascular disease; type 1 diabetes mellitus; obesity of genetic aetiology; pregnancy; current psychopharmacological therapy or estro-progestinic medications; positive history of major psychiatric disorders (schizophrenia spectrum or bipolar disorders).

For each enrolled patient, sociodemographic and clinical information will be collected: height (cm), weight (kg), waist circumference (cm), arterial blood pressure (mmHg), low and high density lipoprotein cholesterol (mg/dL), triglycerides (mg/dL), fasting plasma glucose (mg/dL), c-reactive protein and serum cytochines (IL-1, IL-6, TNFa, IL-10, Kynurenine).

MetS will be diagnosed according to the International Diabetes Federation criteria (2005).

Dietary intake and food-related behaviours will be investigated by means of the Healthy Eating Index (HEI), the ORTO-15 and the OCI-R (Symptoms of Obsessive-Compulsory Inventory – Revised) questionnaires.

Symptoms of anxiety and depression will be measured by means of the Hospital Anxiety and Depression Scale (HADS); personality traits will be assessed by means of the Temperament and Character Inventory (TCI), the Personality Diagnostic Questionnaire (PDQ-4) and the DS14 for assessing Type D Personality.

Finally, data about self-perceived quality of life (Short Form 36 Items scale, SF36), self-perceived stress (Perceived Stress Scale, PSS), psychosomatic comorbidity (the DCPR interview schedule) and bio-psycho-social complexity (INTERMED Self-Assessment, IMSA) will be collected.

Multiple linear and logistic regression analysis will be performed with the software STATA 14.

**Expected results**

This study project is expected to investigate the association between dietary habits, stress, quality of life and anxious-depressive symptoms in an outpatient sample with MetS. If an association between dietary habits, stress, quality of life and anxious-depressive symptoms will be found, this would suggest that the assessment of psycho-social wellbeing should be taken into account in the prevention and treatment of MetS and its consequences in outpatients, in addition to focusing on traditional cardiovascular risk factors.

***Dr. Vittoria Tarantino***

*CEM Curriculum: Translational Medicine*

*Tutor: Prof. Stefano Luminari*

## HIGH RISK INDOLENT LYMPHOMA: HOW TO IDENTIFY AND HOW TO TREAT

### Background

The term indolent lymphoma is generally adopted to described subtypes of Non Hodgkin lymphomas (NHL) characterized by a slow growth pattern, a indolent clinical course and prolonged survival nowadays measured in decades. Among them Follicular lymphoma (FL) is the most common indolent B cell NHL followed by Marginal zone lymphomas (MZLs). Despite the advances achieved in term of overall survival in the modern immunochemotherapy era, the clinical behavior of these diseases is highly heterogeneous and some patients continue to have an unremitting course of relapse or experience histological transformation into an aggressive lymphoma. These events are suggestive of the presence of a high-risk group of patients, with unfavorable outcome despite the standard chemoimmunotherapy approaches and irrespective of initial prognostic stratification. Beside the well-established clinical prognostic indices studied so far, the use of molecular based predictors (minimal residue disease), the length of remission (progression of disease at 24 months and complete remission at 30 months) and diagnostic imaging ( PET) are gaining momentum as more sensitive methods in order to identify patients with a particularly poor outcome . Nevertheless, at present, there are still some issues to be addressed relate to earlier identification of high risk patients and no data are currently available to understand how a different definition of risk could be effectively translated into a clinically useful decisional tool. Consequently, the early identification, characterization and prognostic evaluation of high-risk indolent lymphoma patients represent an unmet medical need.

### Objectives

The aim of the project is to investigate and validate the role of novel prognostic and predictive tools and their integration for follicular and non-follicular indolent lymphomas in order to better identify patients at high risk of relapse or transformation in aggressive lymphoma and predict

subsequent survival. Moreover the purpose of the study is provide the rationale to personalized approach for patients with expected unfavorable prognosis.

**Methods**

The cohort of patients analyzed in the project comprises patients enrolled in multicenter, prospective, randomized Italian studies sponsored by Fondazione Italiana Linfomi, FOLL05 trial (ClinicalTrial.gov Identifier: NCT00774826) and FOLL12 (ClinicalTrial.gov Identifier:NCT02063685) , already completed, and the prospective observational study NF10 still ongoing.

A total of 504 patients represent the cohort from FOLL05 and 807 derive from FOLL12. Both trials include patients with a histological confirmed diagnosis of follicular lymphoma stage II-IV addressed to immunochemotherapy at diagnosis.

The NF10 Project is prospective observational study specifically conceived to investigate the outcome of Indolent Non-Follicular B-Cell Lymphomas (INFL). Consecutive adult patients with newly diagnosed, histologic confirmed diagnosis of INFL were eligible without any exclusion criteria. Eligible INFL included SMZL, ENMZL, NMZL, lymphoplasmacytic lymphoma (LPL), Small lymphocytic lymphoma (SLL), and CD5 negative chronic lymphocytic leukemia. More than 1500 cases have been collected so far.

Moreover very recently an international prospective, longitudinal, observational study, LUPIAE trial, sponsored from EHA lymphoma group was launched for the identification of features leading to progression in patients with histological diagnosis of follicular lymphoma refractory/relapsed after first line therapy. Once starting with the accrual, patients characteristics will be collected and analyzed.

Registration of patients and data collection have been performed on-line in an Electronic Case Report Forms (eCRFs)

**Expected results**

Several prognostic factors are currently available to identify a subgroup of approximately 30% of patients with FL and 20% of MZLs whose lymphoma shows an aggressive clinical behavior with a significant reduction in the life expectancy of the patient. The identification and combination of strong clinical radiological and biological features, at baseline, at the end of treatment and at the relapse can be used to define the prognostic profile earlier during the course of the disease

impacting the outcome. Moreover it could be helpful to guide the clinician's decision and establish the role of individual treatment in a context of a precision medicine approach.

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# STUDY ON THE ASSOCIATION BETWEEN AXIAL SYMPTOMS, COGNITIVE IMPAIRMENT, CLINICAL-INSTRUMENTAL VARIABLES OF MOTOR FUNCTION AND BRAIN AMYLOID BETA-PEPTIDE DEPOSITION IN PARKINSON'S DISEASE PATIENTS WITH BILATERAL SUBTHALAMIC NUCLEUS DEEP BRAIN STIMULATION

## Background

Subthalamic nucleus deep brain stimulation (STN-DBS) represents a short and long-term effective treatment in advanced Parkinson's disease (PD) patients. Several randomized controlled trials have confirmed the superiority of STN-DBS compared to the best medical treatment in complicated PD patients, improving motor disability and global quality of life. In the long-term STN-DBS allows a stable improvement of motor complications, tremor and rigidity with a less relevant effect on axial symptoms (i.e. gait and balance symptoms, speech and swallowing troubles) and cognitive decline, which are the main causes of long-term impairment and disability in PD patients treated with STN-DBS. A large number of studies have analysed axial symptoms in PD patients with an instrumental approach focusing only on gait and postural alterations or speech disturbances. The very few studies that have instrumentally assessed the whole spectrum of axial symptoms in PD have showed the presence of similarities between spatial-temporal gait and speech parameters. Anatomopathological data have confirmed that the neurodegeneration of central dopaminergic pathways, considered the hallmark of PD, is accompanied by a contemporary involvement of other neurotransmitter pathways (i.e. cholinergic, serotoninergic). Prevalent involvement of cholinergic system (pedunculopontine nucleus and nucleus basalis of Meynert) is associated with a clinical "cholinergic" phenotype dominated by axial symptoms, early cognitive deterioration and cerebral Amyloid-β (Aβ) deposition. However, so far there are no studies that have analysed the correlation between axial symptoms, Aβ deposition and cognitive alterations in PD patients who have undergone STN-DBS.

**Objectives**

- To evaluate the correlation between axial symptoms, cognitive alterations and brain Aβ deposition in a cohort of advanced PD patients operated on with bilateral STN-DBS.
- To assess the evolution of axial symptoms in a group of advanced PD patients who have undergone STN-DBS surgery.
- To evaluate the influence of anatomical location and parameters setting of the active STN-DBS contact on axial symptoms.
- To compare the efficacy of STN-DBS and levodopa on axial symptoms.

**Methods**

At first, we are evaluating retrospectively data from 30 PD patients operated on with bilateral STN-DBS at the Neurology Unit of Nuovo Ospedale Civile Sant'Agostino Estense, Modena, from January 2012 to December 2018. Demographic variables, PD characteristics, cognitive and clinical-instrumental data (speech therapist, physiotherapist and neurological evaluations) will be collected by reviewing medical records. Each patient will be reevaluated two to seven years after surgery: axial symptoms will be studied applying a standardized clinical-instrumental approach with the contemporary analysis of speech, gait and postural parameters in order to cover the whole spectrum of PD axial alterations. Each patient will be studied in different stimulation and drug conditions in order to evaluate the selective influence of the two treatments on axial symptoms. The above mentioned analyses will be carry out at the Motion Analysis Laboratory (LAM), Neuromotor and Rehabilitation Department, Correggio Hospital, AUSL-IRCCS of Reggio Emilia. Furthermore, each patient will undergo a complete neuropsychological assessment and a [18F]flutemetamol positron emission tomography (PET) with the aim to quantify cognitive alterations and cerebral Aβ deposition. The anatomical location of the active STN-DBS contact will be calculated merging postoperative computed tomography (CT) imaging with preoperative magnetic resonance imaging (MRI) through a dedicated planning software.

**Expected results**

This project is expected to allow a better understanding of the relationship between speech and gait parameters and cognitive alterations in PD patients treated with bilateral STN-DBS applying a standardized clinical-instrumental approach. Based on the PD cholinergic hypothesis, we can also assume that the evolution of cognitive and axial symptoms after surgery may be partially related

to brain Aβ deposition quantified through the [18F]flutemetamol PET. If confirmed, this correlation will support the possible role of Aβ PET imaging as a biomarker of progression of axial symptoms and cognition in PD. Bearing in mind that axial and cognitive symptoms are the main causes of long-term impairment and disability in PD patients treated with STN-DBS, it appears extremely interesting to find new biomarkers predictive of the evolution into a “cholinergic PD phenotype”, less responsive to stimulation. In the context of personalized medicine, it is crucial to discuss with patients the foreseen outcomes of STN-DBS surgery in the short- and long-term, on the base of his/her clinical characteristics before surgery. Furthermore, we can assume that anatomical location and parameters setting of the active contact within the sensory-motor area of the Subthalamic Nucleus may also influence the response of axial motor symptoms to STN-DBS.

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# DE NOVO HEPATOCELLULAR CARCINOMA IN PATIENTS WITH CIRRHOSIS: DEFINITION OF ITS INCIDENCE ACCORDING TO PORTAL HYPERTENSION MODIFICATIONS AND ANGIOGENIC TRANSCRIPTOMIC SIGNATURE

## Background

The severity of fibrosis/cirrhosis and parameters indicative of portal hypertension are recognized prognostic factors for Hepatocellular Carcinoma (HCC). A close relationship between portal hypertension measured by hepatic venous pressure gradient (HVPG) and HCC has been described, identified baseline HVPG as independent predictors of HCC development. Patients with HVPG $\geq$10 mmHg have a 6-fold increase in the incidence rate of HCC.

The neo-angiogenic mechanisms associated with structural changes in the liver throughout fibrosis progression and during the development and worsening of portal hypertension are critical in predisposing the hepatic microenvironment to HCC development. Angiopoietin-2 expression in liver samples together with liver stiffness and presence of esophageal varices are key factors in HCC development.

Only few studies carried out so far have tried to understand if modifications of portal hypertension induced by non-selective beta-blockers (NSBB) may change the risk of HCC development. These studies demonstrated that patients on NSBB had a lower incidence of HCC, however none of them has provided evidence about the HVPG level modifications during treatment to understand if the lower risk of HCC is related to the hemodynamic response more than to other pleiotropic effects of NSBB. Moreover, very limited data, mainly collected in retrospective cohorts, are present in the literature regarding the potential impact of Transjugular Intrahepatic Portosystemic Shunt (TIPS), which represents the most effective way to decrease portal hypertension, on HCC development, with a suggestion of a possibly slightly increased HCC risk.

Interestingly, none of these studies had correlated the modified incidence of HCC related to portal pressure modifications with neo-angiogenic molecules.

**Objectives**

Primary objective: To evaluate whether either NSBB treatment for primary or secondary prophylaxis of variceal bleeding or positioning a TIPS may have an impact on HCC relative risk in cirrhotic patients.

Secondary objective: To understand whether any induced modification in portal pressure induces a modification of neo-angiogenic molecules, sampled in the local hepatic circulation or in the systemic circulation.

**Methods**

This is a nested study of an already ongoing prospective cohort study looking at predictors of HCC in patients with cirrhosis (ClinicalTrials.gov Identifier: NCT03083002). Patients, recruited from inpatient and outpatient clinics of Gastroenterology Unit will undergo at baseline complete clinical, radiologic, endoscopic and hemodynamic work up (including transjugular liver biopsy and blood samples from the hepatic vein and from the pulmonary artery) and then followed up on 6-months basis with ultrasound in order to identify HCC lesion. Goal of the study is to identify risk factors (clinical or biologic) for HCC development. In this nested study, we envisage to add the evaluation of the modification of these predictors of HCC development in patients requiring NSBB or TIPS insertion.

For patients requiring NSBB therapy or TIPS a second evaluation will be performed after 30 days from baseline (and so from NSBB therapy starting or from TIPS insertion) including a second HVPG with combined right heart catheterization in order to assess the decrease in portal hypertension.

Two groups of patients will be studied according to the potential modification of portal hypertension induced by NSBB or TIPS:

1) cirrhotic patients undergoing evaluation for NSBB therapy (post-hoc analysis will be performed in dependence either being on therapy or not and on achievement or not of a hemodynamic response)

2) cirrhotic patients undergoing TIPS positioning (only for biologic study).

**Expected results**

This study may shed light on relevant issues, i.e.:

1- Understand whether manipulation of portal hypertension, i.e. decreasing it by either NSBB or TIPS, is able to modify factors, like angiogenesis in vivo, which are critical for hepatic

carcinogenesis, would drastically change the indication for these therapies, which would not be only preventive of variceal bleeding but, even more importantly, of HCC;

2- Clarifying, with a dedicated study, whether NSBB play a role in decreasing HCC risk and whether this is due to the primary or secondary actions of NSBB.

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## DEVELOPMENT OF INNOVATIVE ANALYTICAL TECHNIQUES FOR THE CHARACTERIZATION OF NATURAL COMPOUNDS AND THEIR BIOACTIVITY EVALUATION

### Background

Natural products and bio-active compounds extracted from natural sources could be viable and effective alternative to drugs for the prevention of certain diseases and for the maintenance of health conditions. Since the majority of these products are not regulated as drugs, the main concern consists in the quality of the raw material, as well as in the efficacy and safety in their use. In this context, the development of advanced analytical methods for the qualitative and quantitative analysis of active substances in natural products is essential. According to the World Health Organization (WHO) cancer is currently the second leading cause of death world-wide [1]. Considering that the existing screening strategies are not able to reduce the incidence and mortality and also that therapeutic intervention is by itself toxic, an increasing interest is emerging in cancer preventing strategies and possible new treatments with phytochemical agents, also in combination with existing drugs [2].

Non-psychoactive cannabinoids from fibre-type *Cannabis sativa* L. (hemp), which include cannabidiol (CBD), cannabigerol (CBG) and their acid counterparts (CBDA and CBGA), have shown anti-proliferative activity against many cancer cell lines [3]. Despite numerous studies conducted during the last decade, there are still inconsistent data regarding the exact role of the cannabinoid system in cancer development. Although the use of pure cannabinoids is easier to assess the biological effects both *in vitro* and *in vivo* cancer models, still hemp extracts with specified amounts of cannabinoids seem to be valuable resources as potential anticancer agents.

Another important issue that needs to be properly investigated is related to the use of antimicrobial agents as an alternative to conventional antibiotics, considering the implications of multi-drug resistant (MDR) bacteria, that is a global and serious health concern. In this context,

mulberry (*Morus ssp*. L) has been used as a traditional herbal medicine and its extracts, fractions and major constituents have exhibited antibacterial and antifungal properties [4].

**Objectives**

My PhD project is focused on the development of novel and highly efficient techniques for the comprehensive analysis of secondary metabolites present in natural sources, with biological activity and beneficial effects on human health. In this ambit, the main scope is to evaluate the effects on cellular phenotype and proliferation of crude extracts, fractions and purified components, with particular attention to their antioxidant, antibacterial and antiproliferative activity. *In vitro* assays can be used to assess which pharmacological and toxicological effect of the product may be clinically relevant.

The combined results from *in vitro* and *in vivo* studies will assist in the extrapolation of the findings to humans.

**Methods**

The most suitable extraction procedure is necessary in order to obtain as selectively and efficiently as possible the active fraction of the extract. This approach can be based on the application of ultrasound (UAE), microwave (MAE), solid-phase extraction (SPE) or solid-phase microextraction (SPME). The isolation of natural compounds from plant material and complex matrices is usually performed by means of bio-assay guided fractionation. In view of the biological activity data, the fractionation of extracts is carried out, followed by the final purification of active compounds by means of preparative column chromatographic processes. The metabolite profiling and further structural characterization of active compounds can be performed with separative (HPLC or GC, both hyphenated with mass spectrometry) and non-separative techniques (NMR). In particular at this stage of the project, cannabinoids were extracted from hemp inflorescences with absolute ethanol by means of dynamic maceration. An aliquot of plant material has undergone to thermal decarboxylation to obtain richest extracts in CBD and CBG. Each extract was characterized by HPLC-UV/DAD and $^{13}$C-NMR. The method for the quantitative determination of non-psychoactive cannabinoids was previously validated for linearity, sensitivity, accuracy and precision, according to ICH guidelines. For the determination of the number of viable cells in proliferation and cytotoxicity assays, colon adenocarcinoma, glioblastoma and myelogenous leukemia cells were selected (*i.e.* HT29, U87MG and K562) and cultured in DMEM and RPMI respectively. Cells were

seeded in 96-well plates and treated both with pure CBD and dried crude extracts dissolved in medium at different concentration for 24, 48 and 72 hours. The number of viable cells was determined by using a colorimetric assay based on the production of a water-soluble formazan dye upon reduction in presence of dehydrogenases.

As concern the study of mulberry properties, fourteen varieties of *Morus* were extracted and analysed by UHPLC, using a hydrophilic interaction chromatographic method coupled with mass spectrometry (HILIC–MS). The method was enough selective and sensitive for measuring also minor amounts of the active compound 1-deoxynojirimycin (DNJ), and sufficiently simple and convenient to be applied to a large number of samples.

**Expected results**

Hemp extract and pure CBD exerted a significant antiproliferative effect on K562, the most sensitive among the tested cancer cell lines to the treatment. In this case, no major difference in potency and efficacy was observed between the extract and CBD. The effective dose was lower in the 72 h treatment, with respect to 24 and 48 h. For adherent cell cultures (*i.e*. U87MG and HT29) only the highest tested dosage exerted some effect. This finding could suggest the expression of different receptors in these latter cell lines or a different mechanism of action that still remains not fully elucidated. For this purpose, further assays will be performed in serum-free culture condition to verify potential interaction between active compounds and serum proteins. These first results are promising and they will be the subject of an in-depth evaluation, in order to clarify the mechanism and potential of these substances in cancer therapy.

As regards mulberry, at this preliminary phase of the work the content of DNJ was determined in each cultivar in order to select the richest one for a further evaluation of the antimicrobial activity on pathogenic bacteria to assess the bacterial growth inhibition capacity of both the extracts and the pure compounds.

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## NOVEL LONG NONCODING RNA BlackMamba IS ASSOCIATED TO ALK$^-$ ANAPLASTIC T-CELL LYMPHOMA AND CONTRIBUTES TO THE REGULATION OF NEOPLASTIC PHENOTYPE

### Background

Anaplastic Large Cell Lymphoma (ALCL) is the most common subtype of Non Hodgkin Lymphoma. ALCL originates from T-lymphocytes and are stratified based on the presence of ALK gene translocations. Among ALK$^-$ ALCL, distinct subsets can be defined based on transcriptional signatures and/or recurrent genomic alterations. Clinically ALK$^-$ ALCL display a poor outcome compared to ALK$^+$ ALCL patients with the exception of DUSP22 positive and breast implant-associated ALCL (BIA), which have a favorable prognosis. Poor response to therapy and inferior survival have been observed in patients carrying defects of TP53, TP63 and loss of PRDM1 genes. Mechanistically, the molecular mechanisms leading to ALK$^-$ ALCL transformation remain quite elusive.

LncRNAs are molecules longer than 200 nucleotides that play important regulatory functions. LncRNAs were founded to be overexpressed or downregulated in many types of cancer. In lymphocytes, the expression of lncRNAs is a dynamic and cell/stage specific phenomenon. So far, a significant number of studies have identified lncRNAs associated to lymphocyte signatures, and they are predicted to control cell differentiation and identity. Indeed, IFNG-AS1, linc-Ccr2-59AS, Th2LCRR, GATA3-AS1 have been proven to be associated to distinct Th1 or Th2 phenotypes and MAF-4 lncRNA regulates MAF transcription promoting T lymphocyte differentiation by interacting with chromatin modifiers LSD1 and EZH2. Although in normal lymphocytes, functional properties of several lncRNAs have been identified, their expression and/or role in lymphoid malignancies are not fully understood.

By performing a deep expression profiling in conjunction with de novo transcriptome assembly, we described a panel of novel lncRNAs expressed by ALCL. Among these, we discovered and characterized a new chromatin-associated lncRNA, selectively expressed by ALK$^-$ALCL, that we named *BlackMamba*. We showed that BlackMamba is regulated by JAK/STAT3 axis and its

expression is required to sustain ALK$^-$ ALCL cell proliferation and clonogenicity. Short-hairpin mediated BlackMamba knockdown (KD) led to an increase of polynucleated cells and actin cytoskeleton rearrangements. RNA-Sequencing analysis on BlackMamba KD ALK$^-$ALCL cells identified a list of genes involved in migration, integrin-mediated cell adhesion and regulation of actin cytoskeleton reinforcing the hypothesis that BlackMamba controls the neoplastic phenotype through the regulation of cell cytoskeleton.

**Objectives**

Our aims are:

- ✓ To elucidate how BlackMamba regulates ALK$^-$ALCL neoplastic phenotype
- ✓ To characterize BlackMamba target genes involved in the cytoskeleton organization
- ✓ To identify transcriptional partners of BlackMamba in the regulation of ALK$^-$ALCL phenotype.

**Methods**

To characterize the contribution of *BlackMamba* to ALK$^-$ ALCL phenotype we will deep the role of its target genes in these cells. Thus, we will utilize a pharmacological approach where possible and confirm our data with stable shRNA downregulation o selected BlackMamba target in ALK$^-$ALCL cellular models. We will evaluate cell proliferation and clonogenicity (by viability assay and methylcellulose experiments), nuclear-cytoskeleton rearrangements (by immunofluorescence) and the induction of polynuclated phenotype (May Grunwald-Giemsa). In particular, we will focus on BlackMamba target genes responsible for cytoskeleton organization. For these proteins we will also perform immunofluorescence and morphological analysis to establish the impact of BlackMamba target genes on basic processes in the definition of cellular architecture and cell division.

To identify the molecular mechanism through which *BlackMamba* controls the neoplastic phenotype we will correlate the expression of *BlackMamba* with those of transcriptional factors in ALCL patient's samples and ALCL cell models. Next, we will perform RNA-Immunoprecipitation(RIP) experiments to investigate the physical association between *BlackMamba* and transcriptional factors(TFs). Then, we will perform Chromatin Immunoprecipitation(ChIP) experiments to verify the TFs binding on target genes promoters. Once identified the transcriptional factors we will

perform loss of function and ectopic expression experiments to study their biological functions and confirm their key role in supporting ALK$^-$ALCL neoplastic phenotype.

**Expected results**

We will expect: to deep characterize BlackMamba relevance in ALK$^-$ ALCL biology, to define its role in controlling cytoskeleton organization, to identify new molecular players that functional cooperate with BlackMamba to support ALK$^-$ ALCL aggressive features. These may either represent potential markers for patients' stratification and/or target for new pharmacological approaches in this therapy orphan disease.

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## PREVALENCE AND PREDICTORS OF CHRONIC CRITICAL ILLNESS IN PATIENTS WITH ACUTE RESPIRATORY FAILURE

**Background**

Chronic Critical Illness (CCI) is a condition associated to patients surviving an acute phase of disease and respiratory failure (ARF) although remaining dependent on mechanical ventilation (MV). The prevalence and the underlying mechanisms of CCI have not been elucidated in this population.

**Objectives**

The aim of this study was to describe the prevalence of CCI and the risk factors associated with its development in patients with de novo ARF subjected to mechanical ventilation in a specialized Respiratory Intensive Care Unit (RICU).

**Methods**

An observational prospective cohort study was undertaken at the Respiratory Intensive Care Unit (RICU) of the University Hospital of Modena (Italy) from January 2016 to January 2018. Patients mechanically ventilated with ARF in this unit were enrolled. Demographics, diagnosis, severity scores (APACHEII, SOFA, SAPSII) and clinical conditions (septic shock, pneumonia, acute respiratory distress syndrome [ARDS]) were recorded on admission. Respiratory mechanics and inflammatory-metabolic blood parameters were recorded on admission and within the first seven days of stay. All these variables were tested as potential predictors of CCI through appropriate univariate and multivariate analysis. (ClinicalTrials.gov Identifier: NCT03851822).

**Results**

CCI development occurred in 33% of patients consecutively observed. Severity scores (APACHE II, SAPS II, SOFA), septic shock, and the presence of diaphragm dysfunction at sonography on admission were associated to the onset of CCI (RR=3.5 p<0.001, RR=3.9 p=0.002, RR=3.9 p=0.01, RR=14 p<0.0001, RR=3.17 p<0.0001, respectively). The onset of a multidrug resistant (MDR) bacterial infection and the occurrence of a second infection during stay significantly correlated with the risk of developing CCI (RR=4.7 p=0.009 and RR=3.17 p=0.031, respectively). The trend of C-reactive protein (CRP) serum levels in between admission and day-7 was different in patients with or without CCI (p = 0.031). Septic shock, MDR infection, diaphragm dysfunction by sonography, APACHE II score on admission, time course of CRP from admission to day-7, and the onset of a second infection were factors independently associated to CCI (RR=7.2 p<0.001, RR=4.7 p<0.021, RR=3.7 p<0.003, RR=3.5 p<0.002, RR=2.1 p<0.036, RR=1.9 p<0.027, respectively). Septic shock was the strongest predictor of CCI development (AUC=0.92 p < 0.0001) following the ROC analysis.

**Conclusion**

Trajectory towards CCI development is not rare in patients admitted to RICU for ARF. Infection-related factors seem to play the major role as predictors of this clinical syndrome.

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## IDENTIFICATION OF KEY MOLECULES AND MECHANISMS DURING CELLULAR AND TISSUE REGENERATION

### Background

Systemic sclerosis is an immune-mediated rheumatic disease characterized by fibrosis of the skin and of different internal organs, and by vasculopathy, and causes a severe and persistent reduction quality of life. The etiology of the disease is unknown, and several genetic and environmental factors are likely involved. At least 50% of patients suffer from vascular disorders with the appearance of digital ulcers, which are often refractory to classical drugs treatment (vasodilators and antiplatelet agents). New therapeutic approaches involve surgery (skin grafting), injection of growth factors (Platelet-Rich Plasma) or injection of fat tissue (lipofilling) containing mesenchymal stromal cells. Recent scientific evidences show that in almost all treated patients mesenchymal stromal cells, due to their immunomodulatory and anti-inflammatory ability, were successful in the treatment of digital ulcers refractory to classical drugs. Autologous fat tissue was extracted from thighs, abdomen or glutes and after centrifugation was injected at level of injury. Advantages of the use of autologous adipose tissue are due their biocompatibility and long-term stimulation of tissue regeneration.

### Objectives

In the current study we will profile gene expression from punch biopsy of the skin from patients affected by systemic sclerosis treated or not with injection of autologous adipose tissue. The main purpose of the project is to identify key-molecules that are involved in cellular and tissutal mechanisms of regeneration.

**Methods**

Punch biopsy of the skin will be obtained from patients and immediately frozen in liquid nitrogen at −196°C and stored until use. Tissues will be disrupted using TissueLyser II (Qiagen) and homogenized using QIAshredder (Qiagen) to reduce viscosity. RNA will be purified by using RNeasy Mini Kit (Qiagen). Quantification will be performed by using RNA 6000 Nano Kit (Agilent Technologies) on Agilent 2100 Bioanalyzer. Total RNA will be sequenced by state of the art methodologies, and data will be analyzed by RStudio software.

**Expected results**

We expect to observe a differential gene expression between patients treated or not with autologous MSCs injection. An eventual gene signature could allow us not only to identify which pathways are involved in the regeneration of damaged tissue, but also to select key molecules able to improve mechanisms of repair that could enter in the clinical use.

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**EFFECTIVENESS OF AUDIT AND FEEDBACK INTERVENTIONS TO IMPROVE HEALTHCARE PRACTICE IN TYPE 2 DIABETES MELLITUS AND CHRONIC HEART FAILURE**

**Background**

Type 2 diabetes mellitus (T2DM) and Chronic Heart Failure (CHF) are serious and growing common chronic conditions that are increasingly managed by health professionals in outpatient and community settings. Over the last few years, the Emilia-Romagna Regional Health Care System promoted a reorganization of primary care, largely based upon the principles of the Chronic Care Model and the adoption of formally structured clinical pathways. This reorganization departs from the traditional hospital-centered models of care towards new innovative clinical and organizational approaches largely based on outpatient services in the community. The development and implementation of these pathways require the involvement of different health professionals and demand strong clinical as well as managerial responsibility in constantly monitoring processes and outcomes of care. Providing those responsible for the organization and management of clinical pathways with timely and exhaustive information on relevant dimensions of the quality of care delivered (safety, effectiveness, appropriateness and equity) is of utmost importance. With this aim, a great deal of attention is currently paid nationally and internationally both on the implementation of chronic diseases clinical pathways and on the potential of audit&feedback (A&F) interventions to drive health professionals to the adoption of effective and appropriate patterns of care. In an A&F intervention, an individual's professional practice or performance is measured and then compared to professional standards or targets; subsequently, the results of this comparison are fed back to the individual.

**Objectives**

- To assess the impact on the quality of care of an A&F intervention based upon information drawn from the administrative databases available.

- To explore the opportunities offered by additional sources of information and to fully address relevant dimensions of quality of care and health services performance through the use of qualitative research methods (*focus group*).

**Methodologies and statistical analyses**

Reggio Emilia is coordinating in the Emilia Romagna Region a project founded by the Ministry of Health to define the characteristics of an optimal A&F intervention applied to T2DM and CHF clinical pathways. In order to design an optimal A&F system, an analysis of the existing clinical pathways will be undertaken among the Local Health Authorities of Reggio Emilia, Bologna, Imola and Piacenza, relying on the available formal documentation integrated with structured interviews with local managers and clinicians. Particular attention will be paid to examining how managerial and clinical responsibilities are allocated, to their relationship and accountability structure, to the characteristics of the information systems available at the local level. Process and outcome indicators measurable on administrative data available will be selected, also relying on a review of the literature in the field. Moreover, a qualitative analysis, through the construction of focus groups and interviews, will be conducted among health professionals and patients, to identify the determinants and the dynamics that regulate the hospital-territory continuity of care. The analysis will make it possible to verify in detail which subjects and groups are particularly disadvantaged, what are the unmet needs that can be answered by part of the services and which community resources are already locally present. It will also allow the identification of additional information and indicators required in an optimal A&F intervention for the management of effective clinical pathways. The analysis will include the type of indicators to be fed back, the report format and performance display and the key health professionals to be targeted. Consensus on selected indicators will be achieved through the adoption of formal working group methodologies, involving all the pertinent stakeholders and using the relevant literature in the field as reference. Special attention will be deserved to the possibility of developing indicators able to describe the level of integration and coordination of health services and professionals achieved across different services, the quality of relationship both among health services and between health services and patients, patients social background and equity in the provision of health services. Finally, to compare the performance of health professionals exposed to A&F interventions and control group, data will be collected at multiple instances over time before and after the intervention. This

evaluation will be complemented by analyses on the process undertaken in the exposed sites in using the information provided locally by the A&F intervention.

**Expected results:**

While there is a realistic expectation that A&F may have a positive impact on performance, if well designed and complemented by additional interventions, it is hard at this stage to make explicit on which specific dimensions of quality of care such an impact will be observed. Within the context of the already mentioned working groups a consensus will be reached on which indicators the effect of A&F is more likely expected, as well as on the realistic estimate of the effect size. Overall, the preliminary expectation is that an effect (if any) will be observed more likely and earlier on process (i.e. diagnostic test) , rather than on outcomes indicators (such as hospital admission rate) for the two specific diseases investigated in this program.

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# DIAGNOSTIC ACCURACY OF VELCRO SOUND DETECTOR (VECTOR) FOR INTERSTITIAL LUNG DISEASE IN RHEUMATOID ARTHRITIS PATIENTS. THE InSPIRAtE VALIDATION STUDY

## Background

Rheumatoid arthritis (RA) is a chronic inflammatory disease often complicated by extra-articular manifestations. Among them, interstitial lung disease (ILD) is one of the most frequent complication with negative impact on both therapeutic approach and overall prognosis.

Considering the possible severity of ILD and the therapeutic implications, to investigate a better approach to obtain an early diagnosis is mandatory.

High resolution computed tomography (HRCT) represents the gold standard for the diagnosis of this extra-articular manifestation, but ILD can appear in any stage of RA entailing the need for systematic assessment of lung involvement, and a routine use of HRCT for screening program is not advisable for both the high cost and X-rays exposure.

In this background, a delayed diagnosis could be responsible for possible severe complications. Therefore, the use of reliable and non-invasive tools may improve early diagnosis and a better management of the disease, and could allow us to perform prospective studies in order to clarify the epidemiology and the clinical characteristics of RA-ILD.

Velcro sound is a pulmonary sound defined as a fine crackle soft and short in duration. The detection of this typical sound has been proposed as an easy and repeatable screening for the early diagnosis of idiopathic pulmonary fibrosis and other forms of ILD. Recently, we developed an algorithm, named VECTOR (VElcro Crackles detecTOR), to detect the presence of velcro crackles in pulmonary sounds recorded by an electronic stethoscope (ES).

## Objectives

The aim of the study is to validate the diagnostic accuracy of VECTOR in a larger population of RA, compared with the reference standard of HRCT, from a multicenter study.

Moreover, in the near future, we'll aim to investigate the epidemiological, clinical and serological characteristics of RA-ILD using VECTOR to perform prospective studies on this topic.

**Methods**

The study InsPIRAtE (INterStitial Pneumonia in Rheumatoid ArThritis with an Electronic device) involved seven Italian tertiary rheumatologic Centers with clinical experience in rheumatic disorders and interstitial lung diseases.

All consecutive RA patients, classified according to 1987 or 2010 ACR classification criteria, with a recent HRCT evaluation were eligible for the study and enrolled in a six-month period. According to clinical history, the HRCT should have been performed within 12 months in absence of subsequent appearance or variation of signs or symptoms suggestive for lung disease (cough, dyspnea, velcro sound at routine clinical examination).

Respiratory sounds were recorded in 4 pulmonary fields bilaterally in a silent environment with a commercial ES (Littmann 3200TM 3M, USA). Then, audio files acquired for each patient were analyzed by mean of VECTOR and the result (presence or absence of velcro crackles) was compared in a blind manner by an expert thoracic radiologist with HRCT images (presence or absence of ILD). Moreover, for all patients the value of forced vital capacity (FVC), diffusion lung of CO (DLCO), the result of x-Ray thorax, the presence of velcro crackles at thorax examination and the presence of cough or dyspnea at baseline were recorded.

**Results**

To date, 140 RA patients were enrolled for the study. Three patients were excluded for the low quality of the recorded lung sounds.

ILD was detected in 59 patients (43.1%). No differences were observed between patients with or without ILD with regard to sex, autoantibodies, smoking habit, spirometry (FVC), mean age at disease onset, and disease duration. On the contrary, patients with ILD were older, and showed a lower DLCO than non-ILD patients.

VECTOR correctly classified 115/137 patients (83.9%), showing a sensitivity and specificity of 93.2% and 76.9%, respectively. Only 4/59 patients with ILD were not identified by VECTOR, while false positive cases were 18/78. Diagnostic accuracy of VECTOR in detecting ILD was higher than dyspnea (64.6%), cough (58.3%), DLCO (54.9%), chest X-ray (71.3%) and the rheumatologist's pulmonary evaluation.

The diagnostic accuracy of VECTOR was not influenced by the duration of lung disease and by the extension of lung involvement.

**Conclusion**

This multicenter study confirmed the high sensitivity, specificity and diagnostic accuracy of VECTOR on the presence of ILD in patients with RA.

RA-ILD is a field of great interest for both rheumatologists and pulmonologists. In the last years, many studies have been conducted to elucidate different facets of this harmful clinical problem, but they are all retrospective with substantial bias due to diagnostic methodologies.

An early diagnosis of RA-ILD is mandatory since it represents one of the most severe and challenging extra-articular manifestations in RA patients, associated to very low quality of life and poor overall prognosis. Given its significant impact, there is a need to develop strategies to increase the diagnosis of ILD before symptoms occurrence. Moreover, ILD can occur at any stage of the disease; for this reason, lung evaluation should always be included in the clinical assessment of RA patients, regardless the disease duration or activity.

At the moment, a screening for RA-ILD is not feasible mainly because of the low diagnostic accuracy of any method other than HRCT, resulting in mis- or delayed diagnosis.

VECTOR could represent the first validated tool for the screening of RA patients suspected for ILD and who should be directed to HRCT for the diagnosis.

Our software, in combination with an ES, showed a sensitivity and specificity of 93.2% and 76.9%, respectively, and a very good diagnostic accuracy (83.9%), higher than any other method available to date, except for the HRCT, but without any radiological exposure. Of interest, in our population, less than half of the patients correctly identified by VECTOR showed clinical symptoms or a reduction of functional lung test, confirming the usefulness of VECTOR in the identification of subclinical forms of ILD.

RA is the most frequent inflammatory rheumatic disease with a high risk of developing ILD, but up-to-date a systematic approach to this problem is not a routinely engaged in clinical practice. The algorithm proposed could represent an opportunity for all rheumatologists to improve the screening of patients to direct to HRCT.

The main limit of our study is its retrospective design. Although VECTOR performed well in the studied population, further researches are needed to confirm its efficacy as a screening tool in patients with early lung disease.

VECTOR, associated to an ES, could allow at rheumatologist a real-time screening of patients with ILD and it could be really helpful in the design of prospective studies, in which RA-ILD patients are identified with the help of this non-invasive method and followed during all disease course.

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## EFFECTS OF DYSMETABOLISM ON CARDIOVASCULAR AND REPRODUCTIVE SYSTEMS: POSSIBLE INTERVENTIONS IN EXPERIMENTAL AND CLINICAL MODELS

### Background

Metabolic syndrome (MS) and obesity are growing causes of morbidity and mortality worldwide and major risk factors for cardiovascular disease. In pregnancy, these metabolic changes affect maternal and fetal health and act as a catalyst for their future health. Studies have shown that pregnancies complicated by MS and obesity have increased levels of oxidative stress and chronic inflammation which are known factors contributing to the onset of cardiovascular disease, gestational diabetes and preeclampsia in pregnancy, and predispose the offspring to an increased risk of cardiovascular and metabolic disease later in life. Different experimental and clinical studies have tested several interventions in pregnancy complicated by metabolic derangement in order to ameliorate the maternal metabolic-vascular profile and consequently maternal and offspring's long-term health. Among those, the supplementation with natural compounds, specifically with inositols and lifestyle interventions have shown the most promising results in improving metabolic profile during pregnancy.

### Objectives

The primary objective is to evaluate whether a combination of insulin sensitizer compounds (myoinositol and D-chiro-inositol) and antioxidant substances (fucoxanthin and hydroxytyrosol), will synergistically lead to an improvement on maternal cardiovascular profile in pregnant mouse models of MS and obesity, reducing therefore the "hostility" of the intrauterine environment, thus preventing the "fetal mal-programming" that will lead the offspring to long-term cardiovascular and metabolic diseases in the adult life.

The secondary objective is to evaluate the metabolic fingerprint and perinatal outcomes in pregnant women with MS and obesity undergone to a lifestyle intervention during gestation.

**Methods**

The pregnant murine models of MS and Obesity are obtained using a transgenic eNOS-/+ female and a WT female, respectively. Those animals are fed with high fat diet for 4 weeks to induce MS and obese phenotype, then bred with WT males. On gestational day (GD) 1, dams are randomly allocated to the treatment or control groups. Maternal weight and systolic blood pressure are obtained at GD 18, using a calibrated, 8-chamber, tail-cuff system, then dams are sacrificed for fetuses and placentas collection. Carotid arteries are dissected out for the in vitro-vascular studies using a wire-myograph system. The vessels are mounted in the chamber between two force transducers and bathed in Krebs solution at 37 degrees Celsius and pH of 7.4. The tension generated by the vascular tissue is recorded and acquired by a power lab computer software. Responses to the contractile agent, phenylephrine (α1 adrenergic agonist) in the presence and absence of a non-specific nitric oxide synthase inhibitor (NG-nitro-L-arginine methyl ester) are obtained. For the vasorelaxation responses, acetylcholine (endothelium-dependent vasorelaxant agent) and sodium nitroprusside (endothelium independent vasorelaxant agent) are evaluated. Masson's trichrome staining is used to evaluate connective deposition in maternal heart.

For the human metabolomic approach, the measurement of extracellular metabolites will be performed through the metabolic fingerprinting analytical technique, which mostly uses spectroscopic methods for the classification of samples based on their origin or biological relevance.

**Expected results**

With the experimental study we expect to improve maternal cardiovascular profile and ameliorate the intrauterine environment in order to reduce the sequelae of the dysmetabolism in the mother and the offspring. Moreover, it is of remarkable importance that anti-obesity drugs, such as orlistat and sibutramine, have serious side effects including valvular heart disease. While the natural compounds tested in our experiment might provide safe, natural and cost-effective alternatives to synthetic drugs.

With the human metabolomic research, we expect to detect and identify the endogenous and exogenous metabolites that changes after a lifestyle intervention in pregnant women with MS and obesity. The metabolic fingerprinting, by evaluating hundreds of metabolites that are more closely

related to the phenotype, can help us in understanding a detailed analysis of complex reaction networks and uncovering new drug targets and possible therapeutic interventions.

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# GLYCOSAMINOGLYCAN ANALYSIS OF BIOLOGICAL FLUIDS AND ORGANS IN AN ANIMAL MODEL OF MUCOPOLYSACCHARIDOSIS II (HUNTER SYNDROME): EFFECT OF TREATMENTS WITH ENZYME REPLACEMENT THERAPY AND GENISTEIN

## Background

Mucopolysaccharidoses (MPS) are progressive and multisystem Lysosomal storage diseases (LSDs) caused by genetic defects in glycosaminoglycans (GAGs) catabolism [1]. GAGs are linear polysaccharides constructed of repeating disaccharide units with the primary configurations containing an amino sugar and an uronic acid. Most GAGs form complexes with proteins as proteoglycans, participating in important physiological processes such as ligand–receptor interactions and cell proliferation. There are five identified GAGs chains: chondroitin sulfate (CS), dermatan sulfate (DS), heparan sulfate (HS), keratan sulfate (KS) and hyaluronan (HA). The deficiency of the enzymes required to degrade GAGs leads to an accumulation of partially degraded GAG within lysosomes. There are 11 known enzyme deficiencies, resulting in seven distinct forms of MPS: type I, II, III, IV, VI, VII, and IX. The collective incidence is more than 1 in 25,000 live births [1,2].

MPS II (Hunter syndrome) is an X-linked recessive disorder that generally affects only males, caused by a deficiency of the lysosomal enzyme iduronate-2-sulfatase (I2S). The lack of this enzyme causes CS, DS and HS to accumulate in all body tissues, causing abnormalities in many organs, including the skeleton, heart, and respiratory system. In severe cases, this leads to death during the teenage years [3].

Currently, several treatments such as enzyme replacement therapy (ERT), hematopoietic stem cell transplantation (HSCT), gene therapy and Substrate reduction therapy (SRT) are being evaluated for MPS therapy. In MPS II ERT appears to be the most effective treatment. Patients treated with ERT show clinical improvement of somatic manifestations and improved quality of life. However, major limitations of ERT include high cost, rapid clearance from the circulation, limited effect on skeletal and CNS symptoms, inability to cross the blood-brain barrier and immunological issues [4].

The isoflavone genistein has been studied as a potential therapy for the MPS because of its putative ability to inhibit GAG synthesis and subsequent accumulation. Furthermore, genistein is able to cross the blood-brain barrier in murine models, but previous studies on cell, animal, and human showed variable outcomes [5].

**Objectives**

The aim of this project will be focused on GAGs qualitative and quantitative characterization in a murine MPS II model (iduronate-2-sulfatase knock-out) non-treated, treated with ERT or treated with genistein, analyzed at different weeks after the beginning of therapy.

Our first aim is to validate the MPS II murine model comparing the GAGs concentration in urine, plasma and different tissue versus wild type mice.

Our second aim is to evaluate the effects of these different therapeutic approaches on HA, CS, DS and HS total concentration, disaccharide composition, chemical properties and overall structure.

**Methods**

University of Padova, in collaboration with University of Modena and Reggio Emilia, provided to our laboratory samples of urine, plasma and organs (liver, brain, kidney, spleen, heart and lung) of 225 mice. The analyzed mice were divided in: 45 non treated wild type mice, 45 wild type mice treated with genistein, 45 MPS II murine model non treated, 45 MPS II murine model treated with ERT and 45 MPS II murine model treated with genistein. For each category 6 or 7 subject were sacrificed at different weeks after the beginning of therapy (0, 2, 4, 6, 12, 18, 24 weeks). All samples have been stored frozen in polypropylene tubes at -20°C.

GAGs will be extract from urine, plasma and organs sample according to a standardized protocol guidelines. The protocol step include protein digestion with specific protease, treatment with sodium borohydride and sodium hydroxide (Sigma-Aldrich) to release GAGs from their core proteins, purification on anion-exchange resin and filtration on YM-3 centrifugal filters having a molecular mass cutoff of 3000 Da (Amicon). The crude retained GAG fraction will be digest with chondroitinase ABC and heparinase I, II, III (Sigma-Aldrich) to isolate HA, CS, DS and HS disaccharide units. Disaccharides will be lyophilize and tag with AMAC fluorophore (Sigma-Aldrich). Finally, derivatized disaccharides will be separate and quantify by capillary electrophoresis (Agilent) interfaced to laser induced fluorescence.

**Expected results**

The results of this project will help us to validate this MPSII mouse model, evaluating the actual accumulation of GAGs in the murine model samples versus wild type samples. Furthermore, we will have more indications on the molecular effectiveness of different therapies in reducing GAGs accumulation, providing the basis for future clinical trials on humans.


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## DATA-DRIVEN TECHNOLOGIES FOR DRUG REPURPOSING

### Background

Drug repurposing is defined as the process of identifying novel therapeutic applications for already marketed drugs or drug candidates that passed phase 1 clinical evaluation. This gives access to molecules with favorable pharmacokinetic characteristics and a carefully investigated toxicity profile (1). Moreover, similar repurposing strategies can be applied to already synthesized classes of compounds to identify their biological targets. Successful cases of repurposed drug are well known but were often achieved by serendipitous observations. Recently, novel interesting opportunities for drug repurposing arose from the increase of publicly available biological and chemical data. In this context, integrated *in silico* approaches have proven to be efficient strategies to analyze such amounts of information and rationally predict the activity of known ligands for new targets (2).

### Objectives

The main objective of the project is to devise and to apply effective computational strategies for drug repurposing campaigns, by mining publicly available databases of bioactivity and chemical data, as well as promising drug candidates from other sources (such as academic screenings). The project will make use of the joint application and integration of ligand- and receptor-based approaches, and the development of machine learning models.

As a first task, an especially devised *in silico* ligand-based protocol was developed to identify new targets for libraries of synthesized molecules previously identified by our research group. (3)

### Methods

1. Dataset set up: structural information from the DrugBank database, and bioactivity records from ChEMBL (release 24) were downloaded (4,5). A library containing the investigated set of compounds was drawn using Maestro from the Schrödinger suite.

2. Data preparation: OpenEye suite was used to calculate 2D fingerprints (MACCS and ECFP4) and to generate 3D conformations for all molecules. Physico-chemical properties and molecular descriptors were calculated for all relevant compounds.
3. Repurposing process: OpenEye suite was used to perform 2D fingerprint-based and 3D shape-based similarity of the libraries of previously identified compounds against DrugBank molecules; machine learning models were built and tested using KNIME; models were trained on a subset of bioactivity data extracted and filtered from ChEMBL and tested against the investigated libraries.

**Expected results**

The final aim of the project is to build a robust protocol to unveil previously unknown therapeutic applications of known drugs (or potential candidates) on novel targets. This will be achieved by integrating multiple sources of information and combining different computational strategies.

Results of a drug repurposing campaign on libraries of molecules previously identified by our research group unveiled a high degree of similarity for some of the explored compounds against novel, previously un-explored targets. Preliminary *in vitro* testing of the most promising candidate confirmed the *in silico* results, yielding a low nanomolar inhibitor.

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## INVESTIGATION INTO THE ENVIRONMENTAL RISK FACTORS OF ALZHEIMER'S DEMENTIA AND OTHER NEURODEGENERATIVE DISEASES IN THE MODENA AND REGGIO EMILIA POPULATION

### Background

Neurodegenerative dementias are well recognized, severe medical conditions that are prevalent worldwide and expected to rise in the coming years [1]. There is increasing evidence that the cognitive and functional decline characterizing all clinical forms of dementia and commonly occurring in later life, is generally the result of a process that begins earlier in the life course [2]. In addition to genetic susceptibility, the environmental and nutritional risk factors have been suggested to play an important role in dementia etiology [3]. Particularly, environmental factors that have been implicated in dementia onset include exposure outdoor air pollutants, electromagnetic fields, and residential and occupational exposure to pesticides and solvents. Among nutritional risk factors, recent findings highlighted the role of dietary habits [4], including the different adherence to specific dietary patterns, e.g. the Mediterranean diet [5], or the specific intake of metals, e.g. aluminum and manganese [6-7], as well as other metalloids such as selenium, especially in its inorganic forms [8].

### Objectives

This project aims at investigating the association between putative risk and protective factors on the onset and progression of neurodegenerative dementia, particularly the most common form of Alzheimer's dementia, with particular reference to:

- Environmental risk factors: traffic related outdoor air pollutants, electromagnetic fields, pesticides, heavy metals and metalloids.
- Dietary habits and other nutritional factors.

**Methods**

In this project, we will include subjects with mild cognitive impairment (MCI), a clinical disorder characterized by difficulties in thinking and memory loss that can be demonstrated by cognitive test. Affected subjects, however, are still able to compensate and perform all their usual everyday activities successfully, although the cognitive decline is generally progressive and most of them, but not all, develop a form of clinical dementia. Using a prospective cohort study design, subjects newly diagnosed with MCI and referring to Neurology Units of Modena and Reggio Emilia hospitals will be recruited in the period 2019-2020. At the recruitment subjects will be asked to fulfill a demographic questionnaire collecting information on life-styles and other personal characteristics. In particular, we will collect their complete residential history in order to model exposure to several environmental risk factors at subjects' address of residence using Geographic Information System (GIS) methodology. Moreover, we will assess dietary habits of study participants by administration of the EPIC food frequency questionnaire (FFQ) specifically developed for the Central-Northern Italy population. The EPIC-FFQ is a semi-quantitative and validated FFQ that was designed to estimate frequency and amount of consumption of 188 food items over the previous year. Dietary intake of individual nutrients and contaminants will be calculated using *ad hoc* routine previously developed [9]. After 18-24 months, the progression to clinical dementia will be assessed during the follow-up visits at the Neurology units using a Cox-proportional hazard regression model.

**Expected results**

Based on the epidemiology data of MCI in the study area and on an annually estimated conversion rate from MCI to clinical dementia, mainly Alzheimer's dementia, of 20-30% [10], we are expected to recruit a total of 100 MCI subjects from both Modena and Reggio Emilia Neurology Units, for a total of 200 newly-diagnosed subjects to be followed-up for 18-24 months or till the end of the project in 2021, with an estimated number of 40-60 new dementia cases.

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## METABOLIC, CARDIOVASCULAR AND LIVER-RELATED COMPLICATIONS IN GAUCHER DISEASE

### Background

Gaucher disease (GD) is one of the most frequent disease among the rare lysosomal storage disorders with an estimated prevalence of 1:40.000 in the general population; GD is transmitted as an autosomal recessive disorder. GD is caused by mutations in the GBA gene which cause a deficiency of the lysosomal enzyme glucocerebrosidase (GBA) and a consequent glycosphingolipids accumulation in macrophages (Gaucher cells) of the reticuloendothelial system of all tissues, mainly liver, spleen and bone marrow. Type 1 phenotype (non-neuronopathic) is the most frequent form of GD and is characterized by hepato-splenomegaly, skeletal and hematological alterations due to bone marrow involvement. Moreover, GD patients present a peculiar metabolic profile characterized by an increased energy expenditure due to systemic inflammation, glucose metabolism alterations with insulin resistance and lipid metabolism disorders with hypolipidemia and low HDL-cholesterol. Several treatment strategies, i.e. enzyme replacement therapy (ERT) and substrate reduction therapy (SRT), are currently available and have proven effective in reducing liver and spleen volume, in improving hematological and skeletal alterations and in beneficially impacting on quality of life. Moreover, ERT is able to partially revert the metabolic disorders associated with GD by partially restoring the basal metabolic rate at the expense of weight gain and by normalizing the lipid profile. Treatment of GD has increased the life expectancy of GD patients and modified the natural history of the disease; if in the pre-ERT era the main causes of death were infections, hemorrhage and post-surgical complications, now GD patients mainly die because of cardio-cerebrovascular events, malignancies (i.e. multiple myeloma and hepatocellular carcinoma-HCC) and chronic liver disease.

Given that in the ERT/SRT era quality and expectancy of life of GD patients have significantly improved, the ageing GD population may be exposed to unhealthy lifestyle with potential impact on GD natural history and on morbidity and mortality. Little is known on the role of lifestyle on

metabolic alterations, cardiovascular and neoplastic risk and liver disease burden in GD patients either at baseline or during long-term ERT/SRT.

**Objectives**

We aim at:

1. Characterizing the metabolic profile, the cardiovascular risk and the liver disease burden in a large cohort of adult type 1 GD patients mostly on stable ERT/SRT;
2. Evaluating the role of GD severity, ERT/SRT and/or lifestyle on the metabolic profile, i.e. body composition, energy balance, glucose and lipid profile;
3. Identifying the parameters (among metabolic profile, GD severity, ERT/SRT and/or lifestyle) associated with cardiovascular risk and liver disease;
4. Evaluating the changes in metabolic profile and the progression of cardiovascular and liver disease during follow-up and the impact of GD-severity, ERT/SRT and lifestyle on metabolic changes and disease progression.

AIM 1-3:

- Cross-sectional baseline evaluation of GD severity, body composition, energy balance, glucose and lipid metabolism and lifestyle and their potential correlations with cardiovascular and liver disease

AIM 4:

- Prospective longitudinal assessment of metabolic features and identification of potential predictors of cardiovascular and liver disease progression and of cancer development (HCC)

**Methods**

This is an observational study including a large cohort of adult type 1 GD patients, either naïve or on ERT/SRT, who are monitored at the Regional Referral Centre for Lysosomal Storage Diseases in Modena.

AIM 1-3: The first part of project is a cross-sectional study. We carefully assessed the following data at baseline for each GD patient:

- GD severity with scores and biomarkers; ERT/SRT status and duration

- Metabolic features: clinical, anthropometric, biochemical and metabolic data, body composition evaluated by DEXA, energy balance evaluated by calorimetry, and standardized questionnaire for nutritional and lifestyle habits
- Cardiovascular risk: cardiovascular risk score and non-invasive diagnostic methods, such as carotid and cardiac doppler ultrasound
- Liver disease: abdominal ultrasound, liver transient elastometry and magnetic resonance imaging

AIM 4: The second part of the project is a prospective longitudinal study. GD patients enrolled at baseline are evaluated every 6 or 12 months with reassessment of clinical, anthropometric, biochemical, metabolic, nutritional and lifestyle parameters and imaging data listed above.

All examinations/assessments and the timing of follow-up are performed according to guidelines/recommendations. Ethics committee approval has been obtained.

**Expected results**

Our research group has preliminary demonstrated that liver fibrosis is significantly associated with GD severity and, in GD patients on stable ERT, with metabolic syndrome components. We expect to better characterize the metabolic status, the cardiovascular risk and liver disease of these patients. Moreover, we hypothesize that, in GD patients on stable ERT, the lifestyle with its potential impact on metabolic status may play an important role in determining cardiovascular and liver disease burden and their progression. If confirmed, these results could improve the therapeutic approach of GD patients and prevent metabolic, cardiovascular and liver complications.

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# FEASIBILITY STUDY OF PHYSICAL EXERCISE AND HEALTHY LIFESTYLE INTERVENTION IN PATIENTS WITH PROSTATE CANCER: EFFECTIVENESS ON QoL AND ADVERSE EFFECTS OF ANDROGENIC DEPRIVATION THERAPY (ADT)

## Background

Androgen deprivation therapy (ADT) is a common treatment shown to increase survival in selected patients with prostate cancer (PCa) but is responsible for several clinically relevant side effects, including fatigue, loss of muscle mass, decreased physical performance, osteopenia, fractures, cognitive decline and metabolic and cardiovascular disease. Current knowledge on biological changes induced by physical exercise (PE) support its role in inducing beneficial effects on cardiorespiratory fitness, bone health, fatigue and cognitive functions and in improving the quality of life (QoL) of patients with PCa. Therefore, preliminary evidence suggests that a healthy lifestyle, which includes regular PE, may have a therapeutic role in men with PCa and can be proposed as a valid strategy to lessen the adverse effects of ADT.

## Objectives

The main objective of this project is to investigate the feasibility of and compliance to a Physical Exercise and healthy LifeStyle (PhELiS) intervention in patients with PCa during ADT. The secondary objectives are to evaluate patients' satisfaction with the intervention and its effectiveness on the main adverse effects of hormone therapy: fatigue, metabolic disease, loss of physical performance, cognitive decline and decrease in QoL. Furthermore, we will investigate the motivation for a change towards healthier lifestyle to verify the impact of PhELiS on long-term lifestyle.

**Methods**

Patients with PCa receiving ADT at the Oncology and Advanced Technologies Department of the Santa Maria Nuova Hospital – Azienda USL-IRCCS of Reggio Emilia will be invited to participate in this study. PhELiS consists in personalized therapeutic education regarding the adoption of a healthy lifestyle, plus 12 weeks of supervised PE (3 times/week), 12 weeks of supervised and unsupervised PE (3 times/week) and 12 weeks of unsupervised PE (3 times/week). Assessment will take place at baseline, when motivation for a change towards healthier lifestyle will also be assessed, and every 12 weeks. At the final 36-week follow up assessment, the level of maintenance of autonomous physical activity and healthier lifestyle will be recorded. The PE protocol will combine aerobic and resistance exercise training, with potential cardiometabolic benefits, as well as coordination and balance exercise to stimulate cognitive functions. The project will be implemented in collaboration with LILT RE - the Italian League for the Fight against Cancer in Reggio Emilia.

Feasibility will be measured by:

- rate of recruitment measured by comparing the number of patients screened to the number of patients recruited. Feasibility will be defined as ≥75% of participants recruited;
- intervention adherence assessed by average number of exercise sessions completed, both supervised and autonomous (by an exercise log book). Adherence will be defined as ≥75% and ≥ 50% of supervised and autonomous session completed, respectively;
- safety of exercise will be assessed by number of adverse events registered;

Compliance will be assessed by patients' self-reported adoption of healthier lifestyles.

Patients' satisfaction with the protocol will be assessed through a specific questionnaire.

The effectiveness of the intervention in improving the QoL and in countering the adverse effects of ADT will be investigated through the following outcome measures (at baseline and after 12, 24 and 36 weeks of intervention): cardiorespiratory capacity, muscle strength of lower and upper limbs, body composition (% of lean mass and fat mass, weight and body mass index - BMI), specific cognitive domains and tumor marker (prostate-specific antigen (PSA)) in the blood, as an indicator of disease progression.

**Expected results**

The expected results are the description of a feasibility profile and patients' compliance to the PhELiS intervention. In addition, we will measure the degree of motivation to change at the beginning of the intervention and verify the impact of PhELiS on the adoption and maintenance of a healthy lifestyle. In patients with sufficient adherence to the protocol we expect improvements in QoL and a lessening in all measures of the adverse effects of the ADT compared to baseline. This preliminary study will provide the data needed to develop a randomized controlled trial of PE and therapeutic education in order to improve QoL and promote healthy lifestyles in patients with PCa undergoing ADT.

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*CEM Curriculum: Translational Medicine*

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# PROSPECTIVE OBSERVATIONAL INTERNATIONAL REGISTRY OF PATIENTS WITH NEWLY DIAGNOSED PERIPHERAL T CELL LYMPHOMA

## Background

Peripheral T-cell non-Hodgkin lymphomas (PTCLs) are a rare heterogeneous group of lymphoproliferative disorders from mature T cells of post-thymic origin at different stages of differentiation with multiple morphological patterns, phenotypes, and clinical presentation. It represents only 10-15% of all lymphomas. More commonly they appeared in male patients, and the median age at diagnosis is 62 years. In the last 2016 WHO classification there are more than 20 subtypes of PTLC. The most common subtypes are PTCL not otherwise specified (NOS; 25.9%), angioimmunoblastic type (18.5%), NKTCL (10.4%), adult T-cell leukemia/lymphoma (ATLL) 9.6%, anaplastic large-cell lymphoma (ALCL) ALK positive (6.6%); ALCL, ALK negative (5.5%); and enteropathy-associated PTCL (4.7%). PTCLs are generally associated with high relapse rates and a poor prognosis compared to B-cell non-Hodgkin lymphomas with a 5-year-survival rate less than 40%.

## Objectives

The purpose of the project is to verify whether a prospective collection of data would allow to achieve more accurate information on T-cell lymphomas and search for more disease oriented prognostic models. In particular, the T-Cell Project will represent a unique opportunity for better understanding the genomic landscape of different subtypes, and to assess the role of new targeted therapies.

## Methods

This is a prospective, longitudinal, international, observational study of patients with histological diagnosis of Peripheral T-cell lymphoma. Eligible patients with first diagnosis of PTCL will be prospectively registered at a dedicated website and baseline demographic, clinical and laboratory data, as well as therapy details and outcome data will be collected. Patients who will be

enrolled in the Registry will undergo routine clinical examinations and receive therapy by physician choice.Registry duration is planned to be completed in 2 years and a half, also with a minimum follow-up of 2 years.

To evaluate the achievement of the study objective, we will use the PFS as primary endpoint, defined as progression-free-survival wich is measured from date of diagnosis until date of disease progression or death from T-cell Lymphoma.

Survival curves will be calculated using the Kaplan-Meier method and compared using the log-rank test. Effect sizes were reported as hazard ratios (HRs) with 95% CIs and estimated using the Cox proportional hazards regression method, adjusted by relevant confounding factors when needed.

**Expected results**

The information will be analyzed in order to understand if there are common characteristics at the time of diagnosis in patients with PTCL that influence the clinical course of the disease, and consequently to understand which are the most accurate therapeutic strategies based on these characteristics.

***Dr. Maria Luisa Introviane***

*CEM Curriculum: Nanomedicine, Medicinal and Pharmaceutical Sciences*

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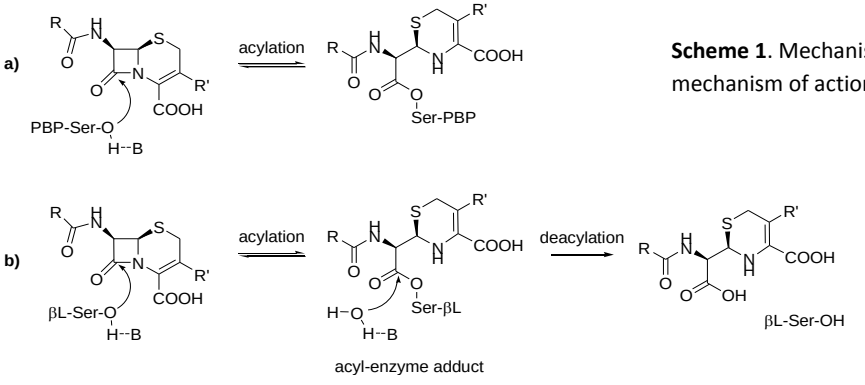
*Co-Tutor: Prof. Emilia Caselli*

## FACE TO FACE WITH ANTIBIOTIC RESISTANCE
## USING BORONIC ACID TRANSITION STATE INHIBITORS

### Background

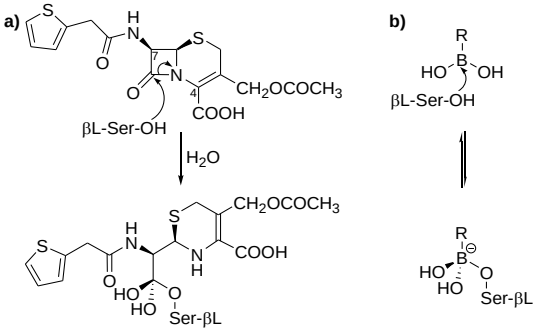
The World Health Organization (WHO) has identified antimicrobial resistance as one of the most important problems facing human health. Resistance is growing among Gram-positive and Gram-negative pathogens and most of them become multidrug-resistant (MDR) bacteria. Rice recently reported these as the "ESKAPE" pathogens (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumanii*, *Pseudomonas aeruginosa*, and *Enterobacter* species) to emphasize that they currently cause the majority of world-wide hospital infections and "escape" the effects of antibacterial drugs.[1] β-Lactam antibiotics remain the most used class of antibacterial drugs due to their safety. Different classes of β-lactams (penicillins, cephalosporins, carbapenem, monobactams) share the same mechanism: their β-lactam ring forms a stable adduct with the bacterial penicillin binding proteins (PBPs), enzymes used for the synthesis of the bacterial cell wall. The bacterium is therefore no longer able to synthesize the cell wall and die. Among the multiple mechanisms of resistance, the most significant is the development of bacterial enzymes, named β-lactamases, capable to neutralize β-lactam antibiotics: they form an adduct with the antibiotic, easily hydrolyzed by a water molecule; thereby, the drug is made ineffective (Scheme 1).[2]




**Scheme 1**. Mechanism of action of β-lactam antibiotics **(a)** and mechanism of action of β-lactamase enzymes **(b)**.

-Lactamase enzymes are sorted in four classes: A, B, C, and D. Class A, C, and D enzymes utilize a serine residue for β-lactam hydrolysis (as in Scheme 1); class B metal-enzymes require one or two zinc ions for substrate hydrolysis.

Boronic Acid Transition State Inhibitors (BATSIs) are known reversible covalent inhibitors of -lactamases, due to the electrophilic character and $sp^2$ geometry of the boronic moiety, which upon attack of the nucleophilic serine, forms a tetrahedral adduct with the enzyme (Scheme 2, **b**), mimicking the one formed with the -lactam antibiotics (Scheme 2, **a**). In this case, hydrolysis is not possible and the enzyme is inhibited (Scheme 2).




**Scheme 2**. **a)** Mechanism of action of a β-lactamase with a β-lactam (the antibiotic Cephalothin). **b)** Mechanism of action of a β-lactamase with a boronic acid.

Selectivity and high potency of specific BATSIs towards -lactamases have been proved in several studies, by means of changing the substituents on the carbon atom attached to the boron[3]: in particular, acylamidoboronic acids (Figure 1, **a**) are characterized by the presence of an amide side chain bearing substituents typical of commercially available β-lactams.

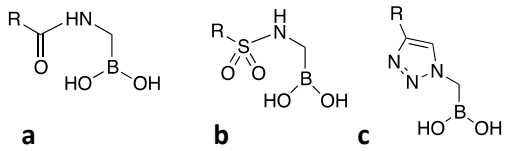



**Figure 1**. General structures of **a)** α-acylamidoboronic acids, **b)** α-sulphonamidoboronic acids and **c)** α-triazolyl-boronic acids.

Starting from acylamidoboronic acids (**a**) our research group replaced the amide group with different bioisosters, such as a sulphonamide (**b**) or a triazole (**c**) and generated compounds active against ADC-7 a highly resistant class C -lactamase in *A. baumannii*. The second step was the synthesis of chiral compounds introducing into the boron-bearing carbon atom a carboxy-substituted ring system, able to interact with different regions of the enzymes. Two compounds demonstrated to bind effectively and inhibit -lactamases: S02030 (Figure 2) and the related MB076. The first recognizes and inhibits class A and class C enzymes; notably, its activity has been demonstrated against class A KPC-2 ($IC_{50}$= 0.084 M) and SHV-1 and class C ADC-7 (Ki = 0.044 M), vastly different lactamases. The latter demonstrated a good activity against ADC-7 (Ki = 0,1 M) too.

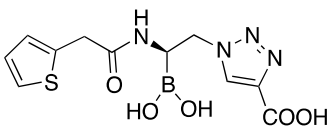


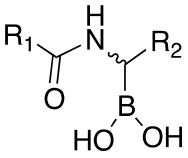
**Figure 2**. S02030

**Objectives**

Starting from the molecular structure of known BATSIs, the general objective of this doctoral project is to design and synthesize wide spectrum β-lactamases inhibitors, thus active not only against class A and class C β-lactamases, but also with class B and/or class D enzymes. These molecules, when associated to a β-lactam antibiotic, will be capable of restoring *in vitro* and possibly *in vivo* the antibacterial activity against β-lactam resistant bacteria.

**Methods**

*Test.* Microbiological analysis and kinetics studies performed by prof. Robert A. Bonomo at the Cleveland Medical Center (Ohio, USA) demonstrated that S02030 recognizes and inhibits class A and class C enzymes.




*Analyze*. In collaboration with the Department of Chemistry of the Grand Valley University (Michigan, USA) the structure of the complex inhibitor/β-lactamase(s) has been determined for S02030 and MB076 after co-crystallization in order to understand the role of substituents, interacting with different regions of the enzymes. In particular the analysis demonstrated that the β-acylamido moiety mimics the interaction of corresponding substituent in β-lactam drugs and the triazole has the same role as the stable cycle present in many antibiotics and inhibitors and precludes water attack.


*Design*. Starting from data obtained for S02030 and MB076, the first goal was to obtain new classes of boronic acids with improved activity profile against class A and/or C. These new compounds were specifically designed keeping into account not only the determinants important for class A and C recognition, but also new substituents able to gain interactions with class B and/or class D enzymes.

*Synthesis*. Synthetic procedures were optimized to obtain new compounds with a different cycle in position R2 and/or with a different R1 configuration. In a second time, molecules with a cyclic boronic acid and able to chelate zinc ions have been synthesised in order to interact with class B metal- β- lactamases.[4]

**Expected results**

During the last six months two strategies have been adopted: to modify the molecular structure of S02030 in order to improve its activity and to create cyclic boronic acid inhibitors able to inhibit

class B metal-enzymes, by chelating zinc ions.[4] These molecules will be tested by prof. Bonomo for the microbiological profile and the complex inhibitor/β-lactamase(s) will be determined and analyzed in *silico* for the best performing compounds identified. Once the structure-activity (SAR) relationship on different β-lactamases will be understood, the next step will be the rational design of a second generation of multi-target inhibitors, thus starting a new cycle *Synthesis-Test-Analysis-Design*.

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***Dr. Clara Lazzaretti***

*CEM Curriculum: Translational Medicine*

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## MOLECULAR AND CELLULAR ACTION OF REPRODUCTIVE HORMONES

### Background

Ovarian physiology is regulated by tandem action of gonadotropins and sex steroids, both necessary for the completion of follicle growth, maturation and ovulation. During the menstrual cycle, the selection of the dominant follicle occurs, as a result of variations in gonadotropins and steroid hormone levels and in receptors expression on the surface of granulosa cells. Nevertheless, mechanisms regulating this event are still poorly understood and it is supposed to be due to low estrogen levels, decline of follicle-stimulating hormone (FSH) levels and receptor (FSHR) expression. Previous studies demonstrated that human granulosa cells (hGLC) and transfected cell lines overexpressing FSHR stimulated with high doses of FSH undergo apoptosis, while estrogens induce anti-apoptotic signals via nuclear receptors and non-genomic action of a G protein-coupled estrogen receptor (GPER). However, it was demonstrated that the FSHR and luteinizing hormone (LH) receptor (LHCGR) genotype impact the ovarian response to hormones. These data suggest the ability of estrogens to modulate FSH/FSHR-dependent apoptotic signals, suggesting new mechanisms regulating the selection and rescue of dominant follicles in an individual-specific manner.

### Objectives

The aim of the project is to better understand the role of estrogens/gonadotropins and their membrane receptors in regulating ovarian physiology and the selection of the dominant follicle. I will evaluate how interactions between GPER- and gonadotropin receptors (GnRs)-mediated signaling cascades regulate pro and anti-apoptotic signals underlying human reproduction.

### Methods

Receptor interactions, expression and dimerization as well as cell signaling events and gene expression will be evaluated *in vitro*. To this purpose, human granulosa cells (hGLC), COS7, HEK293 and other cell lines will be used. GPER and FSHR/LHCGR dimerizations, coupling and displacement with Gα proteins will be assessed by bioluminescence resonance energy transfer (BRET), while their expression and localization will be evaluated by Western Blotting and immunofluorescence. Cell signalling will be also studied by evaluating intracellular endpoints, such as cAMP, Ca2+, pERK1/2, pCREB, pAKT, etc. Life/death signals will be evaluated by analysis of procaspases cleavage, MTT assay and immunostaining. The expression of target genes will be evaluated by real time PCR and using the advanced digital PCR. These analyses will be performed considering the gonadotropin receptors genotype, since they modulate the cell response to the hormone.

The presence of heterodimeric and homodimeric receptor structures will be evaluated by photo-activated localization microscopy (PALM), that will be performed thanks to my 1-year internship at the Dept. of Surgery and Cancer of the Imperial College London (London, UK).

**Expected results**

It is expected to find how estrogens and gonadotropins act in tandem to regulate the endocrine regulation of human reproduction. These results can provide important steps forward in the knowledge of the ovarian physiology and they may have important applications in assisted fertilization protocols.

***Dr. Elia Paradiso***

*CEM Curriculum: Translational Medicine*

*Tutor: Prof. Manuela Simoni*

## ACTION OF LISOSPHINGOLIPIDS AND GONADOTROPINS AS DETERMINANTS OF THE ENDOCRINE REGULATION OF THE OVARIAN FOLLICLE

### Background

Sphingosine-1-phosphate (S1P) is a lysosphingolipid highly represented in plasma and lymph, as well as in the ovarian follicular fluid together with glycoprotein hormone gonadotropins. They are involved in sexual development and reproduction, however, the role of S1P and gonadotropins in gonads is not completely elucidated. Both S1P and gonadotropins exert their physiological functions by binding cognate G protein-coupled receptors (GPCRs). In particular, S1P acts through five specific G protein-coupled receptors (GPCRs), known as S1PR1-5. S1PR1 and S1PR3 are expressed in human primary granulosa lutein cells (hGLC), as well as in the immortalized human primary granulosa cell line hGL5. S1PRs-mediated signals are activated at nanomolar S1P concentrations, resulting in the activation or inhibition of a number of intracellular signaling pathways.

### Objectives

This study aims to characterize the role of S1P- and gonadotropins-induced signaling in determining ovarian follicle development *in vitro*. Receptor homo/heterodimerizations and coupling to intracellular interactors will be also evaluated.

### Methods

Human primary granulosa lutein cells (hGLC), the immortalized human primary granulosa cell line hGL5 and other cell models, such as COS-7 and HEK293 cells, will be used *in vitro*. Intracellular signaling pathways activation, such as production of cAMP, phospho-extracellular-regulated kinase 1/2 (pERK1/2) and protein kinase B (pAKT) activation, as well as intracellular calcium ion increase, will be characterized by treating cells with hormones and/or selective agonists. Receptor homo/heteromerizations and coupling to G proteins and β-arrestins will be assessed. These analysis will be performed by ELISA, Western blotting and bioluminescence resonance energy

transfer (BRET). Moreover, the role of S1P/gonadotropins-dependent steroid hormones synthesis, cell proliferation and viability, as well as gene expression will be investigated in the presence of specific MEK-, PKA- and calcium- inhibitors and ligand antagonists. These intracellular endpoints will be analyzed by immunoassay, MTT assay and real time PCR.

**Expected results**

The role of S1P/gonadotropins in ovarian functioning will be investigated and provide results relevant for understanding human physiology and for assisted reproduction techniques.

***Dr. Sara Castellano***

*CEM Curriculum: Translational Medicine*

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## GENOMIC CHARACTERIZATION OF CHRONIC MYELOPROLIFERATIVE NEOPLASMS

### Background

Myeloproliferative neoplasms (MPNs) are a group of related hematologic cancers characterized by an excessive proliferation of terminally differentiated myeloid cells. The three disorders included in the so-called "classic" MPNs are: Polycytemia Vera (PV), which involves elevated red-cell counts; Essential Thrombocythemia (ET), which is defined by elevated platelets in the blood; and Myelofibrosis (MF), which is characterized by bone marrow fibrosis. PV and ET are chronic conditions that can progress to myelofibrosis (secondary MF). However, MF can also occur without pre-existing conditions (primary MF).

During the past decade, several aberrations have been identified and characterized in MPN patients, such as the dysregulation of JAK2 signalling in 2005 and the CALR mutations in 2013. Still, the molecular pathogenesis of these malignancies remains incompletely understood and for most MPNs there are no known cures, other than stem cell transplants. Diagnostic features and outcomes in patients show strong heterogeneity, probably as a consequence of a complex genetic landscape in addition to changes in gene expression. Currently, risk stratification of MPN patients is based mainly on clinical features and the presence of driver mutations. Gene expression data have already been used to provide prognostic indications in haematological diseases, nevertheless, none of the existing models for MPNs integrates transcriptomic data. Therefore, there is a need to better characterize the transcriptomic profile of these disorders in order to try to elaborate a signature able to add more robustness to clinically widely accepted scoring systems.

### Objectives

The main scope of this project is to identify a molecular signature able to distinguish "high risk" MPN patients with inferior overall survival from "low risk" ones. In particular, we intend to compare it with the current predictive models in terms of prognostic power and investigate how, if

combined to them as an independent prognostic factor, it can improve the ability to direct patients toward the best available therapeutic strategy.

**Methods**

We will analyse the gene expression profile of myelofibrosis primary cells. For this purpose, we will follow two parallel approaches:

In the first one, the dataset will be split into a training and a test set by random sampling and balancing of selected clinical variables. Then the association of the gene expression with the survival will be assessed in the training set using a Cox regression model and the obtained gene signature will be validated on the test set.

In the second approach, “high risk” and “low risk” samples will be selected from the dataset on the basis of clinical criteria, and both SAM (Significance Analysis of Microarrays) and limma (Linear Models for Microarray Data) will be exploited to perform a differential expression analysis for the identification of a molecular signature.

Subsequently, a retrospective analysis of clinical and pathological information will be performed to compare the predictive power of our classification with that of existing prognostic scores (DIPSS and MIPSS70).

**Expected results**

This work is expected to identify a robust molecular signature predicting the patient outcome. In particular, this could be exploited to create a predictive score that would add a further layer of information to the existing models based on clinical features, in order to improve their prognostic significance.

**Dr. Camilla Reggiani**

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*Co-Tutor: Prof. Giovanni Pellacani*

# FLUORESCENCE CONFOCAL LASER MICROSCOPY APPLICABILITY TO THE MICROSCOPICALLY CONTROLLED SURGERY OF SKIN CANCERS, FOR THE INTRAOPERATIVE DIAGNOSIS OF SKIN TUMORS AND INFLAMMATORY SKIN DISEASES

## Background

Microscopically controlled surgery, or Mohs surgery, is a technique used during skin cancer surgery in order to assess surgical margins. The technique provides for the intraoperative histological examination of the skin tumor and its margins. Main fields of applications of the technique in skin oncology are: basal cell carcinomas, squamous cell carcinomas, basal cell carcinomas recurrances, and in situ melanoma. Mohs surgery cure rates are: 99% for basal cell carcinoma, and 94% for squamous cell carcinoma. Other skin tumors applications are: dermatofibrosarcoma protuberans, spindle cells tumors, sebaceous carcinoma, microcystic adnexial carcinoma, Merkel cell carcinoma, atypical fibroxanthoma and leiomyosarcoma.

Fluorescence laser confocal (FCM) involves the use of a laser with different wavelengths and the staining of surgical fresh tissue samples in a DNA fluorescence stain. This procedure allows obtaining, in just few minutes, fresh tissue images with a nuclear-level resolution that is comparable to that of histopathology.

## Objectives

The main objective of the study is to establish the diagnostic accuracy in Mohs surgery of FCM compared to the real time histopathological examination. The second endpoint of the study is to evaluate the applicability of the method in the diagnosis of inflammatory and oncological skin diseases.

## Methods

This project will be an experimental, single center, prospective study. Patients will be enrolled among patients that are routinely going to undergo skin biopsies for inflammatory skin diseases or skin cancers at the Dermatology Department of the University of Modena and Reggio Emilia. Moreover patients affected by complex skin tumors who need Mohs surgery will be enrolled in the project. Bioptic samples of all cases selected will be evaluated with FCM before the analysis with traditional histology. In Mohs surgery cases the central part of the skin cancer and all the margins will be analyzed before the traditional real-time histopathological examination.
FCM diagnostic accuracy will be evaluated through comparison between FCM and histopathology, the gold standard.

**Expected results**

This study project is expected to define the diagnostic accuracy of FCM applied to skin cancers Mohs surgery and in the intraoperative diagnosis of inflammatory and oncological skin diseases.
The main implication related to the application of this method is an immediate diagnostic-therapeutic process with consequently time and cost savings.

***Dr. Gaetano Alfano***

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# MICROORGANISM AND BIOFILM VIRULENCE FACTORS IN CENTRAL-VENOUS-CATHETER-RELATED BLOODSTREAM INFECTIONS

## Background

Central-venous-catheter-related bloodstream infections (CRBSIs) are an important cause of hospital-acquired infections associated with morbidity, mortality, and cost among patients on maintenance hemodialysis[1]. Central venous catheter (CVC) is a medical device that allows access to the central venous vessels to perform hemodialysis. To date, the prevalence of CVCs among patients on maintenance dialysis is estimated to be about 25%[2], [3]. CRBSIs manifest with bacteremia that often evolve in severe septicemia. The incidence of catheter infections varies from about 0.6 to 6.5 episodes per 1000 catheters per day [4]–[6]. The factors favoring an infection include diabetes mellitus, personal hygiene, recent hospitalization and inadequate hemodialysis. The microorganisms that are commonly isolated are Gram-positive (52-84%), followed by Gram-negative (27-36%) and fungal infections (<10%)[7], [8]. Staphylococcus Aureus is the most frequently detected pathogen, its prevalence has been evaluate to vary between 21 and 43% among studies[9]. CRBSIs appear to be preceded by early colonization of the catheter by the biofilm, which is made up of proteins and polysaccharides where bacteria and fungi can survive and proliferate. The interaction between microorganisms and biofilm are therefore recognized as the main virulence factors for CRBSIs. The physical proprieties of the extracellular matrix composing biofilm protect antimicrobials from conventional antimicrobial agents because avoid drugs penetrance within it. Furthermore, the pressure given by the use of antibiotics favors antimicrobial resistance of the microorganisms that arise by mutation or transfer of resistance genes[10].

**Objectives**

Since microorganisms and biofilm concur together to determine CRBIs, the objective of our project is widening the knowledge of their interaction in patients on maintenance hemodialysis. In particular, our goal will be to evaluate whether the microorganisms that cause severe complications including sepsis, endocarditis and osteomyelitis, such as S. Aureus, possess peculiar virulence factors that can explain their increased pathogenicity compared to other bacteria of the same species. Molecular typing of their genome would allow verifying if the germs responsible for CVC infections are the same germs that normally colonize the mucous membranes or the exit side of the CVC of the patients on chronic hemodialysis.

In addition, our study would evaluate the ultrastructural composition of biofilm detected on CVC removed from patients with CRBSIs. This analysis would allow evaluating whether some morphological features of the biofilm (structure, thickness) or localization (intra- or extra-luminal) are associated with poor responsiveness to both systemic and local antibiotics. Finally, the identification of the microorganisms present inside the biofilm would allow assessing the presence of polymicrobial flora, another well-known mechanism of antibiotic resistence.

**Methods**

The study will be conducted at the Nephrology Unit of the University of Modena. We will enroll patients on chronic maintenance hemodialysis patients older than 18 years with CBRI. According to the Infectious Diseases Society of America guidelines of 2009 [11], the diagnosis of CBRI is based on (i) direct microbiological identification of the microorganism from a segment of the catheter if this latter is removed or (ii) on catheter cultures. Approximately 60 patients are expected to be enrolled in 24 months. For each patient demographics, clinical and laboratory data will be collected from the diagnosis of catheter infection. Blood cultures of aerobic and anaerobic germs, nasal swab and exit-side will be analyzed by microbiology laboratory (Department of Diagnostic, Clinical and Public Health Medicine) of the University of Modena for microbial identification. It will occur through microbial culture, thereafter, molecular typing through polymerase chain reaction should identify their virulence factors. In about 30% of the patients, their CVC will be removed for infections nonresponsive to antibiotics. In these cases we will perform an ultrastructural investigation of the biofilm by means of transmission electron microscopy in order to evaluate the spatial organization of biofilm.

**Expected results**

Knowing the microorganism and biofilm virulence factors could provide significant novelties in the management of CRBSi. The results of this study can be an impulse for innovation in material science and pharmacology. The knowledge of the structure of biofilm could favorite the development of surface modification that incorporates technologies preventing microbial adherence and subsequent biofilm formation. On the other side, the development of drugs active in both prophylaxis and treatment could reduce the severe morbidities associated with CRBSi

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***Dr. Kevin Israel Muñoz***

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**AN INTEGRATED PROTEOMICS AND BIOINFORMATICS APPROACH UNCOVERING THE ANTI-CANCER MECHANISM OF ANTI- THYMIDYLATE SYNTHASE DRUGS (LC1343) IN HUMAN COLORECTAL CANCER CELL LINE**

## Background

Thymidylate synthase (TS) targeting drugs are the most widely used drugs in anti-cancer therapy. However, treatment with classical, catalytic-pocket directed TS inhibitors and with other DNA damaging drugs usually induce TS over-expression and the related onset of tumor drug resistance. To avoid such adverse phenomena, we adopted a change of strategy and sought compounds that, unlike all known anti-TS drugs, would bind at the monomer-monomer interface of this homodimeric enzyme and shift its dimerization equilibrium toward the inactive monomers. Continuing the objective of proteomic and bioinformatic studies for the understanding of the biological mechanisms in the investigation of anti-proliferative drugs, these experiments were conducted to investigate the time-dependent effects on the whole proteome of human colorectal cancer cell line, HCT116 after being treated with the LC1343 (E7)— (IC50 on HCT116 35.5μM) a thymidylate synthase (TS) inhibitor compound. The whole cell mass spectrometry (LC MS/MS label-free quantification) differential proteomic study followed by bioinformatics analysis was performed in treated versus untreated cells. Applications of the proteomic and bioinformatic studies encompass experimental design, sample management and global data interpretation to allow to understand this mechanism of action.

**Objectives**

We aim in demonstrating the role of anti-Ts drugs (LC1343) in regulating cancer cell response and provide some insights into the molecular mechanisms through a proteomics-bioinformatics approach. Our attention on biological processes involved in protein metabolism cellular component organization or biogenesis, DNA repair, DNA replication, DNA metabolism, purine nucleobase metabolism, biological regulation, the cell cycle, and apoptosis. In order to explore the relevance and mechanics of these drugs, the use of well characterized colorectal cancer cell lines HCT116, and eventually primary cell lines taken from patients, will be studied pre-treatment and post-treatment.

**Methods**

For the time-dependent experiment, cell lines are seeded for 24h, minimum required for cell adhesion, then treated with LC1343 (35.5 μM). Cells were harvested at two different time points, 6h and 12h. With a control group of untreated cells harvested at time 0h. For comparison to a reference drug, we used 5-fluorouracil (5-FU), at 14 μM, a largely used treatment of colorectal, gastric, pancreas, metastatic breast tumors, among other malignancies, under the same experimental conditions. Filter aided sample preparation (FASP) technique was utilized for the preparation of samples for LC MS/MS label-free quantification analysis. Experimental MS data determined the proteomic profile. The information on modulated proteins were retrieved from UniProt. With a combination of building a literature-based protein network—using STRING database to provide an assessment and integration of protein–protein interactions, including direct (experimental) as well as indirect (co-occurrence/co-expression) associations, to choose relevant proteins of the folate cycle and folate receptor pathway, including the understanding of other relevant and critical proteins pathways. For data management, the full bioinformatics analysis was performed using Progenesis QI which, allows the reliable proteins list to be compiled, and other software such as Panther and R platform for statistical analysis. And finally obtaining a biological validation via Western Blot on identified differentiated proteins.

**Expected results**

Notably, proteomics and bioinformatics are increasingly being applied for revealing the molecular mechanisms of drugs. This approach aims to study the effect of drugs on the whole proteome changes to find key signaling pathways through a bioinformatics method. Results for these experiments are being finalized and are expected to confirm LC1343 having most relevantly, caused a decrease of TS levels due to an increased degradation rate of the TS monomers, found after 12h. It has been observed that after 12h TS protein levels decreased remarkably after exposures to LC1343 in colorectal cancer, HCT116, HT29 cell lines. LC1343 has previously caused a higher reduction of cancer mass and a lower toxicity 5-FU in orthotopic models of pancreatic cancer. Results from these LC-MS/MS label-free quantification and bioinformatic analyses are offering preliminary data that will emerge to be useful for setting up our future experiments in exploiting the use of MS analysis to explore the mechanics of these drugs and understand the effects on the whole proteome of treated cells and will demonstrate the potential of combined proteomic and bioinformatic approaches.

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